FROM THE ORGANIZERS

The term “Bioenergetics” was coined in 1950s by Albert Szent-Gyorgyi who used this word as the title of one of his books. In 1968, a special session of the International Meeting on Oxidative Phosphorylation in Polignano-a-Mare (near Bari, Italy) was devoted to the problem of how to call the new branch of biology dealing with mechanisms of energy transductions in living systems. At this meeting, I attracted the attention of the audience to Szent-Gyorgyi’s term, putting forward only one argument in favor, namely, that I already organized the Department of Bioenergetics at Moscow State University. Surprisingly, the suggestion was accepted. And now I have the great pleasure to welcome numerous participants of the 14th European Bioenergetics Conference to Moscow State University.

In fact, this is the second attempt to organize an EBEC conference in Russia. Originally, the plan was to have EBEC here in 1992, but it was moved to Helsinki due to the severe economic crisis that followed the collapse of the Soviet Union in the very end of 1991. Now I hope the organizers will be happier in carrying out their intention.

Traditionally for EBEC meetings, the conference will deal with two major aspects, i.e. molecular and supramolecular bioenergetics, the latter including physiology of organelles, cells, tissues, and organisms. At the previous 13 EBECs, the majority of the talks were related to molecular bioenergetics. However, now for the first time the physiological aspects approach is slightly dominating. This event indicates a tendency to apply the great success achieved in understanding energy-transducing mechanisms to elucidate principles of functioning of complex biological systems. Most demonstratively, such a tendency was revealed in mitochondrial studies. The number of papers published in this field dramatically increased in 1960-1980, then a plateau was reached, which changed to one more phase of exponential growth in the late 1990s due to discovery of the fact that mitochondrial physiology is essential not only for the life but also for programmed death.

At the Moscow EBEC, symposial (plenary) lectures occupying the first part of each day will be followed by colloquial talks in its second part. The total number of speakers invited by the Organizing Committee is as large as 90 (30 symposial and 60 colloquial). Moreover, 20 speakers were invited by the chairpersons of the participant-led colloquia and 24 were selected by the organizers among the best posters. All the symposial and colloquial speakers had a possibility to contribute to a Special BBA Bioenergetics volume. The great majority of the speakers accepted the invitation, writing excellent papers. I am sure that their contributions will be of importance for the further progress of bioenergetics.

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I am very grateful to all bioenergeticists who will attend; to organizations who financially supported this 14th EBEC; to Monomax professional international congress-organizing agency; to members of the Local Organizing Committee and the International Advisory Committee for their help in organization of this meeting. I am also very grateful to Pat Crowley and all other staff of BBA and Elsevier for their collaboration in the production of two special BBA Bioenergetics volumes.

I wish you all a happy and exciting EBEC in Moscow.

Vladimir Skulachev, Chairman, Organizing Committee, 14th EBEC, Moscow

SCIENTIFIC PROGRAM

MITCHELL MEDAL LECTURE

M1
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ROTARY CATALYSIS OF ATP SYNTHASE

SYMPOSIA (Plenary Sessions)

1. Molecular bioenergetics

Symposium 1.1. Respiratory chain

S1.1.1

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STRUCTURE-FUNCTION RELATIONSHIPS IN TERMINAL OXIDASES

S1.1.2

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STRUCTURE OF THE HYDROPHILIC DOMAIN OF RESPIRATORY COMPLEX I FROM THERMUS THERMOPHILUS

S1.1.3

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REACTION MECHANISM OF BOVINE HEART CYTOCHROME C OXIDASE

Symposium 1.2. Light-driven energy transducers

S1.2.1

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STRUCTURE OF PHOTOSYSTEM II AND ITS IMPLICATIONS FOR UNDERSTANDING THE WATER SPLITTING REACTION IT CATALYSES

S1.2.2

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XANTHORHODOPSIN, A NOVEL RETINAL-BASED PROTON PUMP WITH A CAROTENOID ANTENNA

S1.2.3

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ELECTRON TRANSFER IN BACTERIAL REACTION CENTERS WITH MODIFIED B-BRANCH PIGMENT COMPOSITION

Symposium 1.3. ATP-synthase/ATPase

S1.3.1

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THE RIGHT-HANDED COILED COIL OF THE b DIMER OF ESCHERICHIA COLI ATP SYNTHASE

S1.3.2

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MODULATION OF PROTON PUMPING EFFICIENCY IN BACTERIAL ATP SYNTHASES

S1.3.3

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THE STRUCTURE AND FUNCTION OF ATP SYNTHASE

Symposium 1.4. Porters

S1.4.1

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STRUCTURE AND MECHANISM OF MEMBRANE TRANSPORTERS

S1.4.2

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STRUCTURE OF NHAA Na^+/H^+ ANTIPORTER: INSIGHTS INTO MECHANISM OF ACTION AND REGULATION BY pH

S1.4.3

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METABOLITE TRANSPORTERS OF MITOCHONDRIA

2. Physiology and pathology of mitochondria (*in vitro*, *ex vivo* and *in vivo* studies)

Symposium 2.1. Uncoupling

S2.1.1

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THE UNCOUPLING PROTEINS 1 AND 3: QUESTIONS OF MECHANISM, CONTROL AND PHYSIOLOGICAL FUNCTION

S2.1.2

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THE MITOCHONDRIAL UNCOUPLING PROTEINS: FROM BIOENERGETICS TO PHYSIOPATHOLOGY

Symposium 2.2. ROS and redox regulations

S2.2.1

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MITOCHONDRIAL ROS PRODUCTION AND ITS ATTENUATION BY UNCOUPLING PROTEINS

S2.2.2

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MITOCHONDRIAL ROS-INDUCED ROS-RELEASE: AN UPDATE AND REVIEW

Symposium 2.3. Aging

S2.3.1

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MITOCHONDRIAL OXIDATIVE STRESS, AGING AND CALORIC RESTRICTION: THE PROTEIN AND METHIONINE CONNECTION

S2.3.2

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SUPEROXIDE AGING AND DEATH IN S. CEREVISIAE

S2.3.3

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MITOCHONDRIAL DNA MUTATIONS, OXIDATIVE STRESS AND APOPTOSIS IN MAMMALIAN AGING

Symposium 2.4. Cancer, ischemia and degenerative disorders

S2.4.1

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THE MITOCHONDRIAL PERMEABILITY TRANSITION - FROM MOLECULAR MECHANISMS TO CARDIOPROTECTION

S2.4.2

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CAN A SINGLE SUBUNIT NADH DEHYDROGENASE WORK AS A THERAPEUTIC AGENT FOR COMPLEX I-DEFICIENT DISEASES?

Symposium 2.5. Program death of cells and mitochondria

S2.5.1

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MITOCHONDRIAL PERMEABILIZATION IN CELL DEATH AND MITOPHAGY

S2.5.2

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APOPTOSIS AND NECROSIS, TWO FUNDAMENTAL ALTERNATIVES

S2.5.3

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NUCLEI-MITOCHONDRIA CROSS-TALK IN APOPTOSIS

Symposium 2.6. Mitochondrial dynamics

S2.6.1

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KEEPING MITOCHONDRIA IN SHAPE: A MATTER OF LIFE AND DEATH

S2.6.2

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DYNAMINS, ENDOPHILINS AND BCL-2 FAMILY MEMBERS: INVOLVEMENT IN MITOCHONDRIAL OUTER MEMBRANE DYNAMICS

Symposium 2.7. Therapeutic approach

S2.7.1

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PROPERTIES OF THE PERMEABILITY TRANSITION IN VDAC1$^{-/-}$ MITOCHONDRIA

S2.7.2

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MITOCHONDRIAL NADPH, TRANSHYDROGENASE AND DISEASE

Round Table. Perspectives of Bioenergetics

COLLOQUIA

1. Molecular bioenergetics

Colloquium 1.1. Respiratory chain

C1.1.1

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STRUCTURAL AND FUNCTIONAL INSIGHTS INTO MITOCHONDRIAL COMPLEX I

C1.1.2

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THE MECHANISM OF PROTON PUMPING BY CYTOCHROME C OXIDASE

C1.1.3

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RAPID KINETICS OF CHARGE TRANSLOCATION BY CYTOCHROME C OXIDASE: TRANSITION BETWEEN THE FERYL-OXO AND FERRIC STATES

C1.1.4

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NEW INSIGHTS ON NON-CANONICAL HAEM-COPPER OXYGEN REDUCTASES

C1.1.5

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PROTON TRANSLOCATION BY CYTOCHROME C OXIDASE

Colloquium 1.2. Light-driven energy transducers

C1.2.1

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CONSEQUENCES OF THE STRUCTURE OF THE CYTOCHROME b_6f COMPLEX FOR ITS CHARGE TRANSFER PATHWAYS

C1.2.2

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THE IDENTIFICATION OF F_X IN THE HELIOBACTERIAL REACTION CENTER AS A [4Fe-4S] CLUSTER WITH A GROUND SPIN STATE OF S = 3/2

C1.2.3

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CYCLIC ELECTRON FLOW IN C3 PLANTS

C1.2.4

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HIGH-FIELD EPR ON LOW-SYMMETRY PROTEINS TO REVEAL STRUCTURE-DYNAMICS-FUNCTION RELATIONS

C1.2.5

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INITIAL ELECTRON DONOR AND ACCEPTOR IN ISOLATED PHOTOSYSTEM II REACTION CENTERS IDENTIFIED WITH FEMTOSECOND MID-INFRARED SPECTROSCOPY

Colloquium 1.3. ATP-synthase/ATPase

C1.3.1

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DYNAMICS OF SUBUNIT INTERACTION OF THE Na^+ PUMP BY FRET VOLTAGE CLAMP FLUOROMETRY

C1.3.2

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SUBUNIT MOVEMENTS IN MEMBRANE INTEGRATED EF0F1 BY SINGLE MOLECULE SPECTROSCOPY

C1.3.3

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BIOENERGETICS OF ARCHAEA: ATP SYNTHESIS UNDER HARSH ENVIRONMENTAL CONDITIONS

C1.3.4

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STRUCTURAL AND FUNCTIONAL FEATURES OF YEAST V-ATPASE SUBUNIT C

C1.3.5

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KINETICS OF PROTON-TRANSLOCATING ATP HYDROLYSIS BY PARACOCCUS DENITRIFICANS FO·F1-ATP SYNTHASE

Colloquium 1.4. Porters and some other subjects

C1.4.1

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GROTTHUSS & HOW TO TRACE PROTON TRANSLOCATION IN A MEMBRANE PROTEIN

C1.4.2

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THE REDOX STATE OF CYTOCHROME C REGULATES APOPTOSIS

C1.4.3

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ROLE OF MITOCHONDRIA IN SHAPING A CALCIUM SIGNAL

C1.4.4

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THEORETICAL AND EXPERIMENTAL APPROACH TO MODELING OXPHOS DISEASES

Colloquium 1.5. Mechanisms of energy coupling

C1.5.1

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CYTOCHROME bc_1 COMPLEX AND ITS FUNCTIONAL FUSION VARIANT CYT bc_1-c_Y WITH ITS PHYSIOLOGICAL MEMBRANE ANCHORED ELECTRON ACCEPTOR CYTOCHROME c_Y

C1.5.2

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PROTON TRANSFER BY MEMBRANE PROTEINS: WHETHER THERE ARE RULES?

C1.5.3

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COOPERATIVITY AND FLEXIBILITY OF THE PROTONMOTIVE ACTIVITY OF MITOCHONDRIAL RESPIRATORY CHAIN

C1.5.4

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THE PROTONMOTIVE MECHANISM OF CYTOCHROME C OXIDASE: PROBING THE INTERNAL CHARGE-COMPENSATING PROTONATION BY FTIR SPECTROSCOPY

C1.5.5

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ON THE LOCALIZED COUPLING OF RESPIRATION AND PHOSPHORYLATION IN MITOCHONDRIA

2. Physiology and pathology of mitochondria (*in vitro*, *ex vivo* and *in vivo* studies)

Colloquium 2.1. Uncoupling

C2.1.1

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MODULATORS OF UNCOUPLING

C2.1.2

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UNCOUPLING DUE TO MITOCHONDRIAL UNCOUPLING PROTEINS IN VITRO AND IN VIVO

C2.1.3

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PROTON TRANSPORT MEDIATED BY UNCOUPLING PROTEINS 1 AND 2 RECONSTITUTED IN PLANAR LIPID BILAYERS

C2.1.4

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A NEW LOOK AT UCP1

C2.1.5

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MITOCHONDRIAL UCPS: NEW INSIGHTS INTO REGULATION AND IMPACT

Colloquium 2.2. ROS and redox regulations

C2.2.1

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MITOCHONDRIAL METABOLIC STATES REGULATE NITRIC OXIDE AND HYDROGEN PEROXIDE DIFFUSION TO THE CYTOSOL

C2.2.2

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$P66^{Shc}$, REDOX SIGNALING AND AGING

C2.2.3

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GENERATION OF SUPEROXIDE BY THE MITOCHONDRIAL COMPLEX I

C2.2.4

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S-NITROSYLATION OF AMINOPHOSPHOLIPID TRANSLOCASE: A NEW SIGNALING ROLE IN APOPTOSIS AND PHAGOCYTOSIS

C2.2.5

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FORMATION OF REACTIVE OXYGEN SPECIES IN HEART MITOCHONDRIA: QUINONE ANALOGS AS A TOOL FOR FURTHER INVESTIGATION OF SUPEROXIDE FORMATION BY COMPLEX I AND III

Colloquium 2.3. Aging

C2.3.1

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MELATONIN AS ANTIOXIDANT, GEROPROTECTOR AND ANTICARCINOGEN: LIMITATIONS AND PERSPECTIVES OF CLINICAL APPLICABILITY

C2.3.2

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MITOCHONDRIAL FUNCTIONS IN YEAST AGING AND APOPTOSIS

C2.3.3

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MITOCHONDRIAL METABOLISM AND AGING IN THE FILAMENTOUS FUNGUS PODOSPORA ANSERINA

C2.3.4

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MITOCHONDRIAL THEORY OF AGING: DEAD OR ALIVE?

C2.3.5

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MITOCHONDRIAL DNA MUTATIONS CAUSE RESISTANCE TO OPENING OF THE PERMEABILITY TRANSITION PORE

Colloquium 2.4. Cancer, ischemia and degenerative disorders

C2.4.1

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MITOCHONDRIAL ALTERATIONS AND AUTOPHAGY IN MYOCARDIAL ISCHEMIA/REPERFUSION

C2.4.2

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COORDINATION OF NUCLEAR- AND MITOCHONDRIAL-DNA ENCODED PROTEINS IN CANCER AND NORMAL COLON TISSUES

C2.4.3

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MONITORING MITOCHONDRIAL BIOENERGETICS IN MODELS OF NEURONAL DYSFUNCTION

C2.4.4

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MITOCHONDRIAL POTASSIUM CHANNELS: FROM PHARMACOLOGY TO FUNCTION

C2.4.5

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TARGETS FOR CARDIOPROTECTION AND NEUROPROTECTION

Colloquium 2.5. Program death of cells and mitochondria

C2.5.1

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PRODUCTION OF REACTIVE OXYGEN SPECIES IN MITOCHONDRIA OF HELA CELLS UNDER OXIDATIVE STRESS

C2.5.2

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PROAPOPTOTIC ACTIVITY OF CYTOCHROME C MUTANTS IN LIVING CELLS

C2.5.3

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EXPRESSION OF AN EXPANDED POLYGLUTAMINE DOMAIN IN YEAST CAUSES DEATH WITH APOPTOTIC MARKERS

C2.5.4

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MITOCHONDRIAL MEMBRANE PERMEABILIZATION DURING CELL DEATH

Colloquium 2.6. Mitochondrial dynamics

C2.6.1

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THE FUNDAMENTAL ORGANIZATION OF CARDIAC MITOCHONDRIA AS A NETWORK OF COUPLED OSCILLATORS

C2.6.2

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MITOCHONDRIAL SUBPOPULATIONS AND HETEROGENEITY REVEALED BY CONFOCAL IMAGING; POSSIBLE PHYSIOLOGICAL ROLE?

C2.6.3

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EFFECT OF OXIDATIVE STRESS ON DYNAMICS OF MITOCHONDRIAL RETICULUM

C2.6.4

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MITOCHONDRIAL DIVISION PROTEINS IN C. ELEGANS AND MAMMALS

C2.6.5

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MITOFUSIN-2 AS A REGULATOR OF MITOCHONDRIAL METABOLISM

Colloquium 2.7. Therapeutic approach

C2.7.1

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ANTI-HIV DRUGS AND THE MITOCHONDRIA

C2.7.2

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MITOCHONDRIA AS A RELAY STATION FOR CELL SIGNALING

C2.7.3

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TCA CYCLE INTERMEDIATES AS ONCOGENIC AND THERAPEUTIC SIGNALS

C2.7.4

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HEXOKINASE II AT THE CROSSROADS OF ENERGY METABOLISM AND CELL SURVIVAL: MITOCHONDRIAL BINDING OF HEXOKINASE II IS REGULATED BY PHOSPHORYLATION OF VDAC AND PROTECTS TUMOR CELLS AGAINST CHEMOTHERAPEUTIC DRUGS

C2.7.5

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MITOCHONDRIA IN HEART FAILURE: ADAPTATION, FAILURE & THERAPEUTIC TARGETS

POSTER SESSIONS

1. Molecular bioenergetics

1.1. Respiratory chain
1.2. Light-driven energy transducers
1.3. ATP-synthase/ATPase
1.4. Porters and some other subjects
1.5. Mechanisms of energy coupling

2. Physiology and pathology of mitochondria (*in vitro*, *ex vivo* and *in vivo* studies)

2.1. Uncoupling
2.2. ROS and redox regulations
2.3. Aging
2.4. Cancer, ischemia and degenerative disorders
2.5. Program death of cells and mitochondria
2.6. Mitochondrial dynamics
2.7. Therapeutic approach

3. Miscellaneous (Other topics)

Mitchell Medal Lecture

M1. ROTARY CATALYSIS OF ATP SYNTHASE

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Mechanical rotary motion is scarce in living organism; no animal running with wheel, no bird flying with propeller, no fish swimming with screw. However, this does not mean it is impossible. Rotary motion of FoF1-ATP synthase was first proposed by Boyer some 25 years ago. This proposal became taken seriously by crystal structure of F1 by Walker's group. Then, movement of the central γ subunit relative to surrounding $\alpha_3\beta_3$ in F1 was demonstrated by Cross, Capaldi and Junge, and finally unidirectional rotation was visualized with a single molecule observation. Since then, significant progress in our understanding on the rotary catalysis has been made. Some of recent findings are;

1. Reaction sequence. A single β in F1 binds ATP at 0°, cleaves ATP after ~200° rotation, and undergoes the final catalytic event, presumably the product release, after ~320° rotation. This means that three β subunits cooperate to complete a single 120° rotation; each carries out ATP binding (0°), ATP cleavage (80°), and the final catalytic event (80°).
2. Stochastic nature. When the first β is extremely slow in ATP binding, the second β hydrolyzes ATP at 0°. The order of reaction sequence is not absolute but stochastic.
3. Transition of ε. Bacterial ε subunit can adopt a hairpin form and an extended form depending on ATP/ADP balance and proton motive force. The activity of FoF1-ATP synthase is seriously affected by this transition.
4. ε binds ATP. Bacterial ε is an ATP-binding protein which may monitor the cellular ATP concentration.

SYMPOSIA (Plenary Sessions)

1. Molecular bioenergetics

Symposium 1.1. Respiratory chain

S1.1.2. STRUCTURE OF THE HYDROPHILIC DOMAIN OF RESPIRATORY COMPLEX I FROM THERMUS THERMOPHILUS

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Respiratory complex I plays a central role in cellular energy production and its dysfunction is implicated in many human neurodegenerative diseases, as well as in aging. The crystal structure of the hydrophilic domain (peripheral arm) of complex I from *Thermus thermophilus* has been solved at 3.3 Å resolution (*1*, *2*). The subcomplex consists of eight subunits and contains all the redox centers of the enzyme, including nine iron-sulfur clusters. The primary electron acceptor flavin-mononucleotide is within electron transfer distance to cluster N3, leading to the main redox pathway, and to the distal cluster N1a, a possible anti-oxidant. The structure reveals many new aspects of the mechanism and evolution of the enzyme, which will be discussed.

1. Sazanov, L. A., and Hinchliffe, P. (2006), *Submitted*.

2. Hinchliffe, P., and Sazanov, L. A. (2005) Organization of iron-sulfur clusters in respiratory complex I, *Science 309*, 771-774.

S1.1.3. REACTION MECHANISM OF BOVINE HEART CYTOCHROME C OXIDASE

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Cytochrome *c* oxidase is the terminal oxidase of cell respiration which reduces molecular oxygen (O_2) to water coupled with proton-pumping. X-ray structure of the bovine enzyme at 1.9 Å resolution in the fully reduced state shows a trigonal planer coordination of one of the metal sites, $Cu_{B,}$ in the O_2 reduction site and a covalent link between a tyrosine and one of the three histidine imidazoles ligated to Cu_B. The link fixes the tyrosine near the O_2 reduction site. The former structure could decrease the rate of the two-electron reduction of the O_2 molecule bound at Fe_{a3}^{2+}, while the latter could stimulate the reduction of the bound peroxide (O_2^{2-}) at Fe_{a3}^{3+}. These structures facilitate a four electron reduction of O_2, which avoids production of any active oxygen species. A redox-coupled conformational change in an aspartate residue (Asp51) located near the intermembrane surface of the enzyme molecule and a hydrogen bond network connecting Asp51 with the matrix surface strongly suggests proton-pumping at Asp51 in the bovine enzyme. A peptide bond included in the hydrogen bond network would block reverse proton transfer from the intermembrane space to the matrix space. The net positive charge on heme *a* created upon oxidation and delocalized to the formyl and propionate groups of heme *a* drives the proton transfer through the hydrogen-bond network up to Asp51. Mutational analyses using a gene expression system of a bovine enzyme subunit which includes Asp51 indicate the critical role of Asp51 in the proton pump.

Symposium 1.2. Light-driven energy transducers

S1.2.1. STRUCTURE OF PHOTOSYSTEM II AND ITS IMPLICATIONS FOR UNDERSTANDING THE WATER SPLITTING REACTION IT CATALYSES

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Photosystem II (PSII) is a multi-subunit membrane protein complex which catalyses the oxidation of water to molecular oxygen and reducing equivalents. The reaction occurs at a catalytic centre composed of 4 Mn ions and a Ca ion, is thermodynamically demanding and generates highly oxidised species. Unavoidable side reactions cause detrimental effects on the protein environment leading to the rapid turnover of the reaction centre D1 protein. To understand the mechanisms of water splitting and D1 turnover, structural information is required. Initially the positioning of various protein subunits and their transmembrane helices were determined by electron microscopy (EM) (Barber 2003). More recently a refined structure of the cyanobacterial PSII unit has been elucidated by X-ray crystallography (Ferreira et al 2004) giving details of specific environments of the redox active cofactors. The implications of these structural studies will be discussed in relation to the unique facets of PSII function, particularly the water splitting reaction. EM and Xray data are also used to construct a structural model of higher plant PSII.

Barber J (2003) Photosystem II: the engine of life. Quart Revs Biophys 36, 71-89

Ferreira KN, Iverson TM, Maghlaoui K, Barber J and Iwata S (2004) Architecture of the photosynthetic oxygen evolving center. Science 303, 1831-1838

S1.2.2. XANTHORHODOPSIN, A NOVEL RETINAL-BASED PROTON PUMP WITH A CAROTENOID ANTENNA

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Energy transfer from light-harvesting carotenoids to chlorophyll is common in photosynthesis but such antenna pigments have not been observed in retinal-based ion pumps. We found (1) an integral membrane protein in the eubacterium *Salinibacter ruber*, similar in amino acid sequence to the proton pumps bacteriorhodopsin and proteorhodopsin, but which is a retinal protein/carotenoid complex. We named the purified complex xanthorhodopsin. The two chromophores in the protein, retinal and the carotenoid acyl glycoside, salinixanthin, are in a molar ratio of 1:1. The wavelength dependence of the rate of pumping, difference absorption spectra measured under a variety of conditions, and a highly structured CD spectrum of the bound salinixanthin in the visible region, indicate that the two chromophores strongly interact. The interaction is detected both in the retinal and the carotenoid. Light-energy absorbed by the carotenoid is transferred to the retinal with a quantum efficiency of ca. 40%, and the antenna carotenoid extends the wavelength range of the collection of light for uphill transmembrane proton transport. Conversely, bleaching or removal of the retinal appears to release the bound salinixanthin. Further, the spectrum of the carotenoid exhibits specific changes during the retinal-dependent photochemical cycle. The most important current questions include the geometrical and electrostatic relationship of the retinal to salinixanthin in the complex, and whether there is any functional significance of their interactions beyond the energy migration during the excited state. The xanthorhodopsin complex represents the simplest electrogenic pump with an accessory antenna pigment, and might be an early evolutionary development in utilizing energy transfer for energy capture.

1. S. P. Balashov, E. S. Imasheva, V. A. Boichenko, J. Antón, J. M. Wang and J. K. Lanyi. *Science* 309: 2061-2064, 2005.

S1.2.3. ELECTRON TRANSFER IN BACTERIAL REACTION CENTERS WITH MODIFIED B-BRANCH PIGMENT COMPOSITION

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Transient absorption difference spectroscopy with 20 femtosecond resolution was applied to study the time and spectral evolution of low-temperature (90 K) absorbance changes in isolated reaction centers (RCs) of *Chloroflexus (C.) aurantiacus* and the HM182L mutant of *Rhodobacter (Rb.) sphaeroides*. In both types of RCs the composition of the B-branch chromophores is modified with respect to that of native purple bacterial RCs by occupying the B_B binding site of accessory bacteriochlorophyll by a natural (*C. aurantiacus*) or genetically introduced (HM182L mutant) bacteriopheophytin molecule (Φ_B). It was found that the nuclear wave packet motion induced on the potential energy surface of the excited state of the primary electron donor P* by 20 fs excitation leads to a coherent formation of the states $P^+\Phi_B^-$ and $P^+B_A^-$ (B_A is a bacteriochlorophyll monomer in the A-branch of cofactors). The processes were studied by measuring coherent oscillations in kinetics of the absorbance changes at 900 and 940 nm (P* stimulated emission), at 750 and 785 nm (Φ_B absorption bands), and at 1020-1028 nm (B_A^- absorption band). In both types of RCs, the immediate bleaching of the P band at 880 nm and appearance of the stimulated wave packet emission at 900 nm were accompanied (with a small delay of 10-20 fs) by electron transfer from P* to the B-branch with bleaching of the Φ_B absorption band at 785 nm due to Φ_B^- formation. Only at a delay of 120 fs the electron transfer from P* to the A-branch was observed with a development of the B_A^- absorption band at 1020-1028 nm. This development was in phase with the appearance of the P* stimulated emission at 940 nm. The data on the A-branch electron transfer in *C. aurantiacus* and HM182L mutant RCs are consistent with those observed in native RCs of *Rb. sphaeroides*. The mechanism of charge separation in RCs with the modified B-branch pigment composition is discussed in terms of coupling between the nuclear wave packet motion and electron transfer from P* to Φ_B and B_A primary acceptors in the B- and A-branch, respectively.

Symposium 1.3. ATP-synthase/ATPase

S1.3.1. THE RIGHT-HANDED COILED COIL OF THE b DIMER OF ESCHERICHIA COLI ATP SYNTHASE

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The 156-residue *b* subunit of *Escherichia coli* ATP synthase forms an extended dimer that interacts with the membrane-bound *a* and *c* subunits of F_0 through its N-terminal membrane spanning domain and with the α and δ subunits of F_1 through its C-terminal region. The *b* dimer is essential for assembly of ATP synthase and is believed to act as a stator during rotational catalysis. Much of the length of *b* is in the dimerization domain located between residues 53 and 122. A conserved 11-residue (hendecad) repeat pattern, suggestive of a right-handed coiled coil (RHCC), has been identified in this region. The existence of the RHCC is supported by studies of disulfide formation between cysteines introduced at positions between 61 and 90. These studies revealed that positions 61, 68, 72, 79, 83, and 90, predicted to be interior to the RHCC interaction, preferentially formed disulfides with their neighbors, *e.g.* position 83 formed disulfides effectively with positions 79 or 90 in heterodimers, but less effectively with itself in a homodimer. Analysis of thermal stability by melting in the CD showed the heterodimers to be more stable and to melt with higher cooperativity than homodimers. These results support a RHCC structure with the helices offset by about one half of a hendecad. Interruptions to the RHCC pattern, either by deletion of a residue or by substitution with left-handed coiled coils to produce chimeric *b* subunits, resulted in diminished stability *in vitro*. Cells carrying these mutant *b* subunits were unable to grow on acetate or succinate, indicating defective oxidative phosphorylation. Surprisingly, membranes from these strains showed nearly normal levels of ATP synthase assembly. ATP-Dependent proton pumping by the membranes was reduced but not abolished, and the membrane-bound ATPase activity showed reduced sensitivity to DCCD, implying partial uncoupling. These results indicate that the *b* dimer plays a role beyond assembly and simply preventing rotation of the $\alpha_3\beta_3$ hexamer of F_1 during the proton-driven rotation of the $\gamma\varepsilon c_{10}$ rotor. We suggest that the RHCC is critical to the conservation of energy during rotational catalysis. It is notable that as proton movement through F_0 will tighten the left-handed coiled coil formed by the terminal helices of γ in the central rotor, an

RHCC in the stator will be simultaneously tightened, allowing for the elastic storage of energy, whereas a left-handed coiled coil in the stator would be loosened.

S1.3.2. MODULATION OF PROTON PUMPING EFFICIENCY IN BACTERIAL ATP SYNTHASES

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The ATP synthase in chromatophores of *Rhodobacter caspulatus* can effectively generate a transmembrane pH difference coupled to the hydrolysis of ATP. The rate of hydrolysis was rather insensitive to the depletion of ADP in the assay medium by an ATP regenerating system (phosphoenolpyruvate (PEP) and pyruvate kinase (PK)). The steady state values of ΔpH were however drastically reduced as a consequence of ADP depletion. The clamped concentrations of ADP obtained using different PK activities in the assay medium could be calculated and an apparent $K_d \approx 0.5$ μM was estimated. The extent of proton uptake was also strongly dependent on the addition of phosphate (P_i) to the assay medium. The K_d for this effect was about 70 μM. Analogous experiments were performed in membrane fragment from *Escherichia coli*. In this case, however, the hydrolysis rate was strongly inhibited by P_i, added up to 3 mM. Inhibition by P_i was nearly completely suppressed following depletion of ADP. The K_d's for the ADP and Pi were in the micromolar range and submillimoar range respectively and were mutually dependent from the concentration of the other ligand. Contrary to hydrolysis, the pumping of protons was rather insensitive to changes in the concentrations of the two ligands. At intermediate concentrations, proton pumping was actually stimulated, while the hydrolysis was inhibited. It is concluded that, in these two bacterial organisms, ADP and phosphate induce a functional state of the ATP synthase competent for a tightly coupled proton pumping, while the depletion of either one of these two ligands favours an inefficient (slipping) functional state. The switch between these states can probably be related to the structural change in the C-terminal α-helical hairpin of the ε-subunit, from the extended conformation, in which ATP hydrolysis is tightly coupled to proton pumping, to the retracted conformation, in which ATP hydrolysis and proton pumping are loosely coupled.

S1.3.3. THE STRUCTURE AND FUNCTION OF ATP SYNTHASE

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In order to understand how the ATP synthase works, one of our current aims is to build up its structure by determining accurate structures of its separate domains, and then to build up a mosaic of the complete structure of the enzyme from subcomplexes. Another approach that we are pursuing is to crystallize and solve the structure of the intact enzyme complex. The catalytic F_1 domain is the best understood sector. The structures of the ground state of the enzyme and of intermediate states during a rotary cycle have been established by using substrate analogues to trap them, and the modes of action of all of the common covalent and non-covalent inhibitors of ATP hydrolysis, including azide and the regulatory subunit IF_1, are understood. Symmetries of c-rings, and the interaction of the c-ring with the central stalk have been determined. The structure of the peripheral stalk, which is part of the stator mechanism linking the F_1 domain to the membrane subunits a (ATPase-6) and c, and how it interacts with F_1-ATPase are now emerging. This structure raises new issues about the biophysical properties and functions of the peripheral stalk. The most important issue to be resolved is how the proton-motive force generates rotation of the c-ring and the intimately associated central stalk. The structures of c- and K-rings have revealed how sodium ions bind in Na^+-motive F-ATPases and in the related V-ATPases, thereby providing information about how protons are likely to bind to the ring in the proton-motive enzymes. The missing link is the structure of the a subunit (ATPase-6), and crucially of how it interacts with the c-ring. Once these elements are in place, the molecular basis for the generation of rotation and the synthesis of ATP should be clearer.

Symposium 1.4. Porters

S1.4.1. STRUCTURE AND MECHANISM OF MEMBRANE TRANSPORTERS

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Membrane transport proteins that transduce free energy stored in electrochemical ion gradients into a concentration gradient are a major class of membrane proteins. We have reported the crystal structure at 3.5 Å of the *Escherichia coli* lactose permease (LacY), an intensively studied member of the Major Facilitator Superfamily of transporters. The structure with a bound lactose homologue, β-D-galactopyranosyl-1-thio-β-D-galactopyranoside (TDG), reveals the sugar-binding site in the cavity, and residues that play major roles in substrate recognition and proton translocation are identified. Recently, we determined two novel ligand-free X-ray structures of LacY at acidic (3.3 Å) and neutral pH (2.95 Å) in a different crystal forms. Based on these structures and the TDG complex structure, we propose a model for the mechanism of coupling between lactose and H_+ translocation. No sugar-binding site is observed in the absence of ligand, and deprotonation of the key residue Glu_{269} seems associated with ligand binding. Thus, substrate induces formation of the sugar-binding site, as well as the initial step in H_+ transduction. A possible mechanism for lactose/proton symport of LacY will be discussed. We will also update the structure studies of other membrane transpoter proteins.

S1.4.2. STRUCTURE OF NHAA Na^+/H^+ ANTIPORTER: INSIGHTS INTO MECHANISM OF ACTION AND REGULATION BY pH

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Control by Na^+/H^+ antiporters of sodium/proton concentration and cell volume is crucial for the viability of all cells. Adaptation to high salinity and/or extreme pH in plants and bacteria or in human heart muscles requires the action of Na^+/H^+ antiporters. Their activity is tightly controlled by pH. We determined the crystal structure of the pH down-regulated NhaA, the main antiporter of *Escherichia coli* and many enterobacteria [1]. A negatively charged ion funnel opens to the cytoplasm and ends in the middle of the membrane at the putative ion-binding site. There, a unique assembly of two pairs of short helices connected by crossed, extended chains creates a balanced electrostatic environment. We propose that binding of charged substrates causes electric imbalance inducing movements, which allow for a rapid alternating access mechanism. This ion exchange machinery is regulated by a conformational change elicited by a pH signal perceived at the cytoplasmic funnel entry. Biochemical and genetic [2,3] data fully support the structure-based implications. The NhaA structure represents a novel fold that provides two major insights: First, it reveals the structural basis for the Na^+/H^+ exchange and its unique regulation by pH. Second, it is most important for the general understanding of the architecture of membrane proteins.

[1] C. Hunte, M. Screpanti, M. Venturi, A. Rimon, E. Padan and H. Michel, Structure of a Na^+/H^+ antiporter and insights into mechanism of action and regulation by pH, Nature 534 (2005) 1197-1202.

[2] L. Galili, K. Herz, O. Dym and E. Padan, Unraveling functional and structural interactions between transmembrane domains IV and XI of NhaA Na+/H+ antiporter of Escherichia coli, J Biol Chem 279 (2004) 23104-13.

[3] T. Tzubery, A. Rimon and E. Padan, Mutation E252C increases drastically the K_m value for Na^+ and causes an alkaline shift of the pH dependence of NhaA Na^+/H^+ antiporter of *Escherichia coli*, J. Biol. Chem. 279 (2004) 3265-3272.

S1.4.3. METABOLITE TRANSPORTERS OF MITOCHONDRIA

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The inner membrane of mitochondria is equipped with a family of proteins that provide a link between mitochondria and the cytosol by facilitating the flux of a large variety of metabolites in and out of the matrix. Until now, about twenty members of the family have been identified and biochemically characterized, but many more are present in the genomes of yeast, plants and mammals. The identification and characterization of the mitochondrial carriers for pyrimidine nucleotides and for NAD^+ in *Saccharomyces cerevisiae* will be presented. The main physiological role of the pyrimidine nucleotide carrier (encoded by YBR192W, also named RIM2) is to transport (deoxy)pyrimidine nucleoside triphosphates into mitochondria in exchange for intramitochondrially generated (deoxy)pyrimidine nucleoside monophosphates. The main function of the two isoforms of the NAD^+ transporter (encoded by YIL006W and YEL006W, named Ndt1p and Ndt2p, respectively) is to import NAD^+ into mitochondria by unidirectional transport or by exchange with intramitochondrially generated (d)AMP and (d)GMP. Data showing the identification of the mitochondrial carriers for dicarboxylates, S-adenosylmethionine and basic amino acids in *Arabidopsis thaliana* will also be presented. The results of extensive site-directed and cysteine-scanning mutagenesis studies on the activities of recombinant bovine oxoglutarate carrier and recombinant rat carnitine/acylcarnitine, combined with investigations on the accessibility to SH reagents and ability of the substrates to protect against their inhibition, will be provided. The relationships between specific amino acid residues and the active site of the ornithine/citrulline carrier, purified from rat liver mitochondria, have also been investigated by studying the effect of specific protein-modifying reagents on the transport activity of the carrier. This vast array of functional data interpreted in light of homology models of the above-mentioned carriers, based on the available 3-D structure of the ADP/ATP carrier, yields important insight into the location of the substrate translocation pathway within these models.

2. Physiology and pathology of mitochondria (*in vitro, ex vivo* and *in vivo* studies)

Symposium 2.1. Uncoupling

S2.1.1. THE UNCOUPLING PROTEINS 1 AND 3: QUESTIONS OF MECHANISM, CONTROL AND PHYSIOLOGICAL FUNCTION

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When a phylogenic approach is used, there are only three members of the uncoupling protein family: UCP1, UCP2 and UCP3. Other members of the mitochondrial carrier family have sometimes been referred to as uncoupling proteins (UCP4, UCP5) but they are not more closely related to UCP1 than they are to other carriers and should therefore not be included in the uncoupling protein subfamily. The first identified but evolutionarily youngest member is UCP1 which is only found in mammals (although synteny studies indicate that an ancester is found in fish). Within the mammalian organism, UCP1 is only expressed in brown adipose tissue; reports on expression in other tissues await independent confirmation. Although UCP1 is the uncoupling protein that has been most studied from a bioenergetic point of view, there is still no consensus concerning such basic issues as what the protein really transports over the membrane (protons, fatty acids or hydroxyl ions) and how its activity is regulated: is it constitutively active, are fatty acids necessary for activity or only as antagonists of the accepted inhibitors purine nucleotides, and are additional activators necessary (such as superoxide or other ROS species such as hydroxynonenal)? Physiologically, UCP1 is necessary for nonshivering thermogenesis and for the adaptive adrenergic thermogenesis developed during cold acclimation or in response to certain diets, but its contribution to total energy balance (obesity prevention) has not been unequivocally established (for general reviews see (1) and (2)). The tissue distribution of UCP3 is restricted to muscle and brown adipose tissue which allows for analysis of possible functions and effects. There are reports that an artificial overexpression of UCP3 induces an increase in proton leak of muscle mitochondria, but levels of UCP3 increased through endogenous pathways do not manifest themselves as an increased proton

leak. Furthermore, there are suggestions that UCP3 in some way protects against deleterious effects of free fatty acids, but so far evidence for this is mainly indirect (for general review see (3)).

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S2.1.2. THE MITOCHONDRIAL UNCOUPLING PROTEINS: FROM BIOENERGETICS TO PHYSIOPATHOLOGY

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The permeability of the inner membrane of mitochondria is restricted and is controlled by a number of complexes and membranous carriers such as those referred to as the family of the anion carriers including the adenine nucleotide transporters, the phosphate carrier, the citrate carrier, the oxoglutarate carrier …. The brown adipocyte uncoupling protein UCP, renamed UCP1 in 1997 when UCP2 and UCP3 were identified, undoubtedly belongs to this family although its regulated and physiological transport activity is the transport of protons. UCP1 has very specific features since it is uniquely present in brown adipocyte mitochondria where it strongly uncouples respiration from ATP synthesis allowing the dissipation of fatty acid oxidation energy as heat in situations requiring increased thermogenesis : birth, adaptation to cold environment, arousal from hibernation. The identification of mammalian UCP2 and UCP3 as well as stUCP a plant UCP in 1997, changed drastically the field of research on respiration uncoupling and mitochondrial proton leaks. Actually, the identification of UCP homologues was initially made on the basis of their predicted structural similarity to UCP1. Moreover, several initial observations supported a role for these UCP homologues in energy expenditure and substrate metabolism. In particular, it was proposed that UCP3 and UCP2 were involved in fatty acid oxidation and glucose/fatty acid metabolism shift. Nevertheless, seven years after the discovery of these novel UCPs, no consensus has been reached regarding their exact biochemical and physiological activities. At the biochemical level, some authors reported that UCP2 and UCP3 translocate protons directly or through fatty acid cycling, implying that these novel UCPs function similarly to UCP1 whereas others denied such conclusions. It was postulated that these transporters contribute to mild respiration uncoupling, which implies that they can decrease the mitochondrial membrane potential to some extent and reduce reactive oxygen production by the respiratory chain. Interestingly, it was proposed that ROS activate the uncoupling activity of the UCPs. However, such conclusions were questionned by other laboratories. The physiological function of UCP3 remains rather mysterious since almost no phenotype was observed in transgenic mice excepted a role in thermogenesis induced by ecstasy. On the contrary, the physiological roles for UCP2 appear to be pleiotropic since UCP2 was claimed to be linked to innate immunity, insulin secretion, longevity, inflammation, cancer, oncosis and

neurodegenerescence. A clarification of the functions of UCP2 and UCP3 will come from a better understanding of their biochemical activities.

Symposium 2.2. ROS and redox regulations

S2.2.1. MITOCHONDRIAL ROS PRODUCTION AND ITS ATTENUATION BY UNCOUPLING PROTEINS

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Reduction of oxygen by single low-potential electrons in the mitochondrial respiratory chain produces most of the reactive oxygen species (ROS) in typical cells. Understanding the sites and topology of ROS production can illuminate the pathways of electron and proton transport in this part of the chain. Understanding how mitochondria regulate ROS production and how this varies between species can suggest ways to attenuate cellular oxidative damage and combat degenerative diseases and ageing. Different sites in the electron transport chain produce superoxide to different sides of the membrane. Complex I and ETF-Q oxidoreductase produce superoxide exclusively in the matrix, whereas glycerol phosphate dehydrogenase and centre *o* of Complex III produce it about equally in the matrix and intermembrane space. During reverse electron transport, Complex I produces superoxide ten times faster than any other native site. However, the rate from Complex I during forward electron transport is much less. Analysis of this anomaly suggests that the major site of ROS production in Complex I is a semiquinone in the Q-reduction pocket. This site is active during reverse electron transport when pmf (particularly ΔpH) is high, but equally active during forward electron flow only when pmf is high in the presence of particular Q-site inhibitors, such as piericidin and myxothiazol, that appear to stall electrons on a reactive semiquinone, perhaps SQN_f. Other much less active Complex I sites (e.g. FMN) only predominate under suboptimal conditions. Mitochondrial ROS production is very sensitive to pmf, so mild uncoupling, which reduces pmf slightly, attenuates ROS production at the expense of slight inefficiency. Uncoupling proteins (UCPs) appear to be specialised to do this. Analysis of the activation of the proton conductance of UCPs by ROS and analogues leads to a model in which endogenous superoxide generates carbon-centred radicals on fatty acyl chains of membrane phospholipids. These initiate a cascade of lipid peroxidation reactions that form reactive alkenals, particularly 4-hydroxynonenal, the postulated proximal activator of UCP proton conductance. This simple feedback loop attenuates mitochondrial ROS production by mild uncoupling when it becomes dangerous. Uncoupling by UCP1 may exploit

the inefficiency for adaptive thermogenesis; regulation of pancreatic insulin secretion by UCP2 may exploit it for signalling.

S2.2.2. MITOCHONDRIAL ROS-INDUCED ROS-RELEASE: AN UPDATE AND REVIEW

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Unstable mitochondrial redox transitions can occur following insults including ischemia/reperfusion injury and toxin exposure, with negative consequences for mitochondrial integrity and cellular survival [1-4]. These redox transitions can involve mechanisms such as the recently described process, "Reactive Oxygen Species (ROS)-induced ROS-release" (RIRR) [2], and be generated by circuits where the mitochondrial permeability transition (MPT) [5] pore and the inner membrane anion channel (IMAC) are involved [6]. The exposure to excessive oxidative stress results in an increase in ROS reaching a threshold level that triggers the opening of one of the requisite mitochondrial channels. In turn, this leads to the simultaneous collapse of the mitochondrial membrane potential and a transient increased ROS generation by the electron transfer chain. Generated ROS can be released into cytosol and trigger RIRR in neighboring mitochondria. This mitochondrion-to-mitochondrion ROS-signaling constitutes a positive feedback mechanism for enhanced ROS production leading to potentially significant mitochondrial and cellular injury. This review and update considers a variety of RIRR mechanisms (involving MPT, IMAC and episodes of mitochondrial transient hyperpolarization). RIRR could be a general cell biology phenomenon relevant to the processes of programmed mitochondrial destruction and cell death, and may contribute to other mechanisms of post-ischemic pathologies, including arrhythmias [1,3].

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Symposium 2.3. Aging

S2.3.1. MITOCHONDRIAL OXIDATIVE STRESS, AGING AND CALORIC RESTRICTION: THE PROTEIN AND METHIONINE CONNECTION

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Caloric restriction (CR) decreases aging rate and mitochondrial ROS (MitROS) production and oxidative stress in rat postmitotic tissues. Low levels of these parameters are also typical traits of long-lived mammals and birds. However, it is not known what dietary components are responsible for these changes during CR. It was recently observed that 40% protein restriction without strong CR also decreases MitROS generation and oxidative stress. This is interesting because protein restriction also increases maximum longevity (although to a lower extent than CR) and is a much more practicable intervention for humans than CR. Moreover, it was recently found that 80% methionine restriction substituting it for L-glutamate in the diet also decreases MitROS generation in rat liver. Thus, methionine restriction seems to be responsible for the decrease in ROS production observed in caloric restriction. This is interesting because it is known that exactly that procedure of methionine restriction also increases maximum longevity. Moreover, recent data show that methionine levels in tissue proteins negatively correlate with maximum longevity in mammals and birds. All this suggests that lowering of methionine levels is involved in the control of mitochondrial oxidative stress and vertebrate longevity by at least two different mechanisms: decreasing the sensitivity of proteins to oxidative damage, and lowering of the rate of ROS generation at mitochondria.

S2.3.2. SUPEROXIDE AGING AND DEATH IN S. CEREVISIAE

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Aging is believed to be a non-adaptive process that escapes the force of natural selection. We have shown that yeast undergo an age- , ethanol- and pH-dependent death with features of mammalian programmed cell death (apoptosis). This death is mediated in part by the down-regulation of many protective systems and the generation of mitochondrial superoxide, is associated with the inactivation of the 4Fe-4S cluster enzyme aconitase and is delayed by the overexpression of either SOD1 and SOD2 or human Bcl-2. After 90-99% of the population loses the ability to reproduce and dies, a small sub-population that has acquired mutations that promote early regrowth utilizes the nutrients released from dead cells to grow. This adaptive regrowth occurs in less than 10% of populations of long-lived mutants resistant to superoxide and hydrogen peroxide, in 50% of wild type cultures, but in nearly 90% of cultures of short-lived mutants lacking superoxide dismutase (*sod1*Δ). Thus whereas mitochondrial superoxide contributes to programmed death, cytosolic superoxide causes an increase in the frequency of nuclear mutations and an early release of nutrients which plays an important role in adaptive regrowth. In mixed long-term cultures, wild type yeast out-compete yeast overexpressing *SOD1* and catalase but can be out-competed by *sod1*Δ mutants. Computational simulations confirm that programmed aging and the consequent early death together with a relatively high mutation frequency can result in a major advantage in adaptation to changing environments. These results suggest that superoxide is a mediator of an altruistic aging and death program that improves adaptation to changing environments. The role of similar pathways in the regulation of longevity in organisms ranging from yeast to mice raises the possibility that higher eukaryotes may also undergo programmed aging mediated in part by superoxide.

S2.3.3. MITOCHONDRIAL DNA MUTATIONS, OXIDATIVE STRESS AND APOPTOSIS IN MAMMALIAN AGING

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Mutations in mitochondrial DNA (mtDNA) accumulate in tissues of mammalian species and have been hypothesized to contribute to aging. We show that mice expressing a proofreading-deficient version of the mitochondrial DNA polymerase g (POLG) accumulate mtDNA mutations and display features of accelerated aging. Accumulation of mtDNA mutations was not associated with increased markers of oxidative stress or a defect in cellular proliferation, but was correlated with the induction of apoptotic markers, particularly in tissues characterized by rapid cellular turnover. The levels of apoptotic markers were also found to increase during aging in normal mice. Thus, accumulation of mtDNA mutations that promote apoptosis may be a central mechanism driving mammalian aging.

Symposium 2.4. Cancer, ischemia and degenerative disorders

S2.4.1. THE MITOCHONDRIAL PERMEABILITY TRANSITION - FROM MOLECULAR MECHANISMS TO CARDIOPROTECTION

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Under conditions of grossly elevated matrix [Ca^{2+}], especially when accompanied by oxidative stress and adenine nucleotide depletion, a non-specific pore opens in the inner mitochondrial membrane. Opening of this Mitochondrial Permeability Transition Pore (MPTP) causes mitochondria to become uncoupled and to hydrolyse rather than synthesise ATP. Unrestrained, this leads to the loss of ionic homeostasis and ultimately necrotic cell death. Less severe, or transient opening of the MPTP will not cause catastrophic ATP loss but may be sufficient to cause release of pro-apoptotic factors such as cytochrome c and lead to apoptotic cell death. Opening of the MPTP probably involves a calcium-mediated conformational change of the adenine nucleotide translocase (ANT), facilitated by bound cyclophilin-D (CyP-D). The evidence for this model will be briefly reviewed and re-assessed in the light of recent studies with ANT and CyP-D knockout mice. Mitochondria from the latter lack a CsA-sensitive MPTP, and these mice show no obvious phenotype and no inhibition of apoptosis induced by a variety of stimuli. However, cells from these animals are resistant to necrotic cell death induced by oxidative stress and calcium overload, classic activators of the MPTP. This implies that opening of the MPTP is critical for necrotic cell death but not apoptosis. Cyp-D is the target of two potent inhibitors of the MPTP, cyclosporin A (CsA) and sanglifehrin A (SfA). The heart and brain can be protected from the necrotic damage that occurs following ischaemia and reperfusion by inhibiting the MPTP directly with CsA and SfA. Furthermore, hearts and brains from the CyP-D knockout mice are very resistant to ischaemia / reperfusion induced injury. Other protocols for protecting the heart from reperfusion injury, including ischaemic preconditioning and antioxidants also, lead to inhibition of MPTP opening, but through indirect mechanisms.

S2.4.2. CAN A SINGLE SUBUNIT NADH DEHYDROGENASE WORK AS A THERAPEUTIC AGENT FOR COMPLEX I-DEFICIENT DISEASES?

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Defects of complex I are involved in many human mitochondrial diseases, and therefore we have proposed to use the *NDI1* gene encoding a single subunit NADH dehydrogenase of *Saccharomyces cerevisiae* for repair of respiratory activity. The yeast *NDI1* gene was successfully introduced into mammalian cell lines. The expressed Ndi1 protein was correctly targeted to the matrix side of the inner mitochondrial membranes, was fully functional and restored the NADH oxidase activity to the complex I-deficient cells. The *NDI1*-transduced cells were more resistant to complex I inhibitors and diminished production of reactive oxygen species induced by rotenone. It was further shown that the Ndi1 protein can be functionally expressed in tissues such as skeletal muscles and the brain of rodents, which scarcely induced an inflammatory response. The use of *NDI1* as a potential molecular therapy for complex I-deficient diseases is briefly discussed, including the proposed animal model.

Symposium 2.5. Program death of cells and mitochondria

S2.5.1. MITOCHONDRIAL PERMEABILIZATION IN CELL DEATH AND MITOPHAGY

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In the mitochondrial permeability transition (MPT), the mitochondrial inner membrane becomes non-selectively permeable to molecules of mass up to 1500 Da, which leads to mitochondrial depolarization, uncoupling of oxidative phosphorylation and large amplitude mitochondrial swelling. The MPT causes necrosis from ATP depletion and apoptosis from cytochrome *c* release after swelling. The MPT is implicated in cell death after oxidative stress, Ca^{2+} toxicity, ischemia/reperfusion, cytokine exposure, ethanol and a variety of other stresses to hepatocytes and other cell types. In contrast to necrotic cell death which is a consequence of ATP depletion, ATP is required for the development of apoptosis after the MPT (1-3). In the most widely discussed model, MPT pores form from the adenine nucleotide translocator (ANT) in the inner membrane, the voltage-dependent anion channel (VDAC) in the outer membrane, cyclophilin D (CypD) from the matrix, and possibly other proteins. The validity of this model has been challenged by findings that the MPT still occurs in mouse mitochondria that are deficient in ANT (4). We propose an alternate model of the MPT in which pores form by aggregation of misfolded integral membrane proteins damaged by oxidant and other stresses whose conductance is regulated by chaperone-like proteins (5). Binding of CypD and other chaperones, such as Hsp25/27 (6), to the pore complex blocks conductance and may assist in protein refolding or removal by proteolysis. CypD in this model confers sensitivity to Ca^{2+} such that increased Ca^{2+} opens these regulated MPT pores, an action inhibited by cyclosporin A (CsA). When the nascent pores formed by misfolded protein clusters exceed the number of chaperones available to regulate conductance, unregulated pore opening occurs. Such unregulated pores no longer require Ca^{2+} for opening and are insensitive to CsA inhibition. Recent findings show that heart and liver mitochondria from CypD knockout

mice are desensitized to onset of the Ca^{2+}-induced MPT, consistent with an important regulatory role of CypD in MPT pores (7-9). Various factors regulate the conductance of MPT pores, including divalent cations, matrix pH, membrane potential, and reactive oxygen species (ROS). The ubiquinone analogs, ubiquinone 0 and decylubiquinone, inhibit Ca^{2+}-dependent onset of the MPT, which suggests that one or more mitochondrial respiratory complexes may be part of the MPT pore (10). Numerous studies show that Complexes I and III are also sites for ROS formation by mitochondria, and ROS induce the MPT. Protein phosphorylation is another pathway that can regulate the MPT. Incubation of mitochondria with recombinant protein kinase G (PKG) or hepatic cytosol in the presence of cGMP and ATP inhibits Ca^{2+}-dependent mitochondrial swelling, which is blocked by protein kinase G inhibition (11). These findings implicate that protein phosphorylation by PKG may directly or indirectly regulate the MPT. Proteomic analysis shows that the isoelectric point (pI) of the Rieske iron sulfur protein (RISP) of Complex III increases after MPT induction but not after protonophoric uncoupling (12). CsA prevents this pI shift. Phosphatase treatment causes the same pI shift, suggesting that RISP is dephosphorylated in association with MPT induction. These findings suggest that RISP has functions other than as a core polypeptide of Complex III and may form part of the MPT pore complex and that phosphorylation and dephosphorylation of RISP contributes to regulation of the MPT. In autophagy, portions of cytoplasm are sequestered into autophagosomes and delivered to lysosomes for degradation. Long assumed to be a random process, increasing evidence suggests that autophagy of mitochondria, peroxisomes, and possibly other organelles is selective. For mitochondria, the MPT appears to signal autophagic sequestration and removal of the damaged organelle (13). For selective autophagy of mitochondria, the term “mitophagy” is appropriate in recognition of the non-random nature of the process (14;15). Mitophagy protects against the adverse effects of free radical generation and futile ATP hydrolysis by damaged mitochondria and may also retard accumulation of somatic mutations of mtDNA with aging.

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S2.5.2. APOPTOSIS AND NECROSIS, TWO FUNDAMENTAL ALTERNATIVES

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Apoptosis is the principal cellular process by which cells are eliminated during development and homeostasis in metazoan organisms. Apoptosis is characterized by distinct morphological events, including cell shrinkage, blebbing of the plasma membrane, maintenance of organelle integrity, and chromatin condensation. Early apoptotic cells retain plasma membrane integrity, and are rapidly phagocytosed by macrophages or surrounding cells. This prevents leakage of cellular contents into the surrounding tissue, making apoptosis a 'clean' packaging process that minimizes the risk of inflammation during cell death. Apoptosis is characterized by the activation of cysteine aspartate specific proteases or caspases that cleave crucial substrates associated with the apoptotic process. Necrosis is characterized by swelling of the cell and its organelles leading to loss of plasma membrane integrity. The release of the cytoplasmic contents and accumulation of necrotic cell corpses may damage the surrounding tissue and induce an inflammatory reaction. Necrosis is often considered as a passive cell death process resulting from physico-chemical insults, but it is becoming increasingly clear that necrosis can be elicited by biological stimuli and is governed by defined signal transduction pathways. Necrotic cell death is essentially caspase-independent and implies other signaling events such as RIP1 kinase activity, mitochondrial ROS production, and lysosomal cathepsins in the late phase of necrotic cell death. In this presentation we will elaborate on the signal transduction pathways leading to both types of cell death, the differential role of mitochondria, the crosstalk between both signal transduction pathways, the phagocytosis by macrophages and intercellular communication associated with both types of cell death. Moreover we discuss the possible differential impact of these two cell death pathways on pathophysiology.

S2.5.3. NUCLEI-MITOCHONDRIA CROSS-TALK IN APOPTOSIS

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Initial apoptosis research characterized this form of cell death based on distinct nuclear morphology that was subsequently shown to be associated with the appearance of oligonucleosomal DNA fragments. More recent evidence has indicated that apoptosis depends upon a tightly regulated cellular program for its successful initiation and execution. Molecular participants in this program are present in different subcellular compartments, including the plasma membrane, cytosol, mitochondria and nucleus. The interplay among these compartments and the exchange of specific signaling molecules are critical for the systematic progression of apoptosis. Among all known caspases pro-caspase-2 is only one that is present constitutively in the nucleus. Recent findings indicate that caspase-2 is activated early in response to DNA-damaging agents and is important for the engagement of the mitochondrial apoptotic pathway. This activation was observed only in p53-positive cells. Although no direct interaction between p53 and caspse-2 was found, a functional connection between these two proteins is essential for initiation of DNA damage-induced apoptosis. In cytosol caspase-2 is present in the PIDDosome complex together with PIDD and RAIDD. Both PIDD and RAIDD are required for assembly of this complex, but not sole mediators of caspase-2 activation. Fully processed caspase-2 can permeabilize the outer mitochondrial membrane and cause cytochrome c and Smac release from these organelles. Caspase-2 also might disrupt the interaction of cytochrome c with cardiolipin and thereby enhance the release of the hemoprotein caused by treatment of mitochondria with digitonin or Bax. These results suggest the presence of direct cross-talk between nuclei and mitochondria in apoptosis signaling. We also found that the deficiency in the apoptosis pathway triggered by DNA-damaging agents in non-small lung cancer cells (NSCLC) occurred downstream from (or at the level of) mitochondrial changes and upstream of nuclear apoptotic events. Despite this deficiency, NSCLC undergo cell death in response to staurosporine, which induces caspase activation and mitochondrial dysfunction associated with AIF release. We found that AIF plays a major role in the death of NSCLC via transmission of signal from mitochondria to the nucleus, while caspase-dependent arm of the lethal signal transduction appears to be heavily compromised. Thus, proper dialogue between nuclei and mitochondria is important for both signaling and execution of apoptosis.

Symposium 2.6. Mitochondrial dynamics

S2.6.1. KEEPING MITOCHONDRIA IN SHAPE: A MATTER OF LIFE AND DEATH

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Mitochondria are crucial organelles for life and death of the cell, as they provide most of the ATP needed for endoergonic processes and integrate complex pathways including Ca^{2+} signaling and apoptosis. Their functional versatility is matched by a complex structural organization, both at the cellular and at the ultrastructural level. Moreover, they are remarkably dynamic, continuously fusing and dividing. We are starting to identify the molecular mechanisms regulating the control of mitochondrial morphology as a growing number of "mitochondria-shaping" proteins are being identified in mammals. They include dynamin-related proteins, large GTPases that exert mechanical forces on membranes, like mitofusin-1, -2, DRP-1 and OPA1. Their importance in physiology is confirmed by neurodegenerative genetic diseases associated with mutations in some of these proteins. Several questions remain open: how do mitochondria-shaping proteins function? Do they work in concert, or does each of them have a specific task? How do cellular and mitochondrial signals regulate this machinery? And last but not least, do they (and therefore changes in mitochondrial shape) control mitochondrial and cellular physiology. To address these questions, we integrate genetics, physiology and advanced imaging. Our studies unraveled that these processes are tightly regulated and influence cell life and death. OPA1 has a dual function: it promotes mitochondrial fusion via the outer membrane partner mitofusin-1. Following cleavage and activation by the inner mitochondrial membrane rhomboid protease PARL it regulates apoptosis, by controlling the opening of the tubular cristae junction and therefore the release of cytochrome c from mitochondria. Our studies for the first time revealed a functional difference between the two highly similar mitofusins. While mitofusin-1 is primarily engaged with OPA1 in the control of mitochondrial fusion, mitofusin-2 regulates the shape of another organelle, the endoplasmic reticulum, and the juxtaposition between this and mitochondria. This complex machinery is not left uncontrolled by the cell. For example, we discovered that the cytosolic phosphatase calcineurin regulates mitochondrial fission. Blocking calcineurin inhibits dephosphorylation of DRP-1 and movement of this cytosolic protein to mitochondria, where it binds to its adapter hFis1. This small

molecule of the outer mitochondrial membrane ultimately regulates fragmentation and function of the organelle. A genetic analysis revealed that via a short domain protruding in the intermembrane space it transmits a dysfunction signal to mitochondria, rendering them more vulnerable to opening of the permeability transition pore. In conclusion, mitochondrial morphology is tightly regulated at multiple sites and impacts on mitochondrial and cellular function.

S2.6.2. DYNAMINS, ENDOPHILINS AND BCL-2 FAMILY MEMBERS: INVOLVEMENT IN MITOCHONDRIAL OUTER MEMBRANE DYNAMICS

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Dynamin and endophilin 1 are involved in synaptic vesicle recycling. We have identified an endophilin isoform that, along with the dynamin related protein, Drp1, is specifically involved in mitochondrial morphogenesis. Mitochondria form highly dynamic networks through continuous division and fusion that requires Drp1 for the fission step. Using RNA interference (RNAi) to deplete endophilin B1 followed by immunofluorescence microscopy reveals alterations of the mitochondrial network that are consistent with an inhibition of mitochondrial fission. A striking dissociation of the outer mitochondrial membrane compartment from that of the matrix, and formation of thin, elongated tubules of outer mitochondrial membrane is also observed in endophilin B1 knock down cells indicating that this protein participates in the normal morphogenesis of the outer mitochondrial membrane. Moreover, we find that double knock down of endophilin B1 and Drp1 leads to a mitochondrial phenotype identical to that of the Drp1 single knock down, a result consistent with Drp1 acting upstream of endophilin B1 in the same pathway. Interestingly, endophilin B1 also binds to the pro-apoptosis member of the Bcl-2 family, Bax, during programmed cell death and translocates with Bax to mitochondria. Thus, in addition to the role of certain endophilin family members in plasma membrane endocytosis, other members of the endophilin family participate in intracellular membrane dynamics and play a role in programmed cell death.

Symposium 2.7. Therapeutic approach

S2.7.1. PROPERTIES OF THE PERMEABILITY TRANSITION IN VDAC1$^{-/-}$ MITOCHONDRIA

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Opening of the permeability transition pore (PTP), a high-conductance mitochondrial channel, causes mitochondrial dysfunction with Ca^{2+} deregulation, ATP depletion, release of pyridine nucleotides and of mitochondrial apoptogenic proteins. Despite major efforts, the molecular nature of the PTP remains elusive. A compound library screening led to the identification of a novel high affinity PTP inhibitor (Ro 68-3400), which labeled a ~32 kDa protein that was identified as isoform 1 of the voltage-dependent anion channel (VDAC1) [A.M. Cesura, E. Pinard, R. Schubenel, V. Goetschy, A. Friedlein, H. Langen, P. Polcic, M.A. Forte, P. Bernardi, J.A. Kemp, The voltage-dependent anion channel is the target for a new class of inhibitors of the mitochondrial permeability transition pore, J. Biol. Chem. 278 (2003) 49812-49818]. In order to assess the role of VDAC1 in PTP formation and activity, we have studied the properties of mitochondria from *VDAC1*$^{-/-}$ mice. The basic properties of the PTP in *VDAC1*$^{-/-}$ mitochondria were indistinguishable from those of strain-matched mitochondria from wild-type CD1 mice, including inhibition by Ro 68-3400, which labeled identical proteins of 32 kDa in both wild-type and *VDAC1*$^{-/-}$ mitochondria. The labeled protein could be separated from all VDAC isoforms. While these results do not allow to exclude that VDAC is part of the PTP, they suggest that VDAC is not the target for PTP inhibition by Ro 68-3400.

S2.7.2. MITOCHONDRIAL NADPH, TRANSHYDROGENASE AND DISEASE

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Ever since its discovery in 1953 by N. O. Kaplan and coworkers, the physiological role of the proton-translocating transhydrogenase has generally been assumed to be that of generating mitochondrial NADPH. Mitochondrial NADPH can be used in a number of important reactions/processes, e.g. biosynthesis, maintenance of GSH, apoptosis, aging etc. This assumed role has found some support in bacteria but not in higher eukaryotes, a situation which changed dramatically with two recent but separate findings, both using transhydrogenase knockouts, i.e., in the nematode *C. elegans* and in the mouse, strain C57BL/6J. In the former system the herbicide Paraquat (methylviologen), a known redox cycler, was used to induce oxidative stress. The *C. elegans* knockout showed a pronounced decrease in the overall GSH/GSSG ratio, as well as in the capacity of Paraquat-exposed eggs to survive and develop to adult nematodes. Appently, these nematodes suffer from extensisve oxidative stress. These effects could essentially be mimicked by siRNA treatment, and prevented by re-introducing the *Nnt* gene. In C57BL/6J mice, the knockout is due to a spontaneous deletion mutation in the *Nnt* gene, which was serendipitously found during investigations of the diabetic properties of these mice (Freeman, H. et al (2006) Cell Metab 3, 35-45). In terms of insulin release, these mice show a diminished response to a glucose load, which has been interpreted as being due to a decreased ATP level, which, in turn, attenuates the ATP-regulated K^+-channel, leading to a lower extent of depolarization and Ca^{2+} influx. This lower ATP level is suggested to be due to a partial uncoupling through the uncoupler proteins UCP2/UCP3 (Brand, H. and Esteves, T. C. (2005) Cell Metab. 2, 85-93). The latter channels have been proposed to be opened by products of oxidative stress, generated by leakage of electrons from the respiratory chain, especially at a high redox level of the respiratory chain components. However, at a lower redox level of these components, the leakage of electrons and the level of oxidative stress is lowered. It has been proposed that the role of UCP2/UCP3 is to regulate the level of oxidative stress through a controlled uncoupling. In this context the role of transhydrogenase is believed to be crucial since the lack of transhydrogenase generally increases the level of oxidative stress through a GSH/GSSG ratio, indirectly leading to an attenuation of the release of insulin through the above mechanism. The implications of these findings for the overall role of transhydrogenase in cell metabolism and disease is discussed (supported by the Swedish Research Council).

COLLOQUIA

1. Molecular bioenergetics

Colloquium 1.1. Respiratory chain

C1.1.1. STRUCTURAL AND FUNCTIONAL INSIGHTS INTO MITOCHONDRIAL COMPLEX I

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The molecular mechanism of complex I (NADH:ubiquinone oxidoreductase) is still elusive. Mutational analysis, spectroscopic studies and emerging structural information provide important clues on the parts of the machinery that links electron transfer to proton translocation. Based on its more positive and pH-dependent midpoint potential, iron-sulfur cluster N2 has long been discussed as one of the key components of the pump. However, removal of the redox-Bohr group did not affect the proton pumping stoichiometry. Structural studies with complex I from the strictly aerobic yeast *Yarrowia lipolytica* suggest an unexpected position of the ubiquinone reduction site near the interface between the PSST and the 49 kDa subunits far up in the peripheral arm of complex I.

We propose a two-state mechanism of energy conservation for complex I that is based on long range conformational changes of the enzyme and that is driven by stabilization changes of ubiquinone intermediates. This stabilization change mechanism inherently provides a rationale for the forward and reverse mode of complex I.

C1.1.2. THE MECHANISM OF PROTON PUMPING BY CYTOCHROME C OXIDASE

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Cellular respiration involves electron transfer to oxygen through a series of membrane-bound protein complexes. The process maintains a transmembrane proton electrochemical gradient that is used e.g. for the synthesis of ATP. In mitochondria and many bacteria the last enzyme complex in the electron transfer chain is cytochrome *c* oxidase, which catalyses the four-electron reduction of O_2 to H_2O using electrons delivered by a water-soluble donor, cytochrome *c*. The electron transfer through cytochrome *c* oxidase, accompanied by proton uptake to form H_2O drives pumping of four protons per reduced O_2 across the membrane. To address the molecular mechanism of proton pumping we investigate the specific steps of the catalytic cycle in wild-type and site-directed mutant forms of cytochrome *c* oxidase using a number of biophysical techniques. I will discuss the following topics: (*i*) the order and timing of proton-transfer reactions during the proton-pumping cycle in membrane-reconstituted enzyme, (*ii*) the coupling between electron and proton-transfer reactions, and (*iii*) results from studies of mutant forms of the enzyme in which proton pumping is uncoupled from the oxygen-reduction reaction. Collectively, the results from these studies suggest principles by which the redox-driven proton pump operates and indicate possible molecular mechanisms by which cytochrome *c* oxidase couples electron transfer to proton translocation.

C1.1.3. RAPID KINETICS OF CHARGE TRANSLOCATION BY CYTOCHROME C OXIDASE: TRANSITION BETWEEN THE FERYL-OXO AND FERRIC STATES

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Single-electron photoreduction of the Ferryl-Oxo complex of bovine cytochrome c oxidase by tris-bipyridyl complex of Ru(II) (transfer of the 4-th electron in the catalytic cycle) results in three phases of membrane potential generation with τ values of ca. 40 μs, 1.2 ms and 4.5 ms [1]. Here, the electrogenic phases have been compared with the kinetics of hemes a and a_3 oxidoreduction. In addition, pH and temperature dependences of the resolved electrogenic phases have been studied. The rate of the rapid phase (40-50 μs) does not depend on pH and reveals a very weak temperature dependence (ΔG_a = 3.8 kcal/mol). The rate of the intermediate electrogenic phase (1.2 ms) is pH-independent but shows significant temperature dependence (ΔG_a = 19 kcal/mol) and is assigned provisionally to transfer of the pumped proton from Glu242 in the D-channel to the so-called Proton Loading Site "above the heme" (e.g., carboxylate of the Δ-propionate in heme a_3, or His291 ligand of Cu_B [2]). This phase precedes slightly the major phase of electron transfer from heme *a* to heme a_3. The major part of electrogenic proton transfer (slow electrogenic phase) takes place *after* completion of the redox reaction of the hemes. The phase is highly temperature-dependent (ΔG_a = 17 kcal/mol) and decelerates at alkaline pH with apparent pK of 8.2. We assign this process mainly to electrogenic reprotonation of Glu242 from the N-phase via the D-channel *plus* uptake of a "chemical" proton from the N-phase to the binuclear center. The results do not support models of COX which postulate transmembrane proton translocation in the F→O transition to be sinchronous with ferryl-to-ferric redox transition of heme a_3. We suggest that redox transition of heme a_3 can be assisted by *non-electrogenic* proton donation from the nearest surroundings and is followed by relaxation of the protein coupled to electrogenic uptake and release of protons.

[1]. Zaslavsky,D., Kaulen,A., Smirnova,I.A., Vygodina,T.V. and Konstantinov,A.A. (1993) FEBS Lett. 336,389-393.

[2]. Popovic, D.M, Stuchebrukhov, A.A. (2004) FEBS Lett. 566, 126-30.

C1.1.4. NEW INSIGHTS ON NON-CANONICAL HAEM-COPPER OXYGEN REDUCTASES

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Haem-copper oxygen reductases present a large diversity in terms of amino acid sequences, properties of the haem-copper catalytic site, redox behaviour and proton channels. The study of the more divergent oxidases, i.e., of those far related to the mitochondrial like ones, bring new insights on the minimal essential features of these enzymes. New results on the *aa3* type oxygen reductase from *Acidianus ambivalens*, and enzyme of the B-family, and of the *caa3* oxygen reductase from *Rhodothermus marinus*, and enzyme of the A2-subfamily, will be presented, based on resonance Raman, FT-IR and EXAFS spectroscopies. In particular, it will be shown that, in contrast to the A-type, mitochondrial like oxidases, in the *A. ambivalens* enzyme haem *a3* has a reduction potential higher than that of haem *a*. Also, for the *R. marinus* enzyme, it will be shown that tyrosine residues, besides that linked to the histidine copper ligand, are involve din redox linked proton equilibria.

T. Bandeiras, M. M. Pereira, M.Teixeira, P. Moenne-Loccoz, N. J. Blackburn (2005) *J. Biol. Inorg. Chem.*, 10, 625-35.

S. Todorovic, T. M. Bandeiras, M. M. Pereira, M. Teixeira, P. Hildebrandt and D.H. Murgida (2005) *J. Am. Chem. Soc., 127, 13561-6.*

C1.1.5. PROTON TRANSLOCATION BY CYTOCHROME C OXIDASE

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Proton translocation in the catalytic cycle of cytochrome *c* oxidase proceeds sequentially in a four-stroke manner. Every electron donated by cytochrome *c* drives the activated enzyme from one of the four stable intermediates to another, and each of these transitions is coupled to proton translocation across the membrane, and to uptake of one more proton for charge compensation of the electron in the catalytic site. Using cytochrome *c* oxidase from *Paracoccus denitrificans* we have resolved the kinetics of the electron transfer reactions separately in three of the four reaction steps, and electric potential generation at each step. All four proton translocation cycles during the single catalytic cycle have the same mechanism and are characterized by a cascade of electron transfer equilibria with trapping of the final state by proton transfer to the binuclear site. The extent of electric potential generation during the first wave of electron equilibration between Cu_A and haem *a* shows that this reaction is not coupled to any vectorial proton transfer, whereas oxidation of haem *a* is kinetically linked to the main proton translocation events during functioning of the proton pump. The rate of haem *a* oxidation is different at the different steps of the catalytic cycle, and the same difference can be seen in the rate of electric potential generation. Such correlation of heme a oxidation with the charge translocation discussed in the frame of model, where electron transfer from haem *a* to the O_2 reduction site initiates the proton pump mechanism by being kinetically linked to an internal vectorial proton transfer. This reaction drives the proton pump, and occurs prior to relaxation steps where protons are taken up from the aqueous space on one side of the membrane and released on the other.

Colloquium 1.2. Light-driven energy transducers

C1.2.1. CONSEQUENCES OF THE STRUCTURE OF THE CYTOCHROME b_6f COMPLEX FOR ITS CHARGE TRANSFER PATHWAYS

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At least two features of the crystal structures of the cytochrome b_6f complex from the thermophilic cyanobacterium, *M. laminosus* (1) and green alga, *C. reinhardtii* (2) have implications for the pathways and mechanism of charge (electron/proton) transfer in the complex: (i) The narrow 11 x 12 Å portal between the *p*-side of the quinone exchange cavity and *p*-side plastoquinone/quinol binding niche, through which all Q/QH_2 must pass, is smaller in the b_6f than in the bc_1 complex because of its partial occlusion by the phytyl chain of the one bound chlorophyll a molecule in the b_6f complex. Thus, the pathway for trans-membrane passage of the lipophilic quinone is even more labyrinthine in the b_6f than in the bc_1 complex (3). (ii) A unique covalently bound heme, heme c_n, in close proximity to the *n*-side *b* heme, is present in the b_6f complex. A position of the novel heme in a pathway of photosytem I-linked cyclic electron transport that requires ferredoxin and the ferredoxin: $NADP^+$ reductase is described. This pathway through the *n*-side of the b_6f complex could overlap and interact with the *n*-side of the Q cycle pathway. Thus, regulation is either required at the level of the redox state of the hemes that allows them to be shared by the two pathways, or the two different pathways are segregated in the membrane. (1) Science, 302, 1009-, 2003; (2) Nature, 426, 413-, 2003; (3) Ann. Rev. Biochem. Mol. Biol., 75, in press.

C1.2.2. THE IDENTIFICATION OF F_X IN THE HELIOBACTERIAL REACTION CENTER AS A [4Fe-4S] CLUSTER WITH A GROUND SPIN STATE OF S = 3/2

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Type I homodimeric reaction centers, particularly the class present in Heliobacteria, are not well understood. Even though the primary amino acid sequence of PshA in *Heliobacterium mobilis* has been shown to contain an F_X binding site, the Fe/S cluster has not been detected by EPR spectroscopy. Recently, we reported that PshB, which contains F_A and F_B-like Fe/S clusters, could be removed from the *Heliobacterium modesticaldum* reaction center (HbRC), resulting in 15-ms lifetime charge recombination between $P798^+$ and an unidentified electron acceptor (M. Heinnickel, G. Shen, R. Agalarov, and J. H. Golbeck, (2005) *Biochemistry 44*, 9950-9960). We found that by incubating a HbRC core with sodium hydrosulfite in the presence of light, the 15-ms charge recombination was replaced with a kinetic transient in the sub-μs time domain, consistent with the reduction of an electron acceptor. Concomitantly, a broad and intense EPR signal arises around g = 5 along with a minor set of resonances around g = 2 similar to the spectrum of the $[4Fe\text{-}4S]^{1+}$ cluster in the Fe-protein of *Azotobacter vinelandii* nitrogenase, which exists in two conformations having ground spin states of S = 3/2 and S = 1/2. The Mössbauer spectrum in the as-isolated HbRC core shows that all of the Fe is present in the form of a $[4Fe\text{-}4S]^{2+}$ cluster. After reduction with sodium hydrosulfite in the presence of light, approximately 65% of the Fe appears in the form of a $[4Fe\text{-}4S]^{1+}$ cluster; the remainder is in the $[4Fe\text{-}4S]^{2+}$ state. Analysis of the non-heme iron content of HbRC cores indicates an antenna size of 20-23 BChl *g*/P798. The HbRC therefore contains a [4Fe-4S] cluster assigned to F_X that is coordinated between the PshA homodimer; in contrast to F_X in other Type I reaction centers, this [4Fe-4S] cluster exhibits an S = 3/2 ground spin state.

C1.2.3. CYCLIC ELECTRON FLOW IN C3 PLANTS

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This paper summarized our present view on the mechanism of cyclic electron flow in C3 plants. We propose that cyclic and linear pathways are in competition for the reoxidation of the soluble primary PSI acceptor, Ferredoxin (Fd), that freely diffuses in the stromal compartment. In the linear mode, Fd binds ferredoxin-NADP-reductase and electrons are transferred to $NADP^+$ and then to the Benson and Calvin cycle. In the cyclic mode, Fd binds a site localized on the stromal side of the cytochrome b_6f complex and electrons are transferred to P_{700} via a mechanism derived from the Q-cycle. In dark-adapted leaves, the cyclic flow operates at maximum rate, owing to the partial inactivation of the Benson and Calvin cycle. For increasing time of illumination, the activation of the Benson and Calvin cycle, and thus, that of the linear flow, is associated with a subsequent decrease in the rate of the cyclic flow. Under steady-state conditions of illumination, the contribution of cyclic flow to PSI turnover increases as a function of the light intensity (from 0 to ~ 50 % for weak to saturating light, respectively). Lack of CO_2 is associated with an increase in the efficiency of the cyclic flow. ATP concentration could be one of the parameters that control the transition between linear and cyclic modes.

C1.2.4. HIGH-FIELD EPR ON LOW-SYMMETRY PROTEINS TO REVEAL STRUCTURE-DYNAMICS-FUNCTION RELATIONS

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In photosynthetic organisms the light-induced electron-transfer processes across cell membranes are vectorial in nature. For these processes to be unidirectional, subtle cofactor-protein interactions and/or conformational changes of specific protein segments are functionalized as molecular switches or electron gates. To understand their function, site-specific mutants have been studied to identify functionally important protein sub-domains and cofactors and, moreover, to characterize their structure and dynamics in the transient states of biological action. In the lecture a report will be given on our recent high-field EPR experiments at 95 GHz (wavelength 3 mm) and 360 GHz (0.8 mm) on tailor-made site-directed mutants of photosynthetic reaction centers from the purple bacterium *Rb. sphaeroides.* The EPR results obtained at successively higher Zeeman fields - in comparison to X-band EPR - provide detailed information on structure and dynamics of transient intermediates of the photocycle beyond what is provided by standard X-ray crystallography. Such information is revealed by analysis of the continuous-wave and pulsed high-field EPR spectra and comprises details of molecular structure characteristics in terms of distance and orientation of cofactors in charge-separated radical pairs involved in vectorial transmembrane electron transfer, as well as details of the electronic structure and anisotropic motion of the cofactors in the hydrogen-bond networks of their binding sites. Such pieces of information are essential ingredients for an understanding of the biological transfer process. The lecture will conclude with our most recent developments and applications of high-field PELDOR (pulsed electron-electron double resonance) at 95 GHz to measure distance and orientation of donor and acceptor ions in spin-correlated radical pairs. I thank my co-workers M. Fuchs, M. Plato, A. Savitsky, A. Schnegg as well as my collaboration partners A.A. Dubinskii (Moscow), Y.A. Grishin (Novosibirsk) and W. Lubitz (Mülheim/Ruhr). Financial support by the Deutsche Forschungsgemeinschaft (SFB 498, SPP 1051) is gratefully acknowledged.

C1.2.5. INITIAL ELECTRON DONOR AND ACCEPTOR IN ISOLATED PHOTOSYSTEM II REACTION CENTERS IDENTIFIED WITH FEMTOSECOND MID-INFRARED SPECTROSCOPY

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In spite of the apparent similarity between the plant Photosystem II reaction center and its purple bacterial counterpart, we show in this manuscript that the mechanism of charge separation is very different for the two photosynthetic reaction centers. Using femtosecond visible-pump-mid-infrared probe spectroscopy in the region of the chlorophyll ester and keto modes, between 1775 and 1585 cm-1, with 150 fs time resolution, we show that the reduction of pheophytin occurs on a 0.6-0.8 ps time scale, whereas P+, the precursor state for water oxidation, is formed after ~6 ps. We conclude therefore that in the PS II RC the primary charge separation occurs between the 'accessory chlorophyll' ChlD1 and the pheophytin on the so-called active branch.

Colloquium 1.3. ATP-synthase/ATPase

C1.3.1. DYNAMICS OF SUBUNIT INTERACTION OF THE Na^+ PUMP BY FRET VOLTAGE CLAMP FLUOROMETRY

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The Na^+/K^+-ATPase performs active transport to maintain the physiological Na^+ and K^+ gradients across the plasma membrane in most animal cells. The functional unit of the ion pump is comprised of two mandatory subunits including the alpha subunit which mediates ATP hydrolysis and ion translocation as well as the beta subunit which acts as a chaperone to promote proper membrane insertion and trafficking in the plasma membrane. Although crystal structures of a related protein, the sarcoplasmic reticulum Ca^{2+}-ATPase have provided insight into the conformational flexibility of the alpha subunit, the molecular interaction and orientation between the alpha and beta subunits of the Na^+/K^+-ATPase is not well understood. In order to examine the conformational dynamics between the alpha and beta subunits of the Na^+/K^+-ATPase, we have now used fluorescence resonance energy transfer, under voltage-clamp conditions on oocytes from *Xenopus laevis* in order to determine the relative positions of the alpha and beta subunits by measuring the time constant of irreversible donor fluorophore destruction. We have also investigated the relative movement between the alpha and beta subunits as the ion pump shuttles between the two main conformational states (E_1 and E_2) as described by the Albers-Post scheme. The results from this study have allowed the identification of a model for the orientation of the beta subunit in relation to the alpha subunit. Our data furthermore suggest that a conformational re-arrangement between the alpha and beta subunits occurs during the E_2 to E_1 transition.

Geibel, S., Kaplan, J. H., Bamberg, E. & Friedrich, T. (2003). Conformational dynamics of the Na^+ /K^+ -ATPase probed by voltage clamp fluorometry. */Proc. Natl. Acad. Sci. USA/* 100, 964-969.

Dempski, R. E., Friedrich, T. & Bamberg, E. (2005). The Beta Subunit of the Na+/K+-ATPase Follows the Conformational State of the Holoenzyme. /J. Gen. Physiol./ 125, 505-520.

C1.3.2. SUBUNIT MOVEMENTS IN MEMBRANE INTEGRATED EF0F1 BY SINGLE MOLECULE SPECTROSCOPY

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The H^+-ATPsynthase from *E. coli* was isolated and labelled at the γ- or ε-subunit with tetramethylrhodamine, and at the b-subunits with bisCy5. The double labelled enzymes were incorporated into liposomes. They showed ATP hydrolysis activity, and, after energization of the membrane by ΔpH and Δϕ, also ATP synthesis activity was observed. Fluorescence resonance energy transfer (FRET) was used to investigate the movements of either the γ-subunit or the ε-subunit relative to the b-subunits in single membrane-integrated enzymes. The results show: During catalysis the γε-complex rotates stepwise relative to the b-subunit. The direction of rotation during ATP synthesis is opposite to that during ATP hydrolysis. The stepwise motion is characterized by dwell times (docking time of the γε-complex to one αβ-pair) up to several hundred ms, followed by a rapid movement of the γ- and ε-subunit to the next αβ-pair within 0.2 ms. The same FRET levels (i.e. the same γ-b and ε-b distances) are observed during proton transport-coupled ATP hydrolysis and ATP synthesis, indicating that the reaction proceeds via the same intermediates in both directions.

C1.3.3. BIOENERGETICS OF ARCHAEA: ATP SYNTHESIS UNDER HARSH ENVIRONMENTAL CONDITIONS

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Archaea are a heterogenous group of microorganisms that often thrive at harsh environmental conditions such as high temperatures, extreme pH's and high salinity. As other living cells, they use chemiosmotic mechanisms along with substrate level phosphorylation to conserve energy in form of ATP. Because some archaea are rooted close to the origin in the tree of life, these unusual mechanisms are considered to have developed very early in the history of life and, therefore, may represent first energy conserving mechanisms. A key component in cellular bioenergetics is the ATP synthase. The enzyme from archaea represents a new class of ATPases, the A_1A_O ATP synthases. They are composed of two domains that function as a pair of rotary motors connected by a central and peripheral stalk(s). The structure of the chemically-driven motor (A_1) was solved by small angle X-ray scattering in solution, and the structure of the first A_1A_O ATP synthases was obtained recently by single particle analyses. These studies revealed novel structural features such as a second peripheral stalk and a collar-like structure. In addition, the membrane-embedded electrically-driven motor (A_O) is very different in archaea with sometimes novel, exceptional subunit composition and coupling stoichiometries that may reflect the differences in energy conserving mechanisms as well as adaptation to temperatures at or above 100°C.

C1.3.4. STRUCTURAL AND FUNCTIONAL FEATURES OF YEAST V-ATPASE SUBUNIT C

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V-ATPase is a multi-subunit membrane protein complex; it translocates protons across biological membranes, generating electrical and pH gradients which are used for varieties of cellular processes. V-ATPase is composed of two distinct sub-complexes: a membrane bound V_0 sub-complex, composed of 6 different subunits which is responsible for proton transport and a soluble cytosolic facing V_1 sub-complex, composed of 8 different subunits which hydrolyse ATP. The two sub-complexes are held together via a flexible stator. One of the main features of eukaryotic V-ATPase is its ability to reversibly dissociate to its sub-complexes in response to changing cellular conditions, which arrest both proton translocation and ATP hydrolysis, suggesting a regulation function. Subunit C (vma5p in yeast) was shown by several biochemical, genetic and recently, structural data to function as a flexible stator holding the two sectors of the complex together and regulating the reversible association/dissociation of the complex, partly via association with F-actin filaments. We determined the crystal structure of yeast V-ATPase subunit C (Vma5p) at 1.75 A resolution. Structural features of subunit C that allow smooth energy conversion and interaction with actin will be discussed.

C1.3.5. KINETICS OF PROTON-TRANSLOCATING ATP HYDROLYSIS BY PARACOCCUS DENITRIFICANS FO·F1-ATP SYNTHASE

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$F_o \cdot F_1$-ATP synthase in inside-out coupled vesicles derived from *Paracoccus denitrificans* catalyzes P_i-dependent proton-translocating ATPase reaction if exposed to prior energization that relievs $ADP \cdot Mg^{2+}$-induced inhibition (Zharova, T.V. and Vinogradov, A.D. (2004) *J. Biol. Chem.*, 279, 12319-12324). Here we present evidence that the presence of medium ADP and P_i is required for the steady-state energetically self-sustained coupled ATP hydrolysis. The initial rapid ATPase activity is declined to a certain level if the reaction proceeds in the presence of the ADP-consuming, ATP-regenerating system (pyruvate kinase/phospho*enol* pyruvate). The rate and extent of the enzyme de-activation are inversely proportional to the steady-state ADP concentration which is altered by various amount of pyruvate kinase at constant ATPase level. The half-maximal rate of stationary ATP hydrolysis is reached at ADP concentration of $8 \cdot 10^{-6}$ M. The kinetic scheme is proposed explaining the requirement of the reaction products (ADP and P_i), the substrates of ATP synthesis, in the medium for proton-translocating ATP hydrolysis by *P. denitrificans* $F_o \cdot F_1$-ATP synthase.

Colloquium 1.4. Porters and some other subjects

C1.4.1. GROTTHUSS & HOW TO TRACE PROTON TRANSLOCATION IN A MEMBRANE PROTEIN

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Understanding of proton transfer reactions along the cellular membrane is of fundamental importance for bioenergetics. The purple membrane of Halobacteria represents a sufficiently simple system to study lateral proton transfer with the help of judiciously placed pH-indicators along the surface of bacteriorhodopsin. The analysis of these time-resolved experiments has been inspired by the genius ideas of *Theodor Freiherr von Grotthuss.* Active proton transfer across bacteriorhodopsin is observable by time-resolved FT-IR spectroscopy. Examples will be provided that illustrate how single proton transfer events can be traced in time and space[1,2]. Proton transfer within the electron-driven proton pump cytochrome c oxidase is more difficult to resolve due to the complexity of the catalytic process that involves electron transfer, oxygen fission, and proton translocation. Yet, protonation state changes of a single residue, E286 of the *R. sphaeroides* oxidase, have been detected in catalytically relevant states with the help of perfusion-induced ATR/FTIR difference spectroscopy[3]. Comparison with the oxygen reductase from the thermohalophilic *Rhodothermus marinus,* in which the glutamic acid is spatially replaced by a tyrosine residue (Y256), suggests that proton transfer from a strategically situated donor to the active site is a crucial step in the reaction mechanism of oxygen reductases[4]. Time-resolved FTIR spectroscopy may become feasible by injecting electrons from a metal electrode into the oxidase. We recently developed Surface Enhanced Infrared Difference Absorption Spectroscopy (SEIDAS)[5] to probe potential-induced structural changes of a protein monolayer. A novel concept is introduced to incorporate membrane proteins into solid supported lipid bilayers in an orientated way via the affinity of the His-tag to the Ni-NTA terminated gold surface[6]. Full functionality of the surface tethered cytochrome c oxidase is demonstrated by cyclic voltammetry after binding of the natural electron donor cytochrome c. General applicability of the methodological approach is shown by tethering photosystem II to the gold surface[7]. In conjunction with an hydrogenase, the basis is set for a biomimetic system for H_2-production.

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C1.4.2. THE REDOX STATE OF CYTOCHROME C REGULATES APOPTOSIS

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Ever since the discovery by Xiadong Wang that cyt c release from mitochondria was at the heart of apoptosis, there have been persistent rumours that the redox state of cytochrome c makes a difference. There were two early papers apparently indicating that reduced and oxidised cytochrome c were equally effective at inducing apoptosis, but there have been recent reports that reduced cytochrome c is ineffective at inducing caspase activation. We have used a cytosolic fraction (+deoxyATP) from a macrophage cell line, and shown that added cyt c induces caspase activation only slowly or not at all when it is reduced by ascorbate, TMPD, dithiothreitol, yeast lactate dehydrogenase/cytochrome c reductase, bacterial NADH dehydrogenase/cytochrome c reductase or neuronal NO synthase, whereas added cyt c induces caspase activation rapidly if oxised by hydrogen peroxide or cytochrome oxidase. Scrape-loading macrophages with lactate dehydrogenase/cytochrome c reductase or incubating the cells with ascorbate plus TMPD to reduce intracellular cytochrome c strongly inhibited induced apoptosis and caspase activation but not cytochrome c release. This suggest that reduced cytochrome c is unable or poorly able to activate the apoptosome, and that processes that reduce or oxidise cytochrome c in the cytosol will regulate apoptosis.

C1.4.3. ROLE OF MITOCHONDRIA IN SHAPING A CALCIUM SIGNAL

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Capacitative regulation of calcium entry is a major mechanism of Ca^{2+} influx into electrically non-excitable cells but it also operates in some excitable ones. It participates in the refilling of intracellular calcium stores and in the generation of Ca^{2+} signals in excited cells. The mechanism coupling depletion of the intracellular calcium stores located in the endoplasmic reticulum with opening of the store operated calcium channels in the plasma membrane is not clearly understood. Mitochondria located in the close proximity to Ca^{2+} channels are exposed to high Ca^{2+} concentration and therefore they are able to accumulate this cation effectively. This decreases local Ca^{2+} concentration thus it affects calcium-dependent processes, such as depletion and refilling of the intracellular calcium stores and opening of the store operated channels. Finally, mitochondria modulate the intensity and the duration of calcium signals induced by extracellular stimuli. Ca^{2+} uptake by mitochondria needs these organelles to be in the energized state. On the other hand, Ca^{2+} flux into mitochondria stimulates energy metabolism. Taking together, mitochondria couple cellular metabolism with calcium homeostasis and signaling.

C1.4.4. THEORETICAL AND EXPERIMENTAL APPROACH TO MODELING OXPHOS DISEASES

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Gross alterations in cell energy metabolism underlie manifestations of hereditary and sporadic OXPHOS (oxidative phosphorylation) diseases, many of which depend on proportion of mutant mitochondrial DNA (mtDNA) in tissues that is termed *heteroplasmy*. Cell culture modeling sometimes is ambiguous as high levels of heteroplasmy are needed to obtain respiratory chain insufficiency that in affected tissues of patients develops under much lower mutation load. An animal model of OXPHOS disease with maternal inheritance of heteroplasmy might help understanding the peculiarities of abnormal mtDNA distribution and its effect on pre- and postnatal development. Several mouse models have been proposed worldwide, yet it is hard to predict the yield of affected organisms in generations of transmitochondrial mice. Previously we obtained mice that carry in some tissues both human and murine mtDNA. This heteroplasmy was maternally transmitted to the progeny of animals developed from zygotes injected with human mitochondria. To learn if any regularity exists in such phenomenon we analyzed the probability of obtaining heteroplasmic mice. A large number of experiments with early embryos were carried out, besides, more specimens from F_1 were obtained. Despite somewhat traumatic manipulation with zygotes ca. 33% of those injected with human mtDNA developed into post-implantation embryos (7^{th}-13^{th} days). Only some 21 % of such developed into neonate mice. Percentages of human mtDNA-carriers were ca. 14-16% among post-implantation embryos, the animals in generations F_0 and those in F_1. The latter fact suggests that either no bottleneck filters human from murine mtDNA in germ cell lines of F_0 females or that segregation against foreign mtDNA has its lower threshold that is the observed extent of heteroplasmy. Such percentages are sufficient for modeling maternally inherited heteroplasmy in small animal groups and allow analyzing several generations of mice. Our model is somewhat rigorous, since human mtDNA is really a foreign species for mouse organism rather than mutant murine mtDNA species. Meanwhile it caused no abnormalities and populating mouse tissues was playing a role of mutant mtDNA that in humans is associated with a number of severe OXPHOS diseases. Thus, its distribution can be traced in several generations of phenotypically normal animals. In our experiments heart and skeletal muscles were relatively frequent carriers of human mtDNA as compared to other organs. However, more data are needed to understand the

regularities of tissue distribution of anomalous mtDNA, the process underlying a rapid increase of heteroplasmy and the failure of OXPHOS in selected organs.

Colloquium 1.5. Mechanisms of energy coupling

C1.5.1. CYTOCHROME bc_1 COMPLEX AND ITS FUNCTIONAL FUSION VARIANT CYT bc_1-c_Y WITH ITS PHYSIOLOGICAL MEMBRANE ANCHORED ELECTRON ACCEPTOR CYTOCHROME c_Y

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The membrane integral ubihydroquinone (QH_2) : cytochrome (cyt) *c* oxidoreductase (or the cyt bc_1 complex) is a multisubunit enzyme involved in proton translocation and membrane potential to drive ATP synthesis. The structure and mechanism of function of the cyt bc_1 complex will be discussed in the light of our recent findings that indicate tight coupling of events occurring at its two active sites located on both sides of the energy transducing membranes. Implications of these interactions for the multiple turrnover catalytic mechanism of this enzyme will be described. Moreover, intimate interactions between the cyt bc_1 complex with one its physiological electron acceptors, the membrane-anchored cytochrome c_y (cyt c_y) in the phototrophic bacteria of *Rhodobacter* species, will be described in the light of our recently constructed functional cyt bc_1-c_y fusion complex. This three-subunit cyt bc_1-c_y fusion complex has an unprecedented bis-heme cyt c_1-c_y subunit, instead of the native mono-heme cyt c_1, is efficiently matured and assembled, and sustains cyclic electron transfer *in situ*. The remarkable ability of cells to produce a fully functional cyt bc_1-c_y fusion complex supports the occurrence of structural super complexes in energy production.

C1.5.2. PROTON TRANSFER BY MEMBRANE PROTEINS: WHETHER THERE ARE RULES?

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The energy-converting membrane enzymes, although structurally diverse and often evolutionary unrelated, all have to carry protons across the membrane dielectric barrier and against the backpressure of protonmotive force. To solve this task, on one hand, the desolvation penalty for transferring a proton from water into the hydrophobic part of the membrane has to be lowered. On the other hand, undesirable, futile reactions of transmembrane proton leakage are to be prevented. The common molecular mechanisms that are used by enzymes to accomplish these two, apparently incongruous goals would be discussed.

C1.5.3. COOPERATIVITY AND FLEXIBILITY OF THE PROTONMOTIVE ACTIVITY OF MITOCHONDRIAL RESPIRATORY CHAIN

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Functional and structural data are reviewed which provide evidence that proton pumping in cytochrome c oxidase is associated with extended allosteric cooperativity involving the four redox centers in the enzyme [S. Papa et al., Biochim. Biophys. Acta 1658 (2004) 95-105; S. Papa, Biochemistry (Moscow) 70 (2005) 178-186]. Data are also summarized showing that the H^+/e^- stoicheiometry for proton pumping in the cytochrome span of the mitochondrial respiratory chain is flexible. The ΔpH component of the bulk-phase membrane electrochemical proton gradient exerts a decoupling effect on the proton pump of both the bc_1 complex and cytochrome c oxidase. A slip in the pumping efficency of the latter is also caused by high electron pressure [S. Papa et al, FEBS Lett. 288 (1991) 183-186; T. Cocco et al., Eur. J. Biochem. 209 (1992) 475-481; N. Capitanio et al., Biochemistry 35 (1996) 10800-10806]. The mechanistic and physiological implication of proton-pump slips are examined. The easiness with which bulk phase ΔpH causes, at least above a treshold level, decoupling of proton pumping indicates that for active oxidative phosphorylation efficent protonic coupling between redox complexes and ATP synthase takes place at the membrane surface, likely in cristae, without significant formation of delocalized $\Delta\mu H^+$. A role of slips in modulating oxygen free radical production by the respiratory chain and the mitochondrial pathway of apoptosis is discussed.

C1.5.4. THE PROTONMOTIVE MECHANISM OF CYTOCHROME C OXIDASE: PROBING THE INTERNAL CHARGE-COMPENSATING PROTONATION BY FTIR SPECTROSCOPY

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The electrostatic model of proton/electron coupling in cytochrome oxidases [1] is based on extensive empirical observations that have demonstrated that charge changes in the binuclear centre are accompanied by associated protonation changes so that interconversions between stable states are essentially electroneutral [2]. Two key features of the model are: (1) stable electron transfer from haem *a* to the binuclear centre is associated with intraprotein proton transfer to a site that is physically inaccessible to the oxygen intermediates yet compensates the binuclear centre charge change; (2) protonation of oxygen intermediates is linked to electrostatic repulsion of the charge-compensating protons so that electrical neutrality of stable states is maintained. Although these underlying features have been incorporated into many current mechanistic models, a number of key molecular details remain to be established. One concerns the molecular details of the intraprotein proton transfer reaction that charge-compensates the electron transfer into the binuclear centre: whereas there is good evidence that glutamic acid E-242 is an proton donor, the acceptor site and pathway of proton transfer remain uncertain. Proposals and supporting evidence for the nature of this site will be reviewed. In addition, FTIR data on photolysis of the mixed-valence form of bovine CcO will be presented. After photolysis of CO, ferric haem *a* is reduced by ferrous haem a_3, a reaction that is the reverse of the forward reaction of binuclear centre reduction and is presumably accompanied by a reversal of the charge-compensating proton transfer. Hence, the photolysis-induced FTIR difference spectra of the mixed valence-CO compound provide direct information on the sites involved in the protonation changes that accompany redox changes of the binuclear centre.

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C1.5.5. ON THE LOCALIZED COUPLING OF RESPIRATION AND PHOSPHORYLATION IN MITOCHONDRIA

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This paper is an overview of the experimental studies performed in our laboratory to find out the conditions that favor the hypothetical mechanism of the localized coupling of respiration and phosphorylation postulated by R. Williams in 1961. These studies were undertaken to verify the earlier suggestion that there are two structural and functional states of mitochondria and, respectively, two operation modes of the oxidative phosphorylation system, one of which corresponds to the Williams' model of localized coupling, and the other corresponds to the Mitchell's model of delocalized coupling. There have been numerous attempts to approach the problem. The experiments described in this paper show that the aforementioned models of proton coupling are not necessarily alternative. The paper includes four sections. The first, theoretical, section considers a new modification of the localized coupling of respiration and phosphorylation in mitochondria, which implies the possibility of converting a fraction of energy of oxidative reactions into the thermodynamic potential of hydrogen ions, or free energy of partially desolvated protons. The second section presents experimental evidence for the existence of a relatively high kinetic barrier to proton detachment from the surface of bilayer membranes and intact mitochondrial membranes. The third section demonstrates the formation of nonequilibrium pools of membrane-bound hydrogen ions when the transmembrane flows of hydrogen ions are induced in the above systems. The fourth section establishes a correlation between the size of the pool of membrane-bound H^+ ions on the outer surface of the inner mitochondrial membrane and the productivity of oxidative phosphorylation, evaluated by the ADP/O ratio. A conclusion is made that, depending on the real conditions, either localized or delocalized coupling mechanisms of oxidative phosphorylation come into operation.

2. Physiology and pathology of mitochondria (*in vitro, ex vivo* and *in vivo* studies)

Colloquium 2.1. Uncoupling

C2.1.1. MODULATORS OF UNCOUPLING

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The proton-leak across the inner mitochondrial membrane accounts for a significant part of an animal resting metabolic rate and it represents a potential mechanism for energy dissipation or heat production. We can distinguish two types of proton leak: basal and inducible the former is present in mitochondria in every tissues and, while the mechanism modulating it is not clear, it may be related to the lipidic environment of the membrane (Rolfe et al., 1994 BBA 1118, 405). The inducible proton-leak, on the other hand, occurs through specific Uncoupling Proteins (UCPs) and is tightly regulated (Brand et al., 1999 Int J. Obes. Relat. Metab. Disord. 6, S4). While the role of UCP1 is quite well established, the physiological function of the newly discovered UCP1 homologues, UCP2 and UCP3, still remain uncertain. They have been demonstrated to uncouple mitochondrial oxidative phosphorylation in a number of experimental models such as proteoliposomes, yeast heterologous expression systems and transgenic mice. It is quite clear that heterologous or transgenic expression of these proteins leads to increased proton leak. However, it is less clear whether these uncouplings are due to the activity of UCPs or instead represent a more general perturbation of mitochondrial function. Several evidence suggests that UCPs can be activated in vitro as well as in vivo by exogenous or endogenous effectors among which iodothyronines (such as T3 and 3,5-T2), fenofibrate and fatty acid peroxides, respectively. T3 induces the expression and the activity of UCP3 (for review see, Lanni et al. 2003, FEBS 543, 5). This occurs in association with an induction of a complex network of biochemical pathways leading to increased levels of mitochondrial CoQ, and superoxide and to enhanced mitochondrial lipid handling (all cofactors for UCP3-mediated uncoupling). Fenofibrate administration induces the de-novo expression of UCP3 in rat liver, a tissue that usually lacks UCP3. In analogy with T3,

fenofibrate affects the biochemical pathways involved in mitochondrial lipid handling and CoQ synthesis, that allow UCP3 uncoupling activity to be manifested. 3,5-T2 causes an fatty acids-inducible proton conductance, by activating proteins such as UCP3 and/or Adenin nucleotide translocase in skeletal muscle mitochondria. Fatty acids peroxides and/or their derivatives have been shown to allow UCPs-mediated uncoupling both in liposomes and in isolated mitochondria, thus supporting the recent emerged hypothesis that UCPs could mediate the export of fatty acid peroxides and/or their derivatives allowing protection of the mitochondrial matrix against lipotoxicity.

C2.1.2. UNCOUPLING DUE TO MITOCHONDRIAL UNCOUPLING PROTEINS IN VITRO AND IN VIVO

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Thermogenic uncoupling has been proven only for UCP1 in brown adipose tissue. All other isoforms of UCPs are potentially acting in suppression of mitochondrial reactive oxygen species (ROS) production. UCPs essentially require fatty acids (FAs) including hydroperoxy-FAs, to ensure their uncoupling function, the mechanism of which is still a matter of debate. We prefer Skulachev's FA cycling hypothesis supported by the facts that UCPs are able to provide uniport of long chain alkylsulfonate anions which in turn compete with FA anions and by the existence of inactive FAs which do not flip-flop across the lipid bilayer and do not induce H^+ uniport via UCPs. It is the low abundancy of all UCP isoforms, but UCP1 in BAT mitochondria that makes impossible to uncouple mitochondria more than by several mV of protonmotive force. In contrast, BAT mitochondria can be uncoupled by lauric acid in the range of ~100 nM when endogenous fatty acids are combusted by carnitine cycle and β-oxidation is properly separated from the uncoupling effect. Respiration may increase up to 3 times when related to the lowest fatty acid content (BSA present plus carnitine cycle). Simultaneously, any effect leading to more coupled states leads to enhanced H_2O_2 generation and any effect resulting in uncoupling gives reduces H_2O_2 generation in BAT mitochondria. Attempting quantification, we have found up to four orders of magnitudes differences in mRNA levels of various UCP isoforms (excluding UCP1) in different tissues; e.g. for UCP2 mRNA maximum was quantified in spleen, then for lung, adult rat heart, and pancreatic beta cells, the minimum for the brain. Feedback activation of UCP function by mild oxidative stress is discussed in relation to action of phospholipase A2 and to a possible existence of protein inhibitors of UCPs.

C2.1.3. PROTON TRANSPORT MEDIATED BY UNCOUPLING PROTEINS 1 AND 2 RECONSTITUTED IN PLANAR LIPID BILAYERS

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UCP1 and UCP2 belong to the mitochondrial carrier family and share 59% sequence identity. In contrast to UCP1, specific to brown adipose tissue and responsible for a rapid heat production, UCP2 is localized in brain, lung, spleen, stomach, white adipose tissue and seems to be involved in different pathological states, including atherosclerosis, inflammatory response, diabetes, fever and adaptive thermogenesis. Whereas UCP1 was shown to mediate proton transport exclusively in the presence of free fatty acids (1), the role of cofactors in UCP2 function remains unclear due to the high divergence of data, obtained in cells, intact mitochondria and proteoliposomes. Electrical measurements on planar bilayer membranes reconstituted with UCP1 and UCP2 show that despite the apparently different functions, UCP2 exhibited protonophoric function similar to UCP1 - exclusively in the presence of fatty acids, showing a significant preference for polyunsaturated fatty acids. The preferential activation of UCP by polyunsaturated FA may be of great physiological relevance in vivo.

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C2.1.4. A NEW LOOK AT UCP1

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There has been a resurgence of interest in mitochondrial uncoupling protein 1, due to a desire to understand the regulation of the prominent role it plays in control of metabolic flux in brown adipose tissue and non-shivering thermogenesis, combined with the fact that UCP 1 acts as a paradigm for other novel less abundant uncoupling proteins. In this manuscript we review recent data pertaining to the discovery of UCP 1 in thymus, together with recent evidence on the mechanism UCP 1 function.

C2.1.5. MITOCHONDRIAL UCPS: NEW INSIGHTS INTO REGULATION AND IMPACT

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Uncoupling proteins (UCPs) are mitochondrial inner membrane proteins sustaining an inducible proton conductance. They weaken the proton electrochemical gradient built up by the mitochondrial respiratory chain. Brown fat UCP1 sustains a free fatty acid (FA)-induced purine nucleotide (PN)-inhibited proton conductance. Inhibition of the proton conductance by PN has been considered as a diagnostic of UCP activity. However, conflicting results have been obtained in isolated mitochondria for UCP homologues (i.e. UCP2, UCP3, plant UCP, and protist UCP) where the FFA-activated proton conductance is poorly sensitive to PN under resting respiration conditions. Our recent work clearly indicates that membranous coenzyme Q, through its redox state, represents a regulator of the inhibition by PN of FFA-activated UCP1 homologues under phosphorylating respiration conditions. Several physiological roles of UCPs have been suggested, including a control of the cellular energy balance as well as the preventive action against oxidative stress. In this paper, we discuss new information emerging from comparative proteomics about the impact of UCPs on the mitochondrial physiology, when recombinant UCP1 is expressed in yeast and when UCP2 is over-expressed in hepatic mitochondria during steatosis.

Colloquium 2.2. ROS and redox regulations

C2.2.1. MITOCHONDRIAL METABOLIC STATES REGULATE NITRIC OXIDE AND HYDROGEN PEROXIDE DIFFUSION TO THE CYTOSOL

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Mitochondria isolated from rat heart, liver, kidney and brain (respiratory control 4.0 - 6.5) release NO and H_2O_2 at rates that depend on the mitochondrial metabolic state: releases are higher in state 4, about 1.7-2.0 times for NO and 4-16 times for H_2O_2, than in state 3. NO release in rat liver mitochondria showed an exponential dependence on membrane potential in the range 55 to 180 mV, as determined by Rh-123 fluorescence. A similar behavior was reported for mitochondrial H_2O_2 production by Korshunov et al. (FEBS Lett. 416, 1997, 15-18). Transition from state 4 to state 3 of brain cortex mitochondria was associated to a decrease in NO release (50%) and in membrane potential (24-53%), this latter determined by flow cytometry and DiOC6 and JC-1 fluorescence. The fraction of cytosolic NO provided by diffusion from mitochondria was 61% in heart, 47% in liver, 30% in kidney, and 18% in brain. The data supports the speculation that NO and H_2O_2 report a high mitochondrial energy charge to the cytosol. Regulation of mtNOS activity by membrane potential makes mtNOS a regulable enzyme that in turn regulates mitochondrial O_2 uptake and H_2O_2 production.

C2.2.2. P66^{Shc}, REDOX SIGNALING AND AGING

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P66^{Shc} was the first gene identified whose mutation prolongs life span without defects in mammals [(1)]. Several evidences, reported in the last years, have demonstrated that p66^{Shc} is involved in stress response and in the regulation of intracellular redox balance. More recently p66^{Shc} has been found to catalyze the formation of hydrogen peroxide by mitochondrial respiration through a redox reaction with cytochrome *c* [(2)]. However the means of p66^{Shc} to accelerate aging are still unclear. Fat tissue is supposed to be determinant in aging because of its role in the energetic storage, hormonal secretion and thermoregulation. Indeed many genetic models of altered life span, including invertebrata, rely on alteration of fat tissue. Here we report that p66^{Shc} plays an important role in the control of adipocyte differentiation both *in vitro* and *in vivo*. Primary adipocytes isolated from white or brown fat tissue of p66^{Shc} null mice are impaired to differentiate *in vitro*. Furthermore fat tissue and overall body weight are significantly reduced in p66^{Shc} null mice. Strikingly p66^{Shc} null mice do not accumulated fat and do not increase weight upon high fat diet. The consequences of the function of p66^{Shc} in fat tissue for degenerative disease and aging are discussed.

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C2.2.3. GENERATION OF SUPEROXIDE BY THE MITOCHONDRIAL COMPLEX I

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Superoxide production by inside-out coupled bovine heart submitochondrial particles, respiring with succinate or NADH, was measured. The succinate-supported production was inhibited by rotenone and uncouplers, showing that Complex I is the major contributor in the univalent oxygen reduction. The rate of the superoxide ($O_2^{\cdot-}$) production during respiration at a high concentration of NADH (1 mM) was significantly lower than that with succinate. Moreover, the succinate-supported $O_2^{\cdot-}$ production was significantly decreased in the presence of 1 mM NADH. The titration curves, i.e. initial rates of superoxide production versus NADH concentration, were bell-shaped with the maximal rate (at 50 μM NADH) approaching that seen with succinate. Both NAD^+ and acetyl-NAD^+ inhibited the succinate-supported reaction with apparent $K_i^{'}$s close to their $K_m^{'}$s in the Complex I-catalyzed succinate-dependent energy-linked NAD^+ reduction (reverse electron transfer) and NADH:acetyl-NAD^+ transhydrogenase reaction, respectively. We conclude that: (i) Complex I (most likely FMN in its reduced or free radical form) is the major component in the respiratory chain, participating in the univalent reduction of oxygen; (ii) two different binding sites for NADH (F-site) and NAD^+ (R-site) in Complex I provide accessibility of the substrates-nucleotides to the enzyme red-ox component(s); F-site operates as an entry for NADH oxidation, whereas R-site operates in the reverse electron transfer and univalent oxygen reduction; (iii) it is unlikely that under the physiological conditions (high concentrations of NADH and NAD^+) Complex I is responsible for superoxide generation. We propose that the specific NAD(P)H:oxygen superoxide (hydrogen peroxide) producing oxidoreductase(s) poised in equilibrium with NAD(P)H/$NAD(P)^+$ couple should exist in the mitochondrial matrix, if mitochondria are, indeed, participate in ROS-controlled processes under physiologically relevant conditions.

C2.2.4. S-NITROSYLATION OF AMINOPHOSPHOLIPID TRANSLOCASE: A NEW SIGNALING ROLE IN APOPTOSIS AND PHAGOCYTOSIS

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Plasma membrane aminophospholipid translocase (APT) is mainly responsible for asymmetric distribution of phosphatidylserine (PS) on cell surface. The enzyme can be S-nitrosylated resulting in the loss of its activity. Therefore, we hypothesized that nitrosative stress – acting through APT S-nitrosylation - enhances PS externalization in cells by inhibiting APT activity. This pathway should be particularly important during inflammation whereby oxidative/nitrosative burst generated by macrophages may cause direct nitrosylation or trans-nitrosylation of APT in target cells. To experimentally address this hypothesis we utilized HL-60 cells that express high activity of APT. *S*-nitroso-L-cysteine-ethyl ester (SNCEE) and S-nitroso-glutathione (GSNO) were used as prototypical cell-permeable and cell impermeable trans-nitrosylating reagents. HL-60 cells externalized PS in response to SNCEE or GSNO treatment as evidenced by annexin V binding assay and fluorescence microscopy. No cytotoxic effects were induced by either of the trans-nitrosating agents. RAW 264.7 macrophages elicited enhanced phagocytizing activity towards "nitrosylated" HL-60 cells. Assessments of APT activity revealed that S-nitrosaylation is indeed associated with the changed activity of the enzyme. We speculate that macrophage induced nitrosative stress contributes to effective clearance of apoptotic cells. Consequently, nitrosative stress is involved in regulation of acute inflammatory response and its switch to anti-inflammatory phase as has been observed in the lung and in the brain in in vivo experiments with inhalation of single-walled carbon nanotubes and cortical trauma, respectively. Supported by NIOSH OH008282, NIH HL70755, ES09648, AHA0535365N, Human Frontier Science Program.

C2.2.5. FORMATION OF REACTIVE OXYGEN SPECIES IN HEART MITOCHONDRIA: QUINONE ANALOGS AS A TOOL FOR FURTHER INVESTIGATION OF SUPEROXIDE FORMATION BY COMPLEX I AND III

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It is known that significant part of intracellular reactive oxygen species (ROS) produced by mitochondrial respiratory chain (1). In last thirty years, the importance of ROS production was established for intracellular signaling, oxidative damage of DNA, programmed cell death and a number of deceases (2). Hydrogen peroxide is stable representative of ROS while superoxide anion radical is the primary form of ROS produced by respiratory chain (3). We applied quinone analogs competent in electron transfer and designed in such a way that quinone part of molecule is fixed at different distance from the membrane surface. For this purpose \, triphenylphosphonium derivatives of CoQ with different length of alkyl hydrocarbone linker were employed. Such molecules distribute in the membrane in a membrane potential-dependent fashion (4). Effects of these compounds on the respiratory chain electron transport and H_2O_2 production have been investigated.

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Colloquium 2.3. Aging

C2.3.1. MELATONIN AS ANTIOXIDANT, GEROPROTECTOR AND ANTICARCINOGEN: LIMITATIONS AND PERSPECTIVES OF CLINICAL APPLICABILITY

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The effect of pineal indole hormone melatonin on life span of mice, rats and fruit flies has been studied using various approaches. It has been observed that in female CBA, SHR, SAM and transgenic HER-2/neu mice long-term administration of melatonin was followed by the increase in the mean life span. In rats, melatonin treatment increased survival of male and female rats. In *D.melanogaster*, supplementation of melatonin to nutrient medium during developmental stages have produced contradictory results, but the increase in the longevity of fruit flies has been observed when melatonin was added to food throughout the life span. In mice and rats, melatonin was potent antioxidant both in vitro and in vivo. Melatonin alone turned out neither toxic nor mutagenic in the Ames test and revealed clastogenic activity at the high concentration in the COMET assay. Melatonin has inhibited mutagenesis and clastogenic effect of a number of indirect chemical mutagens. Malatonin has inhibited a development of spontaneous and DMBA- or N-nitrosomethylurea-induced mammary carcinogenesis in rodents; colon carcinogenesis induced by 1,2-dimethylhydrazine in rats, DMBA-induced carcinogenesis of the uterine cervix and vagina in mice; benzo(a)pyrene-induced soft tissues carcinogenesis and lung carcinogenesand induced by urethan in mice. To identify molecular events regulated by melatonin, gene expression profiles were studied in the heart and brain of melatonin-treated CBA mice using cDNA gene expression arrays (15,247 and 16,897 cDNA clone sets, respectively). It was shown that genes controlling the cell cycle, cell/organism defense, protein expression and transport are the primary effectors for melatonin. Melatonin has also increased the expression of some mitochondrial genes (16S,

cytochrome c oxidases 1 and 3 (COX1 and COX3), and NADH dehydrogenases 1 and 4 (ND1 and ND4)), which agrees with its ability to inhibit free radical processes. Of a great interest is an effect of melatonin upon an expression of a large number of genes related to calcium exchange, such as cullins, Dcamkl1, calmodulin, calbindin, Kcnn2 and Kcnn4; a significant effect of melatonin on expression of some oncogenesis-related genes was also detected. Thus, we believe that melatonin may be used for prevention of premature aging and carcinogenesis.

C2.3.2. MITOCHONDRIAL FUNCTIONS IN YEAST AGING AND APOPTOSIS

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It is our aim is to clarify the relationship between oxidative stress, apoptosis and the mother cell-specific aging process in yeast. The transcriptome of senescent wild type yeast mother cells isolated by elutriation centrifugation was studied and compared with the transcriptome of apoptotic yeast cells of *cdc48*^{TS} as well as *orc2-1*^{TS} mutant strains. A large overlap of the old cell and apoptotic cell transcripts was revealed, comprising the functional categories DNA repair, oxidative stress defense and mitochondrial functions. Several of the genes identified in this way were deleted and tested for oxidative stress resistance and longevity. Deletion of genes coding for mitochondrial ribosomal proteins generally led to oxidative stress sensitivity, but we found one example that caused resistance and a marked increase in lifespan. The long-lived yeast cells were not completely unable to undergo apoptosis, but in their final stage of senescence showed the same biochemical markers of apoptosis as the wild type. The *YGR076C*-GFP fusion in old and young cells localized to the mitochondria. Recent experimental results show functional interactions with the TOR signalling pathway. The highly conserved eukaryotic TCTP gene family codes for an antiapoptotic protein. We show here that this protein reversibly shuttles to the outer surface of the mitochondria when the cells are stressed with a mild oxidative treatment which induces apoptosis. The protein is glutathionylated but shuttling does not depend on glutathionylation. The deletion of yeast TCTP causes benomyl hypersensitivity indicating a functional interaction with microtubules. We speculate that TCTP might constitute a link between mitochondria and microtubules. Based on a genome-wide screen for resistance to several oxidants we identified a yeast deletion mutant which renders the cells resistant to the expression of murine Bax. We are now analyzing the function of the gene deleted in this mutant and its relation to yeast apoptosis. Our findings so far indicate that yeast mother cell-specific aging involves an apoptotic process and that mitochondria play a functional role in this process.

C2.3.3. MITOCHONDRIAL METABOLISM AND AGING IN THE FILAMENTOUS FUNGUS PODOSPORA ANSERINA

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The filamentous fungus *Podospora anserina* has a limited lifespan. In this organism, aging is systematically associated to mitochondrial DNA instability. We recently provided evidence that the respiratory function is a key determinant of its lifespan. Loss of function of the cytochrome pathway leads to the compensatory induction of an alternative oxidase, to a decreased production of reactive oxygen species and to a striking increase in lifespan. These changes are associated to the stabilization of the mitochondrial DNA. Here we review and discuss the links between these different parameters and their implication in the control of lifespan. Since we demonstrated the central role of mitochondrial metabolism in aging, the same relationship has been evidenced in several model systems from yeast to mice, confirming the usefulness of simple organisms as *P. anserina* for studying lifespan regulation.

C2.3.4. MITOCHONDRIAL THEORY OF AGING: DEAD OR ALIVE?

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The mitochondrial theory of aging proposes that reactive oxygen species (ROS) generated inside the cell will lead, with time, to increasing amounts of oxidative damage to various cell components. The main site for ROS production is the respiratory chain inside the mitochondria and accumulation of mitochondrial DNA (mtDNA) mutations and impaired respiratory chain function have been associated with degenerative diseases and aging. The theory predicts that impaired respiratory-chain function will augment ROS production and thereby increase the rate of mtDNA mutation accumulation which, in turn, will further compromise respiratory-chain function. Previously we reported that mtDNA mutator mice expressing an error-prone version of the catalytic subunit of mtDNA polymerase accumulate a substantial burden of somatic mtDNA mutations associated with premature aging phenotypes and reduced lifespan. The mtDNA-mutator mice are born in normal Mendelian proportion and have a normal appearance until the age of ~25 weeks when slight kyphosis (curvation of the spine, morphological sign of osteoporosis in mice) and hair loss could be noticed. As the animals got older they developed a wide range of premature ageing phenotypes such as weight loss, reduced subcutaneous fat, alopecia, kyphosis, osteoporosis, anaemia with progressive decrease in circulating red blood cells, reduced fertility, heart enlargement and sarcopeniaMtDNA mutator mice accumulate mtDNA mutations in an approximately linear fashion. The amount of ROS produced was normal and no increased sensitivity to oxidative stress-induced cell death was observed in mouse embryonic fibroblasts from mtDNA mutator mice, despite the presence of a severe respiratory-chain dysfunction. Expression levels of antioxidant defence enzymes, protein carbonylation levels and aconitase enzyme activity measurements indicated no or only minor oxidative stress in tissues from mtDNA mutator mice. The premature aging phenotypes in mtDNA mutator mice are thus not generated by a vicious cycle of massively increased oxidative stress accompanied by exponential accumulation of mtDNA mutations. We propose instead that respiratory-chain dysfunction *per se* is the primary inducer of premature aging in mtDNA mutator mice.

C2.3.5. MITOCHONDRIAL DNA MUTATIONS CAUSE RESISTANCE TO OPENING OF THE PERMEABILITY TRANSITION PORE

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The age-related accumulation of mitochondrial DNA mutations has the potential to impair organ function and contribute to disease. In support of this hypothesis, accelerated mitochondrial mutagenesis is pathogenic in the mouse heart, and there is an increase in myocyte apoptosis. The current study sought to identify functional alterations in cell death signaling via mitochondria. Of particular interest is the mitochondrial permeability transition pore, opening of which can initiate cell death, while pore inhibition is protective. Here, we show that mitochondria from transgenic mice that develop mitochondrial DNA mutations have a marked inhibition of calcium-induced pore opening. Temporally, inhibited pore opening coincides with disease. Pore inhibition also correlates with an increase in Bcl-2 protein integrated into the mitochondrial membrane. We hypothesized that pore inhibition was mediated by mitochondrial Bcl-2. To test this hypothesis, we treated isolated mitochondria with Bcl-2 antagonistic peptides (derived from the BH3 domain of Bax or Bid). These peptides released the inhibition to pore opening. The data are consistent with a Bcl-2-mediated inhibition of pore opening. Thus mitochondrial DNA mutations induce an adaptive-protective response in the heart that inhibits opening of the mitochondrial permeability pore.

Colloquium 2.4. Cancer, ischemia and degenerative disorders

C2.4.1. MITOCHONDRIAL ALTERATIONS AND AUTOPHAGY IN MYOCARDIAL ISCHEMIA/REPERFUSION

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We evaluated autophagy and mitochondrial alterations in a cell-based model of myocardial ischemia/reperfusion (I/R) injury. Using GFP-biosensors and fluorescence deconvolution microscopy we investigated mitochondrial morphology in relation to Bax and Bid activation in the HL-1 cardiac cell line. Mitochondria underwent extensive fragmentation during ischemia. Bax translocation from cytosol to mitochondria was initiated during ischemia and proceeded during reperfusion. However, Bax translocation was not sufficient to induce cell death or mitochondrial dysfunction. Bid processing was caspase-8 dependent, and Bid translocation to mitochondria occurred after Bax translocation and clustering, and minutes before cell death. Mitochondrial fragmentation was a prerequisite for autophagy, which also increased in response to I/R injury. Overexpression of Bnip3 increased apoptosis and was associated with mitochondrial fragmentation and increased autophagy. Mitochondria were targets of autophagy in I/R and Bnip3 overexpression. Inhibition of autophagy increased cell death in response to both I/R and Bnip3 expression, suggesting that the upregulation of autophagy is a protective response.

C2.4.2. COORDINATION OF NUCLEAR- AND MITOCHONDRIAL-DNA ENCODED PROTEINS IN CANCER AND NORMAL COLON TISSUES

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To support the rapid growth of tumors, the cell can respond by increasing the number of mitochondria, in a concerted biosynthesis of mitochondrial constituents (nuclear and mitochondria encoded). Increased transcription, availability and stability of oxidative phosphorylation mRNAs, without increasing mitochondria number could also lead to more rapid energy-yielding effects. Mitochondria biogenesis and *de novo* formation of respiratory chain components imply coordination of nuclear and mt gene transcription. The mitochondrial mass is regulated by a number of physiopathological conditions. In response to external stimuli, mitochondria biogenesis is dependent on an orchestrated crosstalk between the nuclear and the mitochondrial genomes. Based on the higher incidence of glycolysis over oxidative phosphorylation in cancer tissues, we studied by differential proteomics the energy metabolism pathway of matched samples of normal and cancer tissue. Our results indicated that oxidative phosphorylation in cancer cells seemed altered because there is an unbalanced coordination between nuclear- and mitochondria-encoded mitochondrial proteins.

C2.4.3. MONITORING MITOCHONDRIAL BIOENERGETICS IN MODELS OF NEURONAL DYSFUNCTION

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Two general approaches can be taken to investigate mitochondrial physiology. In the first, isolated mitochondria are prepared, but the incubation conditions and experimental protocols are designed to imitate as accurately as possible the environment of the intact cell. The second approach is to work with intact cells, where the gain of physiological relevance is balanced by the greater complexity of the preparation and the difficulty in gaining access to the *in situ* mitochondria. Research in our group has focused on the development of techniques to improve access to the bioenergetics of the *in situ* mitochondria, with particular relevance to the investigation of cultured neurons attached to glass coverslips. Unfortunately, until recently it has not been possible to measure the respiration of cultured cells attached to a substrate on coverslips etc., since the conventional oxygen electrode requires relatively large amounts of mitochondria or cells in suspension. This major limitation has recently been overcome by our development of the 'cell respirometer' where coverslip-attached cells are slowly perfused with medium in a thin, closed imaging chamber, and the downstream depletion of oxygen is quantified by a micro flow-through oxygen electrode. We have used the respirometer to measure the key respiratory rates listed above in neurons undergoing a variety of stresses, distinguishing between changes in respiratory capacity, proton leakiness, ATP demand and spare respiratory capacity. While the respirometer reports the proton current, it is also necessary to know the magnitude of the mitochondrial membrane potential. Membrane-permeant fluorescent cations are nearly always used qualitatively, to detect large changes in potential. There are two pitfalls with the use of these indicators: first the direction of the whole-cell fluorescence response to a change in potential depends of whether the probe is loaded at a concentration sufficient to induce aggregation and quenching in the matrix or whether it is loaded at sub-quenching (low nanomolar) concentrations when mitochondrial depolarization produces a decrease in whole-cell signal. The second complication with the use of these probes is that they also respond, albeit more slowly, to changes in plasma membrane potential. We have recently devised a technique for simultaneously monitoring changes in plasma and mitochondrial membrane potentials with two separate indicators and have combined this with a computer model enabling us to interpret the rather complicated traces that result. These techniques are being applied to investigate mitochondrial dysfunction in models of neuronal dysfunction.

C2.4.4. MITOCHONDRIAL POTASSIUM CHANNELS: FROM PHARMACOLOGY TO FUNCTION

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Mitochondrial potassium channels, such as ATP-regulated or large conductance Ca^{2+}-activated and voltage gated channels were implicated in cytoprotective phenomenon in different tissues. Basic effects of these channels activity include changes in mitochondrial matrix volume, mitochondrial respiration and membrane potential, and generation of reactive oxygen species. In this paper we describe the pharmacological properties of mitochondrial potassium channels and their modulation by channel inhibitors and potassium channel openers. We also discuss potential side effects of these substances.

C2.4.5. TARGETS FOR CARDIOPROTECTION AND NEUROPROTECTION

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Evidence from our lab and others supports that the permeability transition pore complex (PTP) is the end-effector of protection signaling in heart and brain. We proved that the convergence of a multiplicity of upstream pathways via inhibition of GSK-3ß on the end effector, the PTP, to limit its induction is the general mechanism of cardiomyocyte and neuron protection (see: ***J. Clin. Invest***. Juhaszova et al. 2004; 113 (11); 1535-49 and online supplement). Cardiac muscle stretch and loading are important factors controlling cellular growth and survival, but the underlying mechanisms are not fully established. We have found that cardiomyocyte stretch elicits cardioprotective signaling via activation of the PI3K-Akt-eNOS-NO-PKG pathways (independently of the mitoK_{ATP} channel) enhancing PTP resistance to oxidant stress and promoting cell survival. The specific mechanisms of Akt, PKG, and GSK-3ß remain to be elucidated.

Colloquium 2.5. Program death of cells and mitochondria

C2.5.1. PRODUCTION OF REACTIVE OXYGEN SPECIES IN MITOCHONDRIA OF HELA CELLS UNDER OXIDATIVE STRESS

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Mitochondria can be a source of reactive oxygen species (ROS) and a target of oxidative damage during oxidative stress. In this connection, the effect of photodynamic treatment (PDT) with Mitotracker Red (MR) as a mitochondria-targeted photosensitizer has been studied in HeLa cells. It is shown that MR produces both singlet oxygen and superoxide anion upon photoactivation and causes photoinactivation of gramicidin channel in a model system (planar lipid bilayer). Mitochondria-targeted antioxidant (MitoQ) inhibits this effect. In living cells, MR-mediated PDT initiates a delayed ("dark") accumulation of ROS, which is accelerated by inhibitors of the respiratory chain (piericidin, rotenone and myxothiazol) and inhibited by MitoQ and diphenyleneiodonium (an inhibitor of flavin enzymes), indicating that flavin of Complex I is involved in the ROS production. PDT causes necrosis that is prevented by MitoQ. Treatment of the cell with hydrogen peroxide causes accumulation of ROS, and the effects of inhibitors and MitoQ are similar to that described for the PDT model. Apoptosis caused by H_2O_2 is augmented by the inhibitors of respiration and suppressed by MitoQ. It is concluded that the initial segments of the respiratory chain can be an important source of ROS, which are targeted to mitochondria, determining the fate of the cell subjected to oxidative stress.

C2.5.2. PROAPOPTOTIC ACTIVITY OF CYTOCHROME C MUTANTS IN LIVING CELLS

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A non-traumatic electroporation procedure was developed to load exogenous cytochrome *c* into the cytoplasm and to study the apoptotic effect of cytochrome *c* and its mutants in living cells. Murine monocytic WEHI-3 cells were selected for this approach because they surpassed the Jurkat, HL60 and K562 cells in the apoptotic response and demonstrated a linear dependence of the apoptotic cell percentage on the extracellular cytochrome *c* concentration. Apoptosis development in WEHI-3 cells achieved the stage of phosphatidylserine externalization and chromatin aggregation during ~ 1.5 h after cytochrome *c* injection. Living, apoptotic and dead cells were easily discriminated with fluorescence microscopy after staining with Texas Red conjugated annexin V, propidium iodide and Hoechst33342. Under our conditions, up to 80% of WEHI-3 cells demonstrated an apoptotic response to cytochrome *c* electroinjection, and a very low (3-10 %) percentage of cells were positively (dead or apoptotic) responsive to the blank electroinjection. The apoptotic activity of horse, murine and human cytochrome *c*, several K72-substitued mutants and "yeast → horse" hybrid cytochrome *c* were compared quantitatively in living WEHI-3 cells. The minimum apoptosis-activating intracellular concentration of horse heart cytochrome *c* was estimated to be 2.7±0.5 μM (47±9 fg/cell). The equieffective concentrations of the K72A-, K72E- and K72L-substituted mutants of horse cytochrome *c* were five-, 15- and 70-fold higher. The "yeast → horse" hybrid created by introducing S2D, K4E, A7K, T8K, and K11V substitutions (horse protein numbering) and deleting five N-terminal residues in yeast cytochrome *c* did not evoke apoptotic activity in mammalian cells. The apoptotic function of horse cytochrome *c* was abolished by the K72W substitution. The K72W-substituted horse cytochrome *c* possessed reduced affinity to Apaf-1 and formed an inactive complex. The K72W mutant was shown to be competent as a respiratory-chain electron carrier [1]. Relative proapoptotic activity of cytochrome *c* from different species reduced in the row: murine cytochrome *c* > human cytochrome *c* ≈ horse cytochrome *c*. Since K72W-substituted murine cytochrome *c* also possessed no apoptotic activity, this single-point

mutant is well suited for knock-in studies of cytochrome *c*-mediated apoptosis. This research was supported by grants 43.106.11.0018 from RF Ministry of Science and Technology, 01.106.11.0006 from RF Federal Agency for Science and Innovations and the RAS program on molecular and cellular biology.

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C2.5.3. EXPRESSION OF AN EXPANDED POLYGLUTAMINE DOMAIN IN YEAST CAUSES DEATH WITH APOPTOTIC MARKERS

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Huntington's disease is caused by specific mutations in huntingtin protein. Expansion of a polyglutamine (polyQ) repeat of huntingtin leads to protein aggregation in neurons followed by cell death with apoptotic markers. The connection between the aggregation and the degeneration of neurons is poorly understood. Here we show that the physiological consequences of expanded polyQ domain expression in yeast are similar to those in neurons. In particular, expression of expanded polyQ in yeast causes apoptotic changes in mitochondria, caspase activation, nuclear DNA fragmentation and cell death. Interestingly, similar to neurons, at the late stages of expression the expanded polyQ accumulates in the nuclei and seems to affect the cell cycle of yeast. We speculate that the disturbance of the cell cycle might contribute to the development of the apoptotic process in both systems. Our data show that expression of the polyQ construct in yeast can be used to model patho-physiological effects of polyQ expansion in neurons.

C2.5.4. MITOCHONDRIAL MEMBRANE PERMEABILIZATION DURING CELL DEATH

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Mitochondria play an important role in energy production, Ca2+ homeostasis, and cell death. In recent years, the role of the mitochondria in apoptotic and necrotic cell death has attracted much attention. In apoptotic and necrotic death, the mitochondrial membrane permeabilization is considered to be one of the key events, although its detailed mechanism remains elusive. The mitochondrial permeability transition (MPT) is a regulated Ca2+-dependent increase in the permeability of the mitochondrial membrane, resulting in $\Delta\psi$ loss, mitochondrial swelling and rupture of the outer membrane. The MPT is thought to occur after the opening of a channel, termed the permeability transition pore (PTP), which putatively consists of the voltage-dependent anion channel (VDAC), the adenine nucleotide translocator (ANT), cyclophilin D (Cyp D: a mitochondrial peptidyl prolyl–cis, trans-isomerase), and some other molecule(s). We studied a role of MPT in apoptosis and necrosis by generating mice lacking Cyp D. Our results indicate that Cyp D is an essential component of MPT and that the Cyp D-dependent MPT regulates some forms of necrotic death, but not apoptotic death. We also showed that anti-apoptotic proteins, Bcl-2 and Bcl-xL, efficiently block MPT through directly inhibiting VDAC activity. I will summarize these data and describe our more recent data concerning MPT and apoptotic mitochondrial membrane permeabilization.

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Colloquium 2.6. Mitochondrial dynamics

C2.6.1. THE FUNDAMENTAL ORGANIZATION OF CARDIAC MITOCHONDRIA AS A NETWORK OF COUPLED OSCILLATORS

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Mitochondria can behave as individual oscillators whose dynamics may also obey collective, network, properties. Our previous work showed that the mitochondrial membrane potential ($\Delta\Psi_m$) in cardiac cells exhibits high amplitude, self-sustained, and synchronous oscillations together with NADH, reactive oxygen species (ROS), and glutathione when the mitochondrial network is stressed to a critical state[1-3]. Theoretical and computational studies suggested that additional low amplitude high frequency oscillations were also possible[3]. We looked for long-term correlations in time series of $\Delta\Psi_m$ using power spectrum and relative dispersional analysis when cells were under physiological conditions. We found that the collective behavior of the mitochondrial network belongs to statistically fractal, self-similar, processes characterized by a large number of frequencies that scale according to an inverse power law spanning at least three orders of magnitude (from ms to a few min). With oxidative stress the spectrum suddenly narrowed at criticality[1], and a dominant, slow, oscillatory frequency driven by reactive oxygen species (ROS)-mediated coupling between oscillators appeared. This event indicated the transition from physiological to pathophysiological behavior and was visualized as a cell-wide collapse of $\Delta\Psi_m$[2] followed by a bifurcation to oscillatory dynamics[3]. In the heart, this catastrophic event leads to a cascade of failures that scale up to the cellular and whole organ levels impairing electrical propagation and resulting in reentrant cardiac arrhythmias and, ultimately, death[4]. We conclude that, in the physiological domain, the scale-free network behavior confers constancy and flexibility of mitochondrial energetics to changes in energy demand. However, an increase in the coupling between mitochondrial oscillators by ROS leads to bifurcation in dynamics that underlies cardiac dysfunction (e.g. arrhythmias, contractile failure) and cell death.

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C2.6.2. MITOCHONDRIAL SUBPOPULATIONS AND HETEROGENEITY REVEALED BY CONFOCAL IMAGING; POSSIBLE PHYSIOLOGICAL ROLE?

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Heterogeneity of mitochondria has been reported for a number of various cell types. Distinct mitochondrial subpopulations may be present in the cell and may be differently involved in physiological and pathological processes. However, the origin and physiological roles of mitochondrial heterogeneity are still unknown. In mice skeletal muscle, a much higher oxidized state of subsarcolemmal mitochondria as compared with intermyofibrillar mitochondria has been demonstrated. Using confocal imaging technique, we present similar phenomenon for rat soleus and gastrocnemius muscles, where higher oxidative state of mitochondrial flavoproteins correlates also with elevated mitochondrial calcium. Moreover, subsarcolemmal mitochondria demonstrate distinct arrangement and organization. In HL-1 cardiomyocytes, long thread mitochondria and small grain mitochondria are observed irrespectively to the particular cell region, showing also heterogeneous membrane potential and ROS production. Possible physiological roles of intracellular mitochondrial heterogeneity and specializations are discussed.

C2.6.3. EFFECT OF OXIDATIVE STRESS ON DYNAMICS OF MITOCHONDRIAL RETICULUM

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Fragmentation of the mitochondrial reticulum (the thread-grain transition) and following gathering of mitochondria in perinuclear area was induced by oxidative stress. It was shown that the inhibitors of respiratory chain (piericidin and myxothiasol) caused fragmentation of mitochondria in Hela cells and fibroblasts whereas mitochondria-targeted antioxidant (MitoQ) inhibited this effect. Hydrogen peroxide also induced the fragmentation, which was stimulated by the inhibitors of respiration and suppressed by MitoQ. In nontreated cells, mitochondrial reticulum consisted of numerous electrically-independent segments. Prolonged treatment with MitoQ resulted in drastic increase in size and decrease in number of these segments. Local photodamage of mitochondria caused immediate depolarization of the large fraction of mitochondrial network in MitoQ-treated cells. Our data indicated that the thread-grain transition of mitochondria depends on production of reactive oxygen species (ROS) in initial segments of respiratory chain and is a necessary step in the process of elimination of mitochondria (mitoptosis).

C2.6.4. MITOCHONDRIAL DIVISION PROTEINS IN C. ELEGANS AND MAMMALS

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Although a small but growing number of mitochondrial fusion and division proteins are known, it has also become clear that large gaps in our knowledge of mitochondrial membrane dynamics still exist. In the hope of filling some of these gaps, we are using a variety of classical and reverse genetic approaches in C. elegans to search for novel factors that regulate the structure of mitochondria and their cristae. An update on these searches will be given. In addition, we are investigating the functions of *C. elegans* MGM-1 and its human homologue Opa1. The *C. elegans* MGM-1 encoding gene is mutated in *eat-3* mutant animals. C. elegans eat-3 mutants grow slowly and have reduced broodsizes. These phenotypes are partially suppressed by mutations in DRP-1, similar to previous results obtained with yeast. As in other organisms, mutations in C. elegans MGM-1 cause excessive mitochondrial fragmentation. Time-lapse photography made with a temperature sensitive mutation shows that this fragmentation correlates with increased mitochondrial division, possibly triggered by a defect within mitochondria. Human Opa1, like its yeast homologue Mgm1, is localized to the mitochondrial intermembrane space where it is required for fusion between mitochondria. Induction of apoptosis causes mitochondria to fragment and it causes the release of Opa1 into the cytosol. Loss of mitochondrial membrane potential induced by CCCP also causes mitochondria to fragment, but does not cause release of Opa1 into the cytosol. Western blotting shows that CCCP and induction of apoptosis both cause rapid proteolytic cleavage of Opa1. Since the mitochondrial morphology defects induced by CCCP and apoptosis are similar to the effects of Opa1 siRNA, it seems likely that the induced proteolytic cleavage of Opa1 correlates with loss of function, which in turn causes mitochondrial fragmentation and increased susceptibility to apoptosis.

C2.6.5. MITOFUSIN-2 AS A REGULATOR OF MITOCHONDRIAL METABOLISM

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Mitofusin-2 (Mfn2) is a mitochondrial membrane protein that participates in mitochondrial fusion in mammalian cells and mutations in the Mfn2 gene cause Charcot-Marie-Tooth neuropathy type 2A. Mfn2 is a mitochondrial fusion protein and in addition, we have identified a role in mitochondrial metabolism. This is based on a number of observations: a) Mfn2 loss-of-function inhibits pyruvate, glucose and fatty acid oxidation and reduces mitochondrial membrane potential in muscle and non-muscle cells, b) Mfn2 gain-of-function increases glucose oxidation and mitochondrial membrane potential in muscle cells. As to the mechanisms involved, Mfn2 loss-of-function represses nuclear-encoded subunits of OXPHOS complexes I, II, III and V whereas Mfn2 over-expression induces the subunits of complexes I, IV and V. The effect of Mfn2 overexpression on mitochondrial metabolism is mimicked by a truncated Mfn2 mutant that is inactive as a mitochondrial fusion protein. These results indicate that Mfn2 triggers mitochondrial energization, at least in part, by regulating OXPHOS expression through signals that are independent of its role as a mitochondrial fusion protein. An additional support to the concept that Mfn2 plays a metabolic role is related to the observation that Mfn2 expression is dysregulated in skeletal muscle from obese subjects and in obese or non-obese type 2 diabetes patients in parallel to alterations in mitochondrial metabolism. In addition, we have shown that Mfn2 gene expression is induced in skeletal muscle and brown adipose tissue by conditions associated with enhanced energy expenditure such as cold exposure or β_3-adrenergic agonists treatment. In keeping with the role of peroxisome proliferator-activated receptor α coactivator (PGC)-1α on energy expenditure, we have demonstrated a stimulatory effect of PGC-1α on Mfn2 mRNA and protein expression in muscle cells. Alterations in the regulatory activity of PGC-1α and, in consequence, in the expression of Mfn2 may alter mitochondrial function in a variety of pathophysiological conditions.

Colloquium 2.7. Therapeutic approach

C2.7.1. ANTI-HIV DRUGS AND THE MITOCHONDRIA

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Mitochondria are key organelles in energy production. Not only they produce ATP, but they also perform a range of other biologic functions and carry a number of factors involved in cell apoptosis. The infection by the human immunodeficiency virus (HIV) and the therapy for such infection, especially when based upon drugs of the nucleosidic reverse transcriptase inhibitors (NRTIs) category, can alter mitochondrial DNA content and damage other aspects of mitochondrial functions, such as the production of different mtRNAs. This injury and dysfunction can cause several adverse clinical events in a relevant percentage of individuals with HIV infection and on antiretroviral therapy. NRTIs differ in their effects on mitochondria, and dideoxy-NRTIs have the greatest affinity and capacity to inhibit DNA polymerase-gamma, the enzyme responsible for mitochondrial DNA replication. Other drugs (also belonging to other categories of antiretroviral) may however influence mitochondrial function through other mechanisms. A decreased mtDNA content provokes a diminished synthesis of respiratory chain enzymes, leading to alterations in the organelle's functionality, that are in turn responsible for clinically evident side effects. They can range from an asymptomatic hyperlactatemia to lactic acidosis, and to lipodystrophy, a pathology characterized by accumulation of visceral fat, breast adiposity, cervical fat-pads, hyperlipidemia, insulin resistance and fat wasting in face and limbs. The most recent data concerning the effects of different compounds on mitochondria, their role in the pathogenesis of lipodystrophy, as well as problems related to studies on the mitochondrial toxicity of antiviral drugs will be reviewed and discussed.

C2.7.2. MITOCHONDRIA AS A RELAY STATION FOR CELL SIGNALING

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Preconditioning protects the heart via an intracellular signaling pathway in which G_i-coupled surface receptors activate a cascade including PI3K, eNOS, guanylyl cyclase and protein kinase G (PKG) [1]. Activated PKG phosphorylates an outer membrane protein, and this leads to mitochondrial K_{ATP} channel (mitoK_{ATP}) opening by an unknown pathway. We found that exogenous PKG + cGMP induces mitoK_{ATP} opening in isolated heart mitochondria to the same extent as K_{ATP} channel openers such as diazoxide or cromakalim. This effect was blocked by mitoK_{ATP} blockers — 5-HD, glibenclamide, and TPP^+, by the PKG-selective inhibitor KT5823, and by protein kinase C (PKC) inhibitors chelerythrine and the highly selective PKC, peptide antagonist ,$V_{1\text{-}2}$. We also found that mitoK_{ATP} is opened by the PKC activators 12-phorbol 13-myristate acetate (PMA) and H_2O_2. We conclude that PKG is the terminal cytosolic component of the signaling pathway and that it transmits the cardioprotective signal from cytosol to mitoK_{ATP} by a pathway that includes PKC, [2]. MitoK_{ATP} opening causes increased production of reactive oxygen species (ROS) [3] and inhibition of the mitochondrial permeability transition (MPT). Inhibition of MPT opening was prevented by MPG, indicating that the signal was transmitted by a mitoK_{ATP}-dependent increase in H_2O_2. The H_2O_2, in turn, activates a second PKC,, which is presumably associated with and regulates MPT. Thus, endogenous signals activate PKC,1, which opens mitoK_{ATP} causing an increase in H_2O_2. The H_2O_2 activates PKC,2, which inhibits MPT. Because MPT is viewed as the major cause of necrotic cell death after ischemia-reperfusion, this effect is cardioprotective. The increased H_2O_2 act as a second messenger and activate other important kinases, including ERK 1/2 and p38 MAP kinase.

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C2.7.3. TCA CYCLE INTERMEDIATES AS ONCOGENIC AND THERAPEUTIC SIGNALS

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HIFα prolyl hydroxylases (PHDs) are a family of enzymes that regulate protein levels of the α subunit of hypoxia inducible transcription factor (HIFα) according to oxygen levels. Using ferrous iron and ascorbate as cofactors, PHDs catalyse the conversion of molecular oxygen, a prolyl residue and α-ketoglutarate to hydroxy-prolyl, carbon dioxide and succinate. Thus, under normal oxygen conditions PHD hydroxylates HIFα, targeting it for degradation. HIF induction under normal oxygen levels, termed 'pseudo-hypoxia', is an important feature of tumours that result from inactivation of the mitochondrial tumour suppressor succinate dehydrogenase (SDH). Two models have been proposed to explain the link between SDH inhibition and HIF induction. Both models suggest a signal molecule originating in mitochondria is released to the cytosol where it inhibits PHD activity. However, the models differ with regard to the nature of the signal: while the first model implicates mitochondria-generated hydrogen peroxide, our model points to mitochondria-generated succinate. We have also shown that pseudo-hypoxia can be observed in SDH-deficient cells in the absence of oxidative stress and in the presence of potent anti-oxidants. Moreover, our study suggests that cell-permeable α-ketoglutarate derivatives have therapeutic potential for tumours with mutated mitochondrial tumour suppressors.

C2.7.4. HEXOKINASE II AT THE CROSSROADS OF ENERGY METABOLISM AND CELL SURVIVAL: MITOCHONDRIAL BINDING OF HEXOKINASE II IS REGULATED BY PHOSPHORYLATION OF VDAC AND PROTECTS TUMOR CELLS AGAINST CHEMOTHERAPEUTIC DRUGS

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Tumor cells characteristically have a high glycolytic capacity that is active even under aerobic conditions. This is commonly associated with an overexpression of hexokinase II (HXKII), a hexokinase isoform that is capable of binding to mitochondrial outer membrane voltage-dependent anion channel (VDAC). It has been proposed that the mitochondrial localization of HXKII promotes glycolytic metabolism by providing preferential access to mitochondrially generated ATP. However, recent studies from our laboratory and others demonstrated that HXKII binding to the mitochondrial outer membrane also protects against a loss of cell viability by suppressing the mitochondrial outer membrane permeabilization and the release of cytochrome c in response to pro-apoptotic stimuli. This provides tumor cells protection against agents that promote cell death, including chemotherapeutic drugs. Akt activation protects against the onset of apoptosis in part by promoting the binding of HXKII to the mitochondria. The mechanism by which Akt protection mediates this effect requires phosphorylation and inactivation of glycogen synthase kinase 3-β (GSK3-β). Our data demonstrate that GSK3-β is capable of phosphorylating VDAC1 and HXKII is unable to bind to VDAC that is phosphorylated by GSK3-β. Inhibition of Akt potentiates the cytotoxic effects of chemotherapeutic drugs and this potentiation requires GSK3-β activity and the disruption of HXKII binding to the mitochondria. Importantly, detachment of HXKII by other agents potentiates the efficacy of chemotherapeutic drugs even in the absence of Akt inhibition or GSK3-β activation, indicating VDAC phosphorylation acts through its effects on HXKII binding. Thus, Akt and GSK3-β regulate multiple interconnections between cellular metabolism and cell survival that are mediated by HXKII binding to mitochondria and that may have important therapeutic implications for the efficacy of tumor treatment.

C2.7.5. MITOCHONDRIA IN HEART FAILURE: ADAPTATION, FAILURE & THERAPEUTIC TARGETS

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Heart failure is a complex syndrome of numerous dysfunctional components which converge to cause chronic progressive failure of ventricular contractile function and maintenance of cardiac output demand. The aim of this presentation is to highlight the mounting evidence indicating that augmented superoxide, related reactive oxygen species and other free radicals contribute to the oxidative stress evident during the progression of heart failure. While much of the source of increased reactive oxygen species is mitochondrial, there are other intracellular sources, which together are highly reactive with functional and structural cellular lipids and proteins. Bioenergetic defects limiting ATP synthesis in the failing myocardium relate not only to post-translational modification of electron transport respiratory chain proteins but also to perturbation of Krebs Cycle enzyme-dependent synthesis of NADH. Accumulation of pathological levels of lipid peroxides relate to dysfunction in the intrinsic capacity to clear and renew dysfunctional proteins. A number of drugs and other agents with antioxidant and bioenergetic properties currently available show promise in the treatment of post-ischemia and other injury-related heart failure both in animal models and clinical trials. This presentation will also briefly review the key limitations of human heart failure studies and potential clinical therapies that target the elevated oxidative stress that is a hallmark of human heart failure.

POSTER SESSIONS

1. Molecular bioenergetics

Poster session 1.1. Respiratory chain

P1.1.1. A PROTEOMIC STUDY OF COMPLEX I FROM THE AEROBIC YEAST YARROWIA LIPOLYTICA

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NADH:ubiquinone oxidoreductase (complex I) is a very large multiprotein complex of the mitochondrial respiratory chain. In previous studies, the genes for 37 complex I subunits were identified in the *Y. lipolytica* mitochondrial and nuclear genomes. Most of the corresponding proteins could be detected using a combination of doubled SDS PAGE and MALDI-TOF MS [1,2]. A rhodanese-like protein which was found in the purified enzyme may be regarded as the 38th subunits of *Y. lipolytica* complex I [3]. Using MALDI-TOF-MS and tandem mass spectrometry, we have now extended the proteomic study of *Y. lipolytica* complex I, with the following aims: (i) confirm the presence of further subunits in complex I which were found in the genome of *Y. lipolytica*, (ii) search for post-translational modifications of individual subunits, (iii) establish the topology of subunits using limited proteolysis of *Y. lipolytica* complex I reconstituted into proteoliposomes.

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P1.1.2. INHIBITION OF CYTOCHROME C OXIDASE BY NITRIC OXIDE IN INTACT CELLS UNDER NORMOXIA AND HYPOXIA

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High-resolution respirometry provides high sensitivity and reproducibility in measurements of oxygen concentration and respiration under conditions of limited amounts of sample, low oxygen concentrations (hypoxia) and during transitions to anoxia (1). This is in part achieved by on-line correction for instrumental background oxygen dynamics and for the time response of the oxygen sensor. Nitric oxide (NO) binds to and inhibits cytochrome *c* oxidase (COX), the terminal oxygen acceptor in the mitochondrial respiratory chain, in competition with oxygen (2,3) and consequently lowers the affinity of COX for oxygen (4). Using a system in which NO is produced inside the cells in a controlled manner, we performed a detailed respirometric study of inhibition of flux by NO at 'high' physiological oxygen levels (30 μM) and oxygen kinetics in the low oxygen range at concentrations of NO up to 1.8 μM, corresponding to pathophysiological conditions. An NO sensor (ISO-NOP, WPI) was incorporated into the 2 ml chamber of a high-resolution respirometer (OROBOROS Oxygraph-2k). The combined technology allows simultaneous recording of respiration as a function of oxygen concentration and endogenous NO production evaluated by extracellular measurement. Oxygen flux at 30 μM oxygen in control cells was 16 ± 0.9 pmol.s^{-1}.10^{-6} cells (n = 55), decreasing to 4.6 ± 0.9 pmol.s^{-1}.10^{-6} in the presence of 1.8 μM NO (n = 7). Hyperbolic oxygen kinetics of respiration was characterized by the p_{50} of 0.071 ± 0.004 kPa and J_{max} of 15.5 ± 1.6 pmol.s^{-1}.10^{-6} in the absence of NO (n = 5). 1.8 μM NO raised the p_{50} to 4.8 ± 2.2 kPa (n = 5). The decrease in the affinity of COX for oxygen elicited by NO, therefore, significantly alters energy metabolism and may have important pathophysiological consequences.

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P1.1.3. IDENTIFICATION OF TYROSINE-PHOSPHORYLATED PROTEINS OF THE MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION MACHINERY

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Signaling enzymes were found in mitochondria and studies have identified several proteins of OXPHOS whose phosphorylation by PKA (1, 2) or Src kinase family (3) is critical for enzyme activity. Moreover, a cross-talk between both types of kinase can occur, as shown by cAMP-dependant tyrosine phosphorylation of Tyr304 on subunit I associated with the allosteric ATP-inhibition of cytochrome c oxydase (4). Thus it could be of importance to make a systematic study of tyrosine phosphorylated proteins of OXPHOS.

In this study (5), using isolated mitochondria from rat brain, we first confirmed the presence of tyrosine kinases by showing an increase in tyrosine phosphorylation of several mitochondrial proteins with added ATP. We also show that tyrosine kinases can use ATP formed at state 3 to increase tyrosine phosphorylation level compared to state 4, an effect inhibited by the well known Src inhibitor PP2, concomittantly to a decrease in state 3 respiration. As kinases and phosphatases are sensitive to oxidants, we showed that the tyrosine phosphorylation pattern was increased by *in vitro* addition of H_2O_2 and also during an *in situ* production by antimycin inhibition of complex III in respiring mitochondria. Using BN-PAGE to isolate the different complexes followed by SDS-PAGE, western blotting and labeling with an antibody to phosphotyrosine, we detected tyrosine-phosphorylated proteins which were further identified by LC-MS/MS. We found that the 39 kDa subunit of complex I was tyrosine-phosphorylated, in contrast to the β-chain of FoF1-ATP synthase, while we have strong evidence that the α-and γ-chain, and some subunits of the other respiratory complexes were tyrosine-phosphorylated. Furthermore, using Western blotting and immunogold labeling, we identified the prototypic tyrosine phosphatase PTP-1B in mitochondrial inner membrane.

These results showed that kinases and phosphatases participate in the regulation of OXPHOS. This implies that they are potentially involved in mitochondrial pathologies. They also suggested that the mechanism by which mitochondrial ROS production can account for mitochondrial dysfunctionnement could be by affecting kinase and phosphatase activity.

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P1.1.4. EPR CHARACTERIZATION OF THE FLAVIN RADICALS IN THE Na^+-PUMPING NADH: QUINONE OXIDOREDUCTASE FROM VIBRIO CHOLERAE

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Na^+-NQR is the main gateway for electrons into the aerobic respiratory chain of many marine and pathogenic bacteria. Na^+-NQR operates as a primary sodium pump, coupling an electron transfer reaction (NADH to ubiquinone) to Na^+ translocation across the inner cell membrane. It is likely that the enzyme pumps one sodium across the membrane per electron. The activity of Na^+-NQR generates a sodium motive force that is used to do metabolic work. Na^+-NQR has been isolated from the marine bacteria *Vibrio alginolyticus*, *Vibrio harveyi,* as well as from the pathogen *Vibrio cholerae,* and studied extensively by biochemical and biophysical methods. The enzyme is an assembly of 6 different subunits (Nqr A through F) which range in size from 20 kDa to 50 kDa, and it contains several cofactors: four flavins: two non-covalently bound, FAD and riboflavin, and two covalently bound FMN's, a 2Fe-2S center and possibly one tightly bound quinone.

An unusual feature of Na+-NQR is the presence of two spectroscopically distinct flavin radicals. We have shown by EPR and ENDOR that in the air oxidized enzyme (resting state) a neutral flavin semiquinone radical is observed but in the reduced protein, the radical is an anionic flavin semiquinone, deprotonated at the N(5) position of the isoalloxazine ring. The role and identity of the flavin radicals in Na^+-NQR is not known.

In the present work we use site-directed mutants to make spectroscopic assignments of the flavin radicals. Our data suggest that the FMN in subunit B is likely to be the anionic radical. Also, we find that the presence of FMN ligated to a threonine in NqrB is essential for the stability of the enzyme. The data also suggest that the riboflavin is responsible for the formation of the neutral radical. A possible involvement of the flavin radicals in the Na^+ translocation is discussed.

P1.1.5. SITE SPECIFIC MUTAGENESIS OF CONSERVED ARGININE 274 AND HISTIDINES 224 AND 228 IN THE NuoCD SUBUNIT; STUDIES OF QUINONE REDUCTASE ACTIVITY AND EPR SPECTRA IN ISOLATED COMPLEX I FROM ESCHERICHIA COLI

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We have generated point mutations in the NuoCD subunit of Complex I (NDH-1) from *E.coli* (counterpart of the mitochondrial 30 kDa and 49 kDa subunits) substituting histidine 224, histidine 228 and arginine 274 by alanine. These residues are highly conserved in Complex I from various organisms and in the large subunit of some [NiFe] hydrogenases. It was proposed [1] that these residues are close to the interface between NuoCD and NuoB (PSST) and may be involved in the catalytic mechanism of Complex I. *nuoCD* was deleted from genomic DNA and replaced by a zeocin-resistance gene. This deletion was complemented by wild type or mutated NuoCD subunit expressed from a pBAD plasmid. Wild type and mutated Complex I were purified and used for studies of catalytic activity and EPR spectra. Both histidine mutants were fully assembled, but exhibited about 50 % of wild type ubiquinone reductase activity. The R274A mutation caused a reduced amount (66 %) of Complex I with 17 % of quinone reductase activity. Rolliniastatin inhibited wild type and mutated Complex I with almost unchanged values of I_{50}. However, the rolliniastatin-insensitive component of ubiquinone reductase activity was significantly increased in the mutants, it was 10% for wild type and 23%, 23% and 70% for H224A, H228A and R274A, respectively. Although most part of ubiquinone reductase activity in R274A was insensitive to rolliniastatin and piericidin, the reduction of ubiquinone proceeded via the natural path because superoxide production in R274 was not significantly increased in comparison to wild type. Comparative EPR studies of purified wild type and mutated Complex I showed significant but incomplete loss of a [4Fe-4S] signal with g_{xyz}=1.895, 1.904, 2.05, which corresponds to the parameters reported for the N2 cluster in R274A in accordance to [2]. In contrast to [2], the N2 signal was decreased also in the H224A mutant. In addition, the signal derived from one or two fast-relaxing [4Fe-4S] cluster(s) (at g=1.934, 1.875, 2.015) observed at 4.8K, was decreased 2 times in the R274A, H224A and H228A mutants. Assignment of these signals requires further studies. No difference was found between the [2Fe-2S] clusters of wild type and mutated Complex I.

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P1.1.6. THE METASTABLE H STATE IS IMPORTANT FOR PROTON PUMPING BY CYTOCHROME C OXIDASE

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Cytochrome *c* oxidase (C*c*O) is a terminal oxidase in the respiratory chain of aerobic organisms. It catalyses the process of oxygen reduction to water and couples it with creation of an electrochemical transmembrane gradient of protons, which is subsequently used for ATP synthesis. In continuous turnover conditions the catalytic cycle of C*c*O proceeds sequentially in a four-stroke manner via $\mathbf{P_m}$, **F**, **H**, and $\mathbf{E_H}$ intermediates, where each transition is coupled to proton translocation across the membrane. The fully oxidized **H** state, achieved under these conditions, is metastable and in the absence of electron donor it decays into the resting **O** state, which is incapable to pump protons upon reduction. Elucidating the differences in properties and structures of the resting **O** and oxygen-pulsed **H** states may give important information for understanding the functional aspects of proton pumping. Here, we followed the development of absorbance changes and potential generation after photo-induced electron injection into re-oxidized C*c*O from *Paracoccus denitrificans*. In contrast to electron injection into the resting **O**, where injected electrons are mostly stay on heme *a* [Nilsson, (1992), *Proc. Natl. Acad. Sci. USA*, 89, 6497-6501], the photo-induced reduction of **H** results in fast and complete re-oxidation of heme *a* by the binuclear center. Decomposition of optical spectra of this process reveals that the injected electrons are transferred in the latter reaction to Cu_B exclusively. Interestingly, the electron transfer from heme *a* to Cu_B occurs not in one but rather in two sequential steps via some intermediate state. In addition, optical spectra suggest that there may be hydroxide ligands on both heme a_3 and Cu_B centers in the metastable state. Similar experiments of electron injection into **H** on C*c*O incorporated into phospholipid vesicles revealed potential generation due to proton translocation with rates that match heme *a* oxidation. Based on these results we suggest that the crucial differences between **O** and **H** states are in the electron affinity of Cu_B and the presence of an OH^- ligand of heme a_3.

P1.1.7. UTILIZATION OF EXCESS CYTOCHROME C AND COENZYME Q FOR THE MAINTENANCE OF MITOCHONDRIAL ENERGY PRODUCTION IN MUSCLE AND LIVER

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Activity defects in mitochondrial respiratory chain complexes are responsible for a broad range of human diseases. They are also implicated in aging and neurodegeneration. However, repercussions from their deficit on the flux of mitochondrial energy production is variable and follows a threshold pattern of expression. Here, we examined a variety of determinants that affect the biochemical threshold effect in mitochondria isolated from rat muscle and liver. For this, we performed a gradual inhibition of complexes III and IV activities and followed the concentrations of reduced cytochrome c or coenzyme Q, as well as the rate of respiration. We observed an important compensatory increase in these substrate's concentrations that permitted respiratory flux to be maintained even though complex III or IV were strongly inhibited. The magnitude of compensation was in proportion to biochemical threshold values and was determined by the size of a mobilizable pool of cytochrome c or coenzyme Q that was not initially reduced in state 3 respiration. These findings show that there are at least three different pools of intermediate substrates: the "utilized", the "mobilizable" and the "non-mobilizable". In addition, we determined the content of respiratory chain complexes and their maximal activities that revealed a two-fold higher capacity for OXPHOS in muscle than in liver without a difference in stoechiometry. We discussed the role of this "reserve of substrate" in the compensation of pathological defects.

P1.1.8. THERMODYNAMIC PROPERTIES OF THE REDOX CENTERS OF Na^+-TRANSLOCATING NADH: QUINONE OXIDOREDUCTASE

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Redox titration of all optically detectable prosthetic groups of Na^+-translocating NADH:quinone oxidoreductase (Na^+-NQR) from *Vibrio harveyi* at pH 7.5 has been performed. It is shown that the functionally active enzyme possesses only three titratable flavin cofactors, one noncovalently bound FAD and two covalently bound FMN residues. All three flavins undergo different redox transitions during the enzyme function. The noncovalently bound FAD works as a "classical" two-electron carrier with midpoint potential (E_m) of –200 mV. Each of the FMN residues is capable solely of one-electron reduction: one, from neutral flavosemiquinone to fully reduced flavin (E_m = +20 mV), and the other, from oxidized flavin to flavosemiquinone anion (E_m = –150 mV). The lacking second half of the redox transitions for the FMNs cannot be reached under our experimental conditions and is most likely not employed in the catalytic cycle. Besides the flavins, a 2Fe-2S cluster was shown to function in the enzyme as a one-electron carrier with E_m = –270 mV. The midpoint potentials of all the redox transitions determined in the enzyme were found to be independent from Na^+ concentration. Even the components that show very strong retardation in the rate of their reduction by NADH at low sodium concentration [1, 2] had no change in the E_m values when the concentration of coupling ion was changed 1000 times. Based on the present data, plausible mechanisms of transmembrane sodium ions translocation by Na^+-NQR are discussed.

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P1.1.9. NITRIC OXIDE AND THE Cu_B-LACKING BACTERIAL TERMINAL OXIDASE CYTOCHROME bd

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Cytochrome *bd* is a respiratory quinol oxidase, preferentially expressed in bacteria under low O_2 tension or other "stress" conditions, that in contrast to heme-copper oxidases, contains no copper (*1*). Cytochrome *bd* activity is electrogenic, but is not coupled to proton pumping. Interestingly, expression of the enzyme has been shown to allow pathogenic bacteria to colonize O_2-poor environments in the host during infection (*2,3*). Since nitric oxide (NO) is produced by the host to counteract microbial infection, it is of relevance to investigate how NO interacts with *bd*-type oxidases.

Recently, we reported that NO, in competition with O_2, rapidly and potently (K_i = 100 ± 34 nM at ~ 70 μM O_2) inhibits turnover of cytochrome *bd* isolated from *Escherichia coli* and *Azotobacter vinelandii*, the inhibition being quickly and fully reverted upon NO depletion (*4*). In the present study, we characterized by amperometric and time-resolved spectroscopic techniques the reaction of NO with the oxo-ferryl catalytic intermediate (Compound F) of cytochrome *bd* from *A. vinelandii*. Similarly to beef heart cytochrome *c* oxidase (*5,6*), NO reacts with the compound F of cytochrome *bd* with a 1:1 stoichiometry. The reaction follows a second-order behavior, proceeds at 20°C at a rate ($k = 1.2 \times 10^5\ M^{-1}\ s^{-1}$) greater than that measured for the mammalian oxidase ($k = 1 - 2 \times 10^4\ M^{-1}\ s^{-1}$, refs. *5-6*) and leads to an end-point species that displays the optical features characteristic of the oxidized enzyme with nitrite bound to heme *d* at the active site.

These results obtained on the Cu_B-lacking cytochrome *bd* indicate that in the respiratory terminal oxidases, in contrast to previous suggestions (*5-6*), Cu_B is not essential or possibly not involved at all in the reaction of NO with compound F.

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P1.1.10. EXPRESSION AND CHARACTERISATION OF DIFFERENT QUINOL AND CYTOCHROME OXIDASES IN THE ACIDOPHILIC CHEMOLITITROPHIC BACTERIUM ACIDITHIOBACILLUS FERROOXIDANS

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Acidithiobacillus ferrooxidans is an acidophilic chemolithoautotrophic bacterium that can grow in the presence of the weak reductant Fe^{2+} or reducing sulfur compounds. It is used in the industrial extraction of copper, uranium, etc. from ores. We have previously shown the existence of branched electron transfer pathways to the terminal electron acceptor O_2 (1). The expression of the electron transfer components is highly dependent on the growth substrate (iron or sulfur) and on the level of aeration.

- two bc_1 complexes have been described (1-3): one functionning in reverse (4) when the bacterium is grown on iron and the second bc_1 (encoded by a second operon) is present when this bacterium is grown on sulfur and is functioning in the classical mode (1).
- four terminal oxidases have been described: two quinol oxidases (*bd* and bo_3 type) mainly expressed on sulfur, one ba_3 cytochrome c oxidase (with a maximum absorption at 612 nm) expressed only in sulfur and an aa_3 cytochrome c oxidase (with a maximum absorption at 597 nm) which is expressed only in iron (1). In the complete genome of *A. ferrooxidans*, one operon codes for the bo_3 quinol oxidase, one for the *bd* quinol oxidase and only one operon is found to correspond to an aa_3 cytochrome c oxidase, leaving the possibility that the ba_3 cytochrome c oxidase expressed in sulfur corresponds to the aa_3 oxidase expressed in iron with modified hemes groups.

The characterized aa_3 oxidase (5) expressed in iron from two strains of *A. ferrooxidans* showed high level of catalytic activity ($100 s^{-1}$), reaching a maximum at pH 3.5, and was found to share some important properties with the canonical aa_3 cytochrome *c* oxidase: contribution of heme *a* and a_3 to the α and γ bands, the blue shift of the Soret band occurring in the presence of CO. The EPR spectra showed the presence of low spin heme *a* and copper. The cytochrome *c* oxidase is competitively inhibited by ATP, in agreement with the model of the regulation of the downhill and uphill electron transfer pathways in *A. ferrooxidans.* In subunit I, some of the key residues of the

classical D and K proton channels are not present in this oxidase. The phylogenetic tree established for the subunit I shows that this oxidase is positioned between the aa_3 and the ba_3 clusters.

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P1.1.11. MEMBRANE POTENTIAL-DEPENDENCE OF THE METABOLIC FLUX CONTROL EXERTED BY CYTOCHROME C OXIDASE ON THE CELLULAR RESPIRATION RELIES ON ASSEMBLY/DISASSEMBLY OF OXPHOS SUPERCOMPLEXES: POSSIBLE ROLE OF CARDIOLIPIN

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Recent measurements of the low reserve capacity of cytochrome *c* oxidase (COX) on the respiratory activity in intact cells have promoted to a re-appraisal of its "*in vivo*" regulatory function. We have further extended this study in the framework of the metabolic control analysis and evaluated the impact of the mitochondrial transmembrane electrochemical potential ($\Delta\mu H^+$) on the control strength exerted by the oxidase.

Measurements of the control coefficient of COX on cell respiration were carried out, in cultured intact human cell lines, by cyanide titration of the integrated and isolated step. Mitochondrial membrane potential was assessed by laser scanning confocal microscopy using the ratiometric dual- fluorescence emitter specific probe MitoCapture.

Extensive analysis on hepatoma-derived human cell line (HepG2) showed that under respiratory state III condition (i.e. fully phosphorylating or in the presence of uncoupler) the flux control coefficient of COX over endogenous cell respiration was 0.68 ±0.04; in the presence of mt$\Delta\Psi$ (*plus* oligomycin) or mtΔpH (*plus* oligomycin and valinomycin) the control coefficient decreased to 0.25 ±0.03. Consequently, the threshold plot obtained mimicking state III of respiration resulted in a 3-fold increase of the maximal COX reserve. Similar results were obtained with other two additional non-related human cell lines (RD and NHDF-neo) suggesting that the observed effect was general and not depending on the cellular phenotypical background.

Changes in the control coefficient, reserve capacity and threshold of an enzymatic step over a multi-step metabolic flux were modelled as a function of equilibrium between a random collisional- vs an aggregated-controlled state giving results comparable with the experimental evidence.

Measurements carried out on isolated broken mitochondria showed that heart and liver mitochondria behaved differently, with the former exhibiting a much higher flux control coefficient of COX over NADH- or succinate-sustained respiration.

Treatment of the three cell lines with the cardiolipin-ligand nonyl acridine orange (NAO), under conditions unaffecting the endogenous respiration, resulted in a marked decrease of the control strength exerted by COX in the absence of mt$\Delta\mu H^+$.

Our results indicate that mitochondrial $\Delta\mu H^+$ modulates the cellular respiratory flux control exerted "*in vivo*" by cytochrome *c* oxidase. Moreover, this effect might be mediated by a change in the assembly state of the OXPHOS complexes with stabilization of "respirasomes" favoured by collapse of membrane potential with cardiolipin playing a role of a sensor/transducer of the membrane energy state.

P1.1.12. CHARACTERIZATION OF p76, AN EXTERNAL ALTERNATIVE NAD(P)H DEHYDROGENASE FROM NEUROSPORA CRASSA

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The mitochondrial respiratory chain, a main provider of high levels of energy, is known to contain the type I NADH:ubiquinone oxidoreductase or complex I, as well as several alternative non proton-pumping NAD(P)H dehydrogenases. Alternative dehydrogenases are single polypeptide enzymes that oxidize NAD(P)H originating from either the cytosol (external enzymes) or the mitochondrial matrix (internal enzymes) and feed electrons into the respiratory chain without energy conservation. Moreover, they vary widely in number and specificity, suggesting that they may have organism specific roles. Despite the fact that they have been extensively described, we are far from understanding their specific functions and the molecular mechanisms underlying their physiological role.

The filamentous fungus *Neurospora crassa* can be used as a model to study the role of the different mitochondrial NAD(P)H dehydrogenases. It contains a complex I, highly homologous to the human enzyme, and four alternative enzymes, of which we have previously characterized three. Here, we describe the identification and characterization of the fourth alternative NAD(P)H dehydrogenase of *N. crassa*, p76. The enzyme appears to be localized to the mitochondria, as suggested by western blot analysis. The corresponding gene was inactivated by the generation of repeat-induced point mutations, and the resulting null-mutant nde3 was found to be deficient in the oxidation of cytosolic NADH, and to a lesser extent, NADPH. Further characterization of both the mutant strain and a strain expressing the *nde-3* cDNA fused to the fluorescent tag GFP (green-fluorescence protein) is underway and will be presented.

P1.1.13. INVESTIGATION OF TWO ACCESSORY SUBUNITS OF COMPLEX I FROM YARROWIA LIPOLYTICA

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Mitochondrial complex I is the first component of the respiratory chain coupling electron transfer from NADH to ubiquinone to proton translocation across the inner mitochondrial membrane. FMN and eight iron-sulfur clusters serve as redox prosthetic groups. In prokaryotes, complex I is formed from a "minimal" set of 14 subunits, while in eukaryotes the number of subunits is increased significantly by accessory subunits. Complex I from the yeast *Yarrowia lipolytica* is composed of at least 30 hydrophilic and 7 hydrophobic subunits as determined by evaluation of the genomic sequence and by peptide mass fingerprinting [1]. We have investigated two accessory subunits:

Small (~10 kDa) acidic mitochondrial acyl carrier proteins (ACPM) found in eucaryotic complex I were proposed to play a role in fatty acid and polyketide synthesis. We have deleted the genes for two different ACPMs found in *Y. lipolytica*. One deletion lead to loss of complex I assembly, while the other deletion appeared to be lethal.

A novel subunit exhibiting thiosulfate:cyanide sulfurtransferase (rhodanese) activity was found in *Y. lipolytica* complex I. It had been speculated that rhodaneses may take part in iron-sulfur cluster assembly. However, studies of the EPR spectra of complex I from a rhodanese deletion strain showed that the deletion had no effect on the assembly of the iron-sulfur cluster [2]. Further invesigation revealed that assembly and activity of complex I lacking rhodanese was normal.

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P1.1.14. ROLE OF CARDIOLIPIN AND PHOSPHATIDYLGLYCEROL IN THE SYNTHESIS AND ASSEMBLY OF MITOCHONDRIAL PROTEIN COMPLEXES

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The anionic phospholipids cardiolipin and its precursor phosphatidylglycerol are synthesized and localized to the inner mitochondrial membrane. These lipids are essential components of individual complexes required for mitochondrial respiratory function and ATP formation. Mutants of *Saccharomyces cerevisiae* lacking cardiolipin (*crd1* null strains) are only partially compromised for respiratory function and growth on non-fermentable carbon sources. Accumulation of high levels of phosphatidylglycerol in this mutant may partially compensate for the lack of cardiolipin. Complexes III (cytochrome bc_1) and IV (cytochrome *c* oxidase) exist as a supermolecular heterodimer composed of homodimers of each of the individual complexes. Systematic reduction of cardiolipin levels by genetic manipulation results in a proportional decrease in the amount of Complexes III and IV organized into a supermolecular complex. Both biochemical analysis employing native gel electrophoresis on detergent extracts of mitochondria (1) and kinetic analysis of intact mitochondria (2) are consistent with a specific role for cardiolipin in forming the supermolecular complex. Mutants of *S. cerevisiae* lacking both phosphatidylglycerol and cardiolipin (*pgs1* null strains) are completely respiratory deficient due to the lack of both mitochondrial and nuclear encoded subunits of Complexes III and IV. The failure to make these subunits is due to a block in translation of their respective mRNA's rather than a lack of transcription or stability of the final protein products (3). Detailed analysis of the expression the *COX4* gene (nuclear gene encoding subunit 4 of Complex IV) has revealed a 50 nucleotide segment in the 5' untranslated region of the *COX4* mRNA to which a repressor is bound in a *pgs1* null strain but not in a wild type strain. Recessive nuclear gene mutants have been isolated in this repressor that exhibit full expression of COX4 protein. Respiratory deficiency alone does not result in repression of *COX4* expression suggesting a direct response to the lack of mitochondrial anionic lipids. These results strongly point to a novel communication mechanism between the mitochondria and the nucleus responsive to mitochondrial lipid composition. Supported by NIH grant GM56389 to W.D.

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P1.1.15. NEUROSPORA CRASSA MUTANTS AS MODELS TO STUDY COMPLEX I DISEASE-ASSOCIATED MUTATIONS

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Mitochondria are best known as the energy source of the cell. However, in the past decades research has shed light on its pivotal roles in generating reactive oxygen species, calcium metabolism and cell death. Thus, it is not surprising that mitochondrial dysfunction contributes to diverse pathologies including neurodegeneration, diabetes and tumorogenesis. Disturbances in the mitochondrial oxidative phosphorylation pathway most often lead to devastating disorders with a fatal outcome. Of these, complex I deficiency is the most frequently encountered. To date, the cause of about half of the complex I-deficient patients could be traced back to mutations in the structural genes, specifically in those coding for the human homologues of the 75kDa, 51kDa, 49kDa, 30kDa, 24kDa, TYKY, PSST, AQDQ and IP13 subunits. However, and although the mutations are known, very little is understood about the molecular mechanisms underlying the diseases. To achieve a more thorough understanding of the molecular basis of these devastating disorders we are using *Neurospora crassa* as a model system.

We mimicked two compound heterozygous mutations in TYKY (P79L and R102H) and a homozygous mutation in PSST (V122M) in the fungus, as described in patients suffering from Leigh syndrome. Since *N. crassa* contains the internal alternative NADH dehydrogenase NDI1, absent in humans, we have created these mutations in an ndi1 mutant background, therefore demonstrating that the disease causing mutations are not lethal in the fungus. All these strains have similar linear growth in race tubes and comparable to the wild type strain, suggesting that the mutations have no apparent effect on growth. We characterized the five different strains in terms of gene expression from a heterologous inducible promoter, complex I assembly and enzyme function. Our preliminary results indicate a diminished formation/stability of complex I in the mutants, which may be the major factor in the development of the disease, as previously proposed.

P1.1.16. COMPLEX I IN COLOR - CONSTRUCTION OF MICRO-CYTOCHROME C FUSION PROTEINS

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The reason that Complex I (or NADH:quinone oxidoreductase) is not only the largest but also the least understood enzyme in the respiratory chain is most likely that only the promontory part of the enzyme contain detectable prosthetic groups (FMN and iron-sulfur clusters) whereas the membrane-spanning part has no distinct spectroscopic features. We have genetically introduced a chromophore into the hydrophobic domain of *Escherichia coli* Complex I by constructing fusion proteins between membrane spanning subunits and a genetically modified minimal size cytochrome *c*. The original wild type cytochrome c from *Bradyrhizobium japonicum* was in previous work truncated in both ends to get the shortest possible peptide chain that can still bind heme [1]. The resulting microperoxidase consist of 26 amino acids including six c-terminal histidines [2]. The small periplasmically located c-terminal fusion thus adds both a covalently bound heme and a His-tag to Complex I. This facilitates purification of Complex I, but more importantly, allows robust quantification of subunits individually and within the enzyme complex. Since the membrane-spanning subunits usually are poorly antigenic, this represents a major advantage. In addition, the micro-cytochrome c domain form a useful marker to determine the orientation of subcomplex and whole enzyme when reconstituted into liposomes. Last but not least, the modification renders Complex I visible for optical spectroscopy, specifically allowing us to probe heme properties and spectral features at distinct locations on the distal surface of the membrane domain (as further described in [3] of this issue).

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P1.1.17. CONFORMATIONAL CHANGES OF CATALYTIC IMPORTANCE IN ISOLATED COMPLEX I FROM ESCHERICHIA COLI CAUSED UPON ACTIVATION BY PHOSPHOLIPIDS

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The bacterial Complex I (NADH:ubiquinone oxidoreductase type I) or NDH-I is considered a simple version of the mitochondrial enzyme. We have purified Complex I from *E. coli* and showed its intactness by monitoring NADH-dependent electric potential generated by Complex I reconstituted into liposomes. The dependence of solubilized enzyme activity on detergent and phospholipids was studied. Quinone reductase activity was shown to depend drastically on the detergent concentration in the assay medium. The dependence has a bell shape that indicates multiple effects of the detergent; an activation up to 10-fold could be achieved. Treatment with asolectin subsequently yields more than 2-fold activation with a corresponding increase in the apparent V_{max} and without significant changes in apparent K_m. We estimated the ubiquinone reductase activity of isolated Complex I at optimal conditions (turnover approximately of 300 s^{-1}) to be close to that of membrane-bound enzyme. The possibility that the enzyme conformation has been changed upon treatment of Complex I with phospholipids and detergent in a way that some artificial binding sites for ubiquinone become accessible was tested using rolliniastatin. The titration of NADH:ubiquinonone reductase activity of *E. coli* sub-bacterial vesicles, and purified "as prepared" and "activated" Complex I by rolliniastatin, showed similar I_{50} values, which indicates that changes in accessibility or modification of the quinone binding site are not involved in the phospholipid-dependent stimulation of activity [1]. Comparative EPR studies of "as prepared" and activated Complex I showed no changes in [2Fe-2S] clusters but an increase in the signals derived mainly from two [4Fe-4S] clusters in the activated enzyme [1]. One of these clusters could be simulated with an axial spectrum with g_{xyz}=1.895, 1.904, 2.05, which corresponds to the parameters reported for the N2 cluster [2]. This data may indicate conformational rearrangements in the ligands or the immediate surroundings of at least two iron-sulfur clusters during activation, and these rearrangements are essential for the recovery of quinone reductase activity in purified Complex I.

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P1.1.18. EXPRESSION REGULATION OF THE NQR-OPERONS IN VIBRIO HARVEYI AND KLEBSIELLA PNEUMONIAE

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Some marine and pathogenic bacteria exploit unique enzyme called sodium-translocating NADH:quinone oxidoreductase (Na^+-NQR) which couples electron transfer from NADH to quinone with creating sodium electrochemical potential. Sodium gradient is known to be essential for vital functions of microorganisms. Despite this fact, existence of primary sodium pump in some bacteria is amazing as majority of microorganisms forms this gradient by means of secondary processes, such as Na^+/H^+-antiport.

Prokaryote metabolism is considered to be mainly regulated on genes expression level. Consequently dependence of enzyme expression on environmental conditions may elucidate the physiological roles of the protein in a bacterial organism.

We planned to study expression of *nqr*-operons of *V. harveyi* and *K. pneumoniae* in dependence on sodium ions concentrations, pH, uncoupler concentration and type of growth substrate in presence and absence of oxygen. For this purpose, *lac*Z gene was cloned under control of *nqr*-promoters of *V. harveyi* and *K. pneumoniae,* and β-galactozidase activity was measured to estimate *nqr*-genes expression.

In *V. harveyi* level of *nqr* expression was found to be negligibly affected by pH of growth medium, presence of protonophore and NaCl concentration, but it was strongly influenced by electron acceptor type. A pronounced induction of the enzyme synthesis took place during aerobic growth, the largest repression occurred when cells were cultivated without O_2 and other electron acceptors. Some features of this anaerobic repression points out this process to be under the control of ArcAB regulatory system.

Almost invariable expression of Na^+-NQR genes under different pH values and NaCl concentration and in the presence of a protonophore could be explained by the fact that it is the only energy coupled NADH-dehydrogenase in this organism. Therefore, the same series of experiments was carried out for enterobacterium *K. pneumoniae,* which possesses all known NADH-dehydrogenases [1]. Nevertheless the *nqr*-genes expression was not dependent on pH, uncoupler and sodium ions concentration in this bacterium. Furthermore, we failed to observe in *K.*

pneumoniae any dependence of *nqr* expression upon type of terminal electron acceptor for respiratory chain. These data are consistent with absence of genes encoding ArcAB system in *K. pneumoniae*'s genome [2].

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P1.1.19. PROTON TRANSFER IN THE PARACOCCUS DENITRIFICANS NITRIC OXIDE REDUCTASE

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Nitric oxide reductase (NOR) from *Paracoccus denitrificans* is a membrane bound cytochrome *bc* complex and a member of the super-family of heme-copper oxidases (HCuOs). NOR is part of the denitrification pathway where nitrate, NO_3^- is step-wise reduced to di-nitrogen ($N_{2\ (g)}$). NOR catalyses the reduction of NO to N_2O according to $2NO + 2e^- + 2H^+ \rightarrow N_2O + H_2O$. NOR can also catalyse the reduction of O_2 to water ($O_2 + 4e^- + 4H^+ \rightarrow 2H_2O$). NOR harbours four redox active cofactors (two low-spin hemes *c* and *b*, one high-spin heme b_3 and a non-heme iron).

In contrast to the conventional HCuOs, NOR does not contribute to the membrane potential, it does not pump protons, and it does not have the conserved amino acids for this purpose. Instead, NORs have 5 other conserved glutamates within the catalytic subunit, NorB, that are important for catalysis.

In our earlier work, we used O_2 as a substrate for fully reduced NOR in order to study the mechanism for proton transfer into the active site. We could observe proton uptake from the bulk solution coupled to electron transfer into the active site with a τ=25 ms at pH 7.5. This reaction was found to be pH-dependent with an observed pK_a of 6.6. We assume the pK_a=6.6 group to be an important amino acid close to the active site in NOR.

In this work the reduction of NO and O_2 was studied, both in the wild-type enzyme and two Glu-mutants (the E198A and E202A) modeled to be close to the catalytic site, and possibly corresponding to the observed pK_a=6.6 group. The oxidation of the fully reduced enzyme was studied with the flow-flash technique and followed by time-resolved optical absorption spectroscopy. The E198A and the E202A mutant NOR have both been shown to express and fold properly. Our data shows that in the E198A mutant the reaction with O_2 does not proceed after oxygen binding to heme b_3 showing that the proton-coupled electron transfer reaction is severely inhibited. In contrast, the reaction in the E202A mutant NOR proceeds essentially as in the wt NOR,. We suggest that the E198 in the NorB subunit is a candidate for the internal protonatable pK_a =6.6 group that limits the reaction, and a crucial part of a proton pathway into the binuclear site from the periplasm.

P1.1.20. THE LOCAL STRUCTURE OF Zn BINDING SITES IN THE BOVINE CYTOCHROME C OXIDASE: AN X-RAY ABSORPTION SPECTROSCOPY STUDY

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Cytochrome c oxidase (COX) is the terminal component of the respiratory chain: it catalyses the oxidation of cytochrome c reduced by the cytochrome bc_1 complex, reducing O_2 to H_2O and pumping protons across the mitochondrial membrane. Definition of the proton transfer pathways is an open question, crucial for understanding the catalytic mechanism of the enzyme (1).

Zn^{2+} inhibits bovine heart COX (2). The observation that Zn^{2+} inhibits proton uptake in the bacterial cytochrome c oxidase led to propose that Zn^{2+} binds near the H^+ entry point of the D-pathway where a cluster of histidine residues and carboxylates is found (3).

An endogenous Zn ion has been found in the bovine heart COX where it binds to a structurally well defined site (4). To characterize the site at which exogenous Zn binds, inhibiting proton uptake, we performed Zn K-edge X-ray Absorption Spectroscopy (XAS) measurements on three samples of bovine COX characterized respectively by 1, 1.5 and 2 zinc atoms per complex. The last two samples were obtained by incubating the protein with a proper amount of $ZnSO_4$. Metal stoichiometries were measured by ICP-emission spectroscopy.

In agreement with X-ray diffraction data (4), XAS analysis of the endogenous zinc site shows that Zn binds four sulphur atoms. The average Zn-S distance is slightly bigger (2.33 Å) than that reported in the crystallographic model (2.25 Å). On the basis of the XAS spectrum of the endogenous Zn, we extracted the XAS signal of the inhibitory Zn binding site(s). The difference spectra obtained from samples characterized by 1.5 and 2.0 Zn per complex exhibited very similar features. This indicates the presence of a unique, high affinity zinc binding site. First shell analysis of the XAS signal for the exogenous Zn site suggests three nitrogen atoms at 1.99 Å and one O at 1.98 Å as ligands. A complete, multiple-shell, multiple scattering analysis is in progress to possibly localize a cluster of residues consistent with the XAS local structure.

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P1.1.21. OPA1 CONTROLS MITOCHONDRIAL CRISTAE REMODELLING INDEPENDENTLY FROM MITOCHONDRIAL FUSION DURING APOPTOSIS

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Complete release of cytochrome *c* from mitochondria during apoptosis is accompanied by remodelling of the cristae and by fragmentation of the organelle. The latter has been ascribed to "mitochondria-shaping" proteins like dynamin-related protein-1 and mitofusin-1, which are recruited or switched off during cell death. Molecular mechanisms governing the former remain conversely uncertain. Here we show a role for OPA1, a dynamin-related protein of the inner mitochondrial membrane. OPA1 protects from apoptosis at the mitochondrial level, by preventing cytochrome *c* release and dysfunction following intrinsic stimuli, including the "BH3-only" molecule BID. OPA1 does not require mitofusin-1, essential for OPA1-mediated fusion of mitochondria, nor does it interfere with activation of multidomain proapoptotics BAX and BAK. OPA1 regulates remodelling of the cristae and mobilization of cytochrome *c* stores, by maintaining the tubular cristae junction narrow during apoptosis.

P1.1.22. INTERACTION OF DIPHENYLENEIODONIUM WITH COMPLEX I IN BOVINE SUBMITOCHONDRIAL PARTICLES

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The effect of the flavoprotein inhibitor diphenyleneiodonium (DPI) on NADH:ubiquinone oxidoreductase was investigated. As expected, DPI inhibits only the reduced form of the enzyme reacting with reduced flavin and blocking NADH oxidation by all electron acceptors (ubiquinone, hexaaminеruthenium or oxygen). NADPH oxidase activity was affected to the same extent. Analysis of the kinetic behavior of the inhibitor suggested that nucleotide binding hampers DPI entry into the flavin containing pocket. The rate of inhibitor binding to the enzyme catalyzing uncoupled NADH oxidase activity was strongly pH dependent rising steeply after pH 7.5. The second-order rate constant calculated at pH 8.0 was about 10^5 $M^{-1}\times min^{-1}$. Inhibitor binding to the enzyme in coupled conditions was two-times faster when the $FMNH_2$/FMN ratio is higher. Since the flavin of complex I can be reduced also through reverse electron transfer (oxidation of succinate by tightly coupled submitochondrial particles) DPI also binds to complex I during reduction of the enzyme from the terminal end. The kinetic differences between DPI inhibition of the direct and the reverse reaction were analyzed.

P1.1.23. HIGH EXCESS CAPACITY OF CYTOCHROME C OXIDASE IN PERMEABILIZED FIBERS OF THE MOUSE HEART

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Metabolic flux control analysis and the concept of excess capacity of enzymes over pathway flux are related by the functional threshold, at which damage or inhibition of an enzyme reduces excess capacity to a minimum and starts to limit overall flux through the pathway. Excess capacity of cytochrome *c* oxidase (COX) varies between tissues, but little is known about differences between species. In particular, information is lacking on mitochondrial respiratory function in the mouse heart, despite the fact that transgenic mice provide increasingly important animal models. Permeabilized muscle fibers were prepared from the left ventricle of a single mouse heart, and measured in OROBOROS Oxygraph-2k instruments in parallel at 4, 25, 30, 37 and 40 °C ($N \geq 4$). Threshold plots were constructed from azide titrations of flux through the electron transport chain (parallel e-input into complexes I+II with malate+pyruvate+glutamate+succinate and uncoupling by FCCP), versus COX (0.5 mM TMPD+2 mM ascorbate after uncoupling and inhibition by rotenone+malonate+antimycin A). Azide was used, since inhibition of COX by cyanide is reversed by pyruvate particularly at low oxygen levels. The inhibition constant, K_i, of COX for azide was 0.1 mM at 37 °C, increasing from 4 to 40 °C over two orders of magnitude. COX velocity measured with TMPD+ascorbate was 1.3-fold of maximum electron transport capacity of the respiratory chain at 25 to 40 °C, and 3.3-fold at 4 °C. In contrast, linear extrapolations of threshold plots revealed a COX excess capacity of 1.6-fold over pathway flux in the range of 30 to 40 °C, increasing to 1.8- and 7.6-fold at 25 °C and 4 °C, respectively. Application of complex I substrates only, would yield an apparent COX excess capacity of >3-fold over pathway flux (at 30 and 37 °C), since parallel e-input through complex I+II doubled flux compared to complex I substrates. Taken together, COX excess capacity in myocardial fibers of the mouse was significantly higher than in fibers of rat heart or human skeletal muscle. Results obtained under hypothermic incubation conditions of permeabilzed fibers may be extrapolated to physiological temperature of 37 °C with caution only. The very high COX excess capacity under hypothermia (4 °C) may compensate for

hypothermic hypoxia by decreasing the p_{50} of mitochondrial respiration in parallel to the decreased p_{50} of hemoglobin and myoglobin. The present study yields an important baseline for further investigations of mitochondrial function in the mouse heart, including genetic models of acquired and inherited mitochondrial defects.

P1.1.24. X-RAY ABSORPTION STUDIES OF Zn^{2+} BINDING SITES IN BACTERIAL, AVIAN AND BOVINE CYTOCHROME bc_1 COMPLEXES

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The cytochrome (cyt) bc_1 complex is one of the major contributors of the transmembrane electrochemical proton gradient used for ATP synthesis. The mechanism of redox coupled H^+ translocation by the cyt bc_1 complex involves two catalytic sites facing the two opposite sides of the energy transducing membrane: Q_o , at which quinol oxidation is coupled to proton release and Q_i , where quinone reduction is coupled to H^+ uptake.

Zn^{2+} is a well established inhibitor of the bovine mitochondrial bc_1 complex (1), in which it competes with proton binding (2). In bacterial cyt bc_1 complexes Zn^{2+} has been shown to decelerate electron transfer and transmembrane voltage generation (3). It has been proposed that Zn^{2+} binds close to the Q_0 site, blocking the proton release channel(s). Two Zn^{2+} binding sites have been located in the avian cyt bc_1 complex by X-ray diffraction: one of them, located in a hydrophilic area between the cyt b and c_1, might interfere with the egress of H^+ (4).

To determine the coordination geometry of the bound metal we performed Zn K-edge X-ray Absorption Spectroscopy (XAS) measurements on Zn incubated samples of avian, bovine and bacterial (from *Rhodobacter capsulatus* (5)) cyt bc_1 complexes. Zn stoichiometries were measured by ICP-emission spectroscopy, assuming 5 iron atoms per cyt bc_1 complex. Samples were incubated with sub-stoichiometric amounts of Zn to maximize the occupancy of the high affinity site(s) minimizing that of lower affinity site(s).

Preliminary analysis of both the avian and the bovine complexes indicates one histidine nitrogen and two oxygen atoms (at ~ 2.00 Å distance) plus one nitrogen or oxygen atom as metal ligands. This agrees with the crystallographic binding site of the avian complex formed by His121, Asp253 and Glu255 (4).

First-shell analysis in the bacterial complex suggests two nitrogen and two oxygen atoms (at ~2.20 Å) plus one oxygen (or nitrogen) atom as ligands. In order to better define the local structure

and possibly locate the cluster of binding residues a multi-shell, multiple scattering analysis is in progress.

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P1.1.25. REDOX PROPERTIES OF CYTOCHROME C OXIDASE RESOLVED BY FTIR SPECTROSCOPY

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Electrochemical titration of enzyme by Fourier Transform Infrared (FTIR) spectroscopy was performed for the first time. The redox titration of cytochrome c oxidase (CcO) revealed possibility to measure transitions of all four redox centers (including "invisible" CuB) separately. This approach allowed direct estimation of thermodynamic parameters such as midpoint potentials (Ems) and redox interaction which are necessary for the construction of the whole thermodynamic model of CcO functioning.

Redox titration of C*c*O by FTIR was performed at three pHs: 6.5, 8.0, and 9.0. Applied potentials were in range 0:+480 mV *vs* NHE with step of 40 mV. Analyzed infrared region was 4000-1000 cm^{-1}. Surfaces "optical density-potential-wavenumber" were decomposed into separate redox titration curves (dependence of optical density on potential at each peak).

Redox titration of resolved IR peaks showed four types of behavior (Em, pH-dependence, redox interaction) reflecting the redox transitions of four redox centers of the enzyme (Cu_A, heme *a*, heme a_3, and Cu_B).

Most of infrared bands were assigned to specified vibrations of redox centers themselves (heme groups) and their ligands (all four centers).

As a result of band assignment to each of four redox centers, we have found 12 bands which were titrating with the highest midpoint redox potential (E_m) +412 mV at pH 6.5. They had pH dependence (52 mV per pH unit) very close to the theoretical one (60 mV) and could be assigned to "invisible" Cu_B center. To the Cu_A center we assigned bands, titration of which showed pH independent E_m =+250 mV (10 bends resolved). Two other groups reflecting redox transition of the hemes had more complex behavior. Each of them included two parts corresponding to the high and low potential transitions. For the bands representing heme *a* (6 bands), the ratio of high to low potential components was *ca.* 3:2, and for a_3 (8 bends) this ratio was *ca.* 2:3. Taking into account the redox interactions between hemes, such ratio gave the difference in the redox potentials of hemes of 9 mV. The value of interaction was estimated to be negative and pH-dependent. The pH dependence of E_ms for two hemes were the same (30 mV) and practically twice smaller than

theoretical one. This indicates that the hemes have common group which binds proton upon reduction either of the hemes.

Several bands were found to halve their amplitude in pH range 6.5→9.0 what may reflect protonation reactions of the enzyme.

P1.1.26. AZIDOQUINONE PHOTO-LABELING AND DETECTION OF THE LABELED PEPTIDES WITH MASS SPECTROMETRY

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Azidoquinone labeling has been and continues to be a useful method to locate quinone binding sites in proteins c.f. [1, 2]. Typically, labeling studies using photo reactive chemicals such as azidoquinones and photo-reactive quinone binding site inhibitors have been performed using tritiated compounds, allowing the labeled protein or peptide to be identified by tracing the radioactivity. The fewer modifications and the greater resemblance of the photo-reactive substance to the natural compound, the greater the chance of labeling of all the existing binding sites. If the labeled peptides can instead be identified with mass spectrometry, the quinone or inhibitor will only need to be modified in one position, that of the azido group. In addition, using tandem mass spectrometry, we can immediately identify not only the labeled peptide but the exact amino acid. However, mass spectrometric studies of membrane proteins are limited by the low sequence coverage achieved using the standard methods developed for soluble proteins. The most commonly used protease, trypsin, cleaves the peptide bond on the c-terminus of lysine and arginine. Since these amino acids are relatively rare in the membrane spanning parts of proteins a trypsin digest usually produce very large peptides.

In this work, we have developed a system to overcome these problems. Using alternative proteases such as pepsin in a digestion mixture with and without trypsin we can produce peptides from membrane proteins with appropriate masses (from 800-3000 Da), and obtain <80% sequence coverage in most of the membrane-spanning proteins tested. To characterize the compounds formed after illumination we reacted different concentrations of 2,3-dimethoxy-5-azido-6-methyl- 1,4-benzoquinone, 2,6-methyl-5-azido-1,4-benzoquinone and 2,3-dimethoxy-5-(5-azido-pentyl)-6-methyl-1,4-benzoquinone (synthesized previously [3]) with small model peptides and recorded the resulting mass increases. Since this reaction - in contrast to the labeling of a quinone binding site - is unspecific, multiple mass increases separated by the weight of the respective azidoquinone adducts are seen.

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P1.1.27. PURIFICATION, CHARACTERISATION AND CRYSTALLISATION OF THE HYDROPHILIC DOMAIN OF RESPIRATORY COMPLEX I FROM THERMUS THERMOPHILUS

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NADH:quinone oxidoreductase, or complex I, is the first of four enzyme complexes in the respiratory chain. It catalyzes the transfer of two electrons from NADH to quinone coupled to the translocation of four protons across the inner mitochondrial membrane (1). The hydrophilic domain of complex I from the thermophilic organism *T. thermophilus* HB8 has been purified, characterised and crystallised. The subcomplex is stable in sodium dodecyl sulfate at temperatures of up to 80 ^{o}C. The purified enzyme shows high activity with the electron acceptor ferricyanide and residual activity with the menaquinone analogues decylubiquinone and ubiquinone-5. Out of nine iron-sulphur clusters (two binuclear, seven tetranuclear) (2) at least three (one binuclear and two tetranuclear) could be detected by EPR in the NADH-reduced enzyme. Biochemical analysis of the preparation indicated about 30:1 ratio of iron to flavin-mononucleotide (FMN). This indicates that one molecule of FMN is bound per complex I. The preparation consists of eight different polypeptides. Seven of them have been positively identified by peptide mass mapping and N-terminal sequencing as known hydrophilic subunits of *T. thermophilus* complex I. The eighth polypeptide has co-purified with the sub-complex at all stages, is strongly associated with other subunits and has therefore been identified as a novel subunit, which we named NQO15. ORFs encoding homologous protein are present in the genomes of the closest relatives of *T. thermophilus.* Crystals of the subcomplex, used for X-ray data collection, contain all hydrophilic domain subunits, including the previously unknown NQO15.

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P1.1.28. NITRIC OXIDE TRIGGERS THE EARLY REDUCTION OF MITOCHONDRIAL CYTOCHROMES IN RESPIRING MAMMALIAN CELLS

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We have developed a system based on visible light spectroscopy (VLS) to monitor mitochondrial cytochrome redox states simultaneously with oxygen (O_2) and nitric oxide (NO) concentrations in mammalian cells respiring to anoxia in a closed chamber [1]. We have observed in various cell lines that, within a finite O_2 concentration ($[O_2]$) 'window', cytochrome redox changes occur whilst the rate of O_2 consumption (VO_2) remains maximal [1,2]. Thus, as $[O_2]$ decreases within this window cytochromes aa_3 from cytochrome c oxidase (CcO) and cytochromes cc_1, a combined signal from cytochrome c and cytochrome c_1 of complex bc_1, start to become more reduced. This 'early reduction' of the cytochromes leads to an increase in the production of reactive oxygen species (ROS) and to the activation of transcription factor NF-kB, an indicator of hypoxic stress [2]. NO is known to bind reversibly and in competition with O_2 to CcO, thus inhibiting cellular respiration in a concentration-dependent manner [3], an action indicating an important role for NO in regulating cell bioenergetics [4]. By inhibiting endogenous NO synthesis we observed a shift in the early reduction to lower $[O_2]$ and, at a given $[O_2]$, a decrease in ROS production and NF-kB activation [2]. This suggests that NO modulates the $[O_2]$ window over which the early reduction occurs. Our recent results, using a transfected cell line in which the synthesis of NO can be finely controlled, confirm that endogenously generated NO can trigger the early reduction via an interaction with the catalytic centre of CcO. Increasing concentrations of NO shift the onset of early reduction to progressively higher $[O_2]$, thus enhancing the possibility of ROS production under normoxic conditions before energy supply is compromised. We propose a mechanism by which CcO can compensate for the partial inhibition by NO and maintain respiration. We have also separated the spectroscopic contributions from cytochromes c and c_1, and show that neither cytochrome c_1 nor cytochrome b_H is involved in the early reduction: this may implicate complex bc_1 as the site of mitochondrial ROS production [5].

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P1.1.29. EFFECT OF TRANSIENT ISCHEMIA ON RAT BRAIN ENERGY METABOLISM ASSESSED IN VIVO BY 31P MRS AND IN VITRO BY MITOCHONDRIAL OXPHOS

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Oxidative stress contributes to post-ischemic brain damage. The aim of this study was to evaluate the changes in the brain energy metabolism after ischemia (20 min.) and subsequent reperfusion (8 days) in 4-months old Wistar rats. Transient brain ischemia was induced by the occlusion of left common carotid artery and brachiocephalic trunk (without thoracotomy) to eliminate blood flow through both the right common carotid and vertebral arteries **(three – vessel occlusion, 3-VO).** ***In vivo*** **^{31}P-MRS** was performed at 4.7 T SISCO 200/300 imaging spectrometer using 16 mm surface coil with a typical line width of 20-35 Hz in the proton signal. Relative concentrations of phosphate metabolites and pHi were determined from their signals in ^{31}P-MR spectra by AMARES method using JMRUI program. The saturation transfer measurements were accomplished by DANTE pulse sequence. The Kfor was fited by nonlinear regression analysis according to the McConell equation [1]: MPCr = M^0PCr{1-kfor T1sPCr[1-exp(-t/T1sPCr)]}, where M^0PCr is the magnetization of PCr in the absence of γ–ATP saturation, kfor is the forward creatine kinase reaction rate constant, $(T1sPCr)^{-1}$ = Kfor+$(T1PCr)^{-1}$ is the apparent longitudinal relaxation rate in the presence of γ–ATP saturation, and t is the irradiation time. **In vitro mitochondrial respiration** was assessed polarographically using a Clark – type oxygen electrode and glutamate as substrate. After ischemia/reperfusion period there are significant changes of brain energy metabolism as detected **[i]** *in vivo* by ^{31}P-MRS, i.e. decrease in pHi and increase in relative concentrations of phosphate metabolites, as well as **[ii]** *in vitro* by mitochondrial respiration, i.e. decrease in all parameters of oxidative phosphorylation. Our ^{31}P MRS study suggests that 3-VO for 20 min. and reperfusion for 8 days induce significant increase of inorganic phosphate (Pi), decrease of phosphocreatine (PCr) and pHi in the adult rat brain. These results are consistent with *in vitro* oxidative phosphorylation of mitochondria and suggest that global decrease of oxidative phosphorylation rate (OPR) is supplied *in vivo* by anaerobic processes and by increased activity of creatine kinase. Therefore, non-invasive *in vivo* measurement of brain energy metabolism may be used as early indicator of brain disorders

such as cognitive impairment and vascular dementia [1]. Acknowledgement: Supported by the Slovak Science and Technology Agency, Grant APVT-21-022004.

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P1.1.30. ATR-FTIR CHARACTERISATION OF THE P_M INTERMEDIATE OF PARACOCCUS DENITRIFICANS CYTOCHROME C OXIDASE

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Structural changes upon formation of the key ferryl intermediates, P_M and F, of *Paracoccus denitrificans* cytochrome *c* oxidase were investigated by perfusion-induced attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectroscopy. This work aims to identify the IR signatures of ferryl and radical species in haem a_3 and peptide. Different types of isotope labelling were exploited to aid band assignments. After the purified enzyme was deposited as a thin film on the ATR prism, reaction-induced difference ATR-FTIR spectra were measured by switching perfusing buffers containing different chemicals. P_M was generated by perfusion with CO/oxygen and F by perfusion with H_2O_2 (1,2), in both cases applied to fully oxidised, 'fast' enzyme (O). IR spectra of model compounds, such as histidine, tyrosine and haem proteins were also measured to aid band assignments.

In the ATR-FTIR spectrum of P_M -*minus*- O, a prominent trough at 1541 cm^{-1} has been tentatively assigned to the coupled ring mode of covalent His-Tyr as deprotonation of Tyr-OH (1,2). However, the new data exclude this possibility since the band was unchanged by $^{13}C^{15}N$-labeling of either Tyr or His. The trough might instead reflect an amide II (peptide bond) or a haem ring mode. A positive peak at 1233 cm^{-1} could be the CH vibrational mode of ferryl-oxo haem a_3, since it was insensitive to all of the tested isotope substitutions (H_2O/D_2O, universal ^{15}N, $^{13}C_9{}^{15}N$-Tyr, ring-$^{13}C_6$-Tyr, ring-D_4-Tyr and $^{13}C_6{}^{15}N_3$-His). The peak was retained in the F state, and an equivalent peak was observed in ferryl compounds of model haem proteins. Candidates for covalent His-Tyr vibrational modes in P_M -*minus*- O spectra are bands at 1506(-), 1350(-), 1311(-) and 1129(+) cm^{-1}. Bands at 1106(+), 1094(-) and 1082(-) cm^{-1}, which were affected by $^{13}C^{15}N$-His labelling but not by Tyr-labelling, can be assigned to the free His ligands of haem Fe or Cu. Positive peaks at 1637 and 1610 cm^{-1} were identified as amide I vibrational modes of the peptide bond of Tyr and His, respectively since they decreased upon labelling with $^{13}C^{15}N$-Tyr or $^{13}C^{15}N$-His, respectively, being replaced with a new peak at 1620 cm^{-1}. The reaction mechanism and structural change of P_M/F formation will be discussed in the light of structural information from X-ray crystallography.

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P1.1.31. EFFECT OF INTRACELLULAR Ca^{2+} ON MPT INDUCTION IN EXCITABLE CELLS

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Ischemia-reperfusion injury in heart and brain is associated with the induction of the mitochondrial permeability transition (MPT). It is well documented that in isolated mitochondria Ca^{2+} and ROS are principal MPT inducers. In intact cardiomyocytes and neurons, however, normal excitability achieves Ca^{2+} elevations to levels comparable to that believed to induce the mitochondrial permeability transition (MPT) based on in vitro data. Contrary to isolated mitochondria, in cardiac myocytes with intact sarcolemma the MPT-sensitivity to oxidant was not affected by increase of the cytoplasmic Ca^{2+} from ~100nM to >500nM. However, skinned cardiac myocytes maintained in controlled Ca^{2+}/EGTA buffered solutions displayed Ca^{2+} sensitivity similar to isolated mitochondria: 100 nM Ca^{2+} induced the same MPT ROS-threshold as seen in intact cells; however, in skinned myocytes 500 nM Ca^{2+} decreased MPT ROS-threshold by more than half of that observed in 100 nM Ca^{2+}. Thus, once cardiac mitochondria are isolated from the cytoplasm some critical factor is apparently lost and the MPT pore complex now becomes susceptible to high Ca^{2+}. Furthermore, intact cells that are essentially free of intracellular Ca^{2+} after 100% equimolar replacement with Sr^{2+} generated exactly the same MPT ROS-threshold values as seen in cells with physiological Ca^{2+}. Cell death after hypoxia/reoxygenation injury in cardiac myocytes and excitotoxic glutamate stress in neurons is not decreased by the absence of extracellular Ca^{2+}. Finally, both excitotoxic stress (100 μM glutamate) and depolarization (40 mM KCl) induced Ca^{2+} mobilization in neurons. While KCl treated cells exhibited the same MPT ROS-threshold as controls the MPT ROS-threshold was significantly decreased in neurons exposed to glutamate. Thus, cytoplasmic Ca^{2+} (high, low, or virtually absent) probably does not play an important role in mediating MPT-induction in intact cardiac myocytes and neurons.

P1.1.32. THE CYANIDE BINDING TO CYTOCHROME ba_3 IS CONTROLLED BY AN INTRAPROTEIN PROTONATION

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Cyanide is widely used in studies of terminal oxidases for its specific interaction with the heme-copper binuclear centre. The type A heme-copper oxidases bind cyanide both in fully oxidized and reduced states [1, 2], though in the first case the affinity is 3-4 orders of magnitude higher. Cytochrome *ba*$_3$ belongs to type B of the heme-copper superfamily which differs from type A by peculiarities of the binuclear centre and proton channels [3]. The fully oxidized binuclear centre of cytochrome *ba*$_3$ does not interact with any ligand. The arrival of an electron converts it to the open conformation reactive toward exogenous ligands [4]. Cyanide binds to a_3^{+2} heme of one or more electron reduced cytochrome *ba*$_3$ [5].

Some parameters of the reaction between partly reduced cytochrome *ba*$_3$ and cyanide are presented. Based on steady state experiments, the Kd_{app} of the cyanide complex at near neutral pH is about 10^{-8} M. This value is much lower than that known for reduced (~10^{-3} M) and even fully oxidized (~10^{-6} M) type A oxidases. The rate constants k_{on} ($1.25x10^2$ $M^{-1}s^{-1}$) and k_{off} ($3.7x10^{-6}$ s^{-1}) of the reaction between cytochrome *ba*$_3$ and cyanide determined at pH 7.6 give the Kd = $2.95x10^{-8}$ M which is very close to our thermodynamic estimation.

Stability of the cytochrome *ba*$_3$ complex with cyanide strongly depends on pH and this can not be explained by the change of CN^-/HCN ratio. At pH 10.0 the Kd_{app} is ~$5x10^{-6}$ M. Kinetic measurements have revealed that the affinity fall occurs entirely at the expense of the k_{on} value (decreased by two orders of magnitude comparing to pH 7.6), whereas the k_{off} is constant in the pH range from 7.0 to 10.0.

The observed pH dependence is probably connected with a protonation/deprotonation event near the binuclear centre. Involvement of an internal protonated group could explain an unusually high affinity of cytochrome *ba*$_3$ to cyanide at neutral pH. Such a group may be significant in the catalytic cycle of *ba*$_3$ oxidase.

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P1.1.33. STRUCTURAL AND FUNCTIONAL ROLES OF HISTIDINE RESIDUES IN CYTOCHROME b561, A TRANSMEMBRANE ELECTRON TRANSPORTER IN ADRENAL CHROMAFFIN GRANULES

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Cytochrome b561 (cyt b561) transports electrons across the membrane of chromaffin granules (CG) in the adrenal gland medulla, connecting the cytoplasmic and intra-granular ascorbate pools; the latter is depleted during noradrenalin synthesis. The current model has cyt b561, a 28 kDa hydrophobic protein, arranged in 6 transmembrane helixes, carrying two hemes and two ascorbate binding sites. Adrenal cyt b561 is a prototype of a family of animal and plant proteins with diverse functions. Our hypothesis is that electron transport in the CG membrane cyt b561 proceeds against the gradient of redox potentials of the two cyt b561 hemes, with electron transfer aided by a membrane potential created by V_1V_0 type H^+-ATPase. We have developed a system for large-scale expression of wildtype and mutant forms of adrenal cyt b561. The mutations focused on the seven histidine residues of bovine cyt b561. The mutant proteins were characterized by EPR and absorption spectroscopy, and potentiometric and reductive ascorbate titrations. Mutations in the cyt b561 histidine residue that is not conserved in other animal species, His109, had little effect. Mutations in any of the four putative axial ligands (His54, His88, His122, and His161) all produced similar effects: marked decreases in the yield of detergent-extractable recombinant protein, with the remaining solubilized proteins not ascorbate reducible and exhibiting dramatic increases in the high-spin EPR signal near g=6, a new low-spin signal near g=2.96, and very little or none of the native low-spin EPR signals (g=3.1 and 3.7). These results indicate that the two hemes and their axial ligands are linked parts of a major structural unit in cyt b561, with perturbation of any of the axial ligands producing structural changes in the whole unit and coordination changes at both heme centers. We hypothesize that this major structural unit corresponds to the four-helix bundle (helices 2-5) "cross-linked" by the two hemes (an arrangement similar to succinate:quinone oxidoreductase from B. subtilis). Mutations in the other two conserved histidines (His92 and His110), each predicted to be in the vicinity of one of the hemes but not axial ligands, shifted the absorption spectrum and the ascorbate titration behavior of the nearby heme center. These two histidine residues, though not axial ligands, thus appear to modulate the function of the individual heme

centers. Overall, all six conserved histidine residues in bovine adrenal cyt b561 appear to be functionally important.

P1.1.34. INTRA- AND INTERPROTEIN PHOTOINDUCED ELECTRON TRANSFER IN RESPIRATORY CHAIN REDOX PROTEINS

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The photoinduced covalent redox label 8-Thiouredopyrene-1,3,6-trisulfonate (TUPS) [1] has been attached to various lysine residues on the surface of horse heart cytochrome c, as well as to cysteines introduced by site directed mutagenesis replacing some of these lysines and other amino acids. Electron transfer between TUPS and the heme of cytochrome c deviates from the expected monoexponential kinetic behavior. Neither the overall rate, nor the individual exponential components of electron transfer, as followed by kinetic absorption spectroscopy, correlate with the length of the covalent link connecting the dye with the protein. Molecular dynamics calculations show that TUPS can approach the protein surface and occupy several such positions. This heterogeneity may explain the multiexponential electron transfer kinetics. The optimal electron transfer pathways calculated using the program HARLEM [2] (www.kurnikov.org) do not follow the covalent link but involve through space jumps from the dye to the protein moiety, effectively decoupling the length of the covalent link and the electron transfer rates. The correlation of the experimental rates with the calculated pathways and protein packing density which follow from the molecular dynamics simulations will be evaluated [3]. Optimal labeling positions by TUPS have been identified for efficient and rapid reduction of cytochrome c oxidase in its complex with cytochrome c. This system will be shown to be a viable alternative to the ruthenium complex initiated electron transfer studies of the enzyme.

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P1.1.35. MITOCHONDRIAL POTASSIUM CHANNEL MODULATORS INFLUENCE MITOCHONDRIAL FUNCTION OF ACANTHAMOEBA CASTELLANII

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In this study we attempt to identify a cation-selective channel from the mitochondria of non-photosynthesizing ameboid protozoan *Acanthamoeba castellanii*. Potassium channels have been described to be present in inner mitochondrial membranes of animal and plant cells. However nothing is known about cation-transporting systems in mitochondria of unicellular *Eucaryotes*, of which *A. castellanii* is an especially interesting example, as in molecular phylogenesis, it is located at a divergence point of the animal, plant and fungi kingdoms.

It has been proposed that the opening of mitochondrial potassium channels has a direct effect on mitochondrial physiology, causing an increase in steady-state matrix volume, respiratory stimulation, small mitochondrial inner membrane potential depolarization and matrix alkaliniization. We found that substances modulating potassium channel activity influence the bioenergetics of *Acanthamoeba castellanii* mitochondria. The rate of respiration is increased in response to diazoxide (a potassium channel opener) by 11.6 ± 3%, when succinate is used as a substrate, and by 20 ± 3,5 % during NADH based respiration. This effect is partly reversed by glibenclamide (a potassium channel inhibitor). Diazoxide also causes a small depolarization of inner mitochondrial membrane potential (measurement conducted using TPP^+ electrode), regardless the substrate used. The steady state value of $\Delta\Psi$ is restored after treatment with glibenclamide. The other mitochondrial potassium channel inhibitor (5-hydroxydecanoic acid) does not reverse the effect of diazoxide. These results suggest that a cation-transporting system similar to those from other species is present in *A. castellanii* mitochondria.

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P1.1.36. FTIR SPECTROSCOPIC CHARACTERIZATION OF THE Q_O BINDING SITE IN MITOCHONDRIAL AND BACTERIAL bc_1 COMPLEX

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Ubiquinol-cytochrome *c* oxidoreductase (cytochrome bc_1 complex) is one of the fundamental components of respiratory electron transfer chains located in the inner mitochondrial or bacterial cytoplasmic membrane. A x-ray structure of several bc_1 complexes is available (1 and references within). Previously, the infrared spectroscopic characterization of wild type bc_1 revealed the contribution of protonated acidic residues, that can be manipulated by addition of Stigmatellin (3) and variation of quinone content (3). Potential candidates for this interaction have been probed with a combination of site directed mutagenesis and FTIR difference spectroscopy on the mitochondrial and bacterial bc_1 complex. On the basis of our experiments, the involvement of Glu 272 (4), Glu 255 and Asp 71 is suggested (yeast numbering). Interestingly, the case of the bc_1 complex from *Paracoccus denitrificans* where the binding site is fully occupied, signals of the bound quinone are shifted in some of the mutant enzymes including Glu 272, Tyr 279, Asp 66, and Asp 71, indicating a variation of the hydrogen bonding. Structural and functional implications will be discussed.

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P1.1.37. REDOX-LINKED PROTONATION STATE CHANGES IN CYTOCHROME bc_1 COMPLEX IDENTIFIED BY POISSON-BOLTZMANN ELECTROSTATICS CALCULATIONS

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Cytochrome *bc*$_1$ is a key enzyme of biological energy conversion in mitochondria and bacteria. It is a transmembrane complex that transfers electrons from reduced coenzyme Q (CoQ) to a small and mobile redox-active protein. The free energy of the redox reaction is used to generate a proton gradient across the membrane. A modified Q-cycle mechanism is generally accepted to account for the coupling between electron transfer and proton translocation in cytochrome *bc*$_1$. However, the details of the reactions in the two active sites remain largely unresolved and are a matter of ongoing debate.

Structure-based Poisson-Boltzmann electrostatics calculations (1-3) in combination with Monte Carlo algorithms for the evaluation of energy profiles are a valuable tool to identify low energy protonation and redox states in large systems such as cytochrome *bc*$_1$ complex. By performing Poisson-Boltzmann/Monte Carlo titration calculations on completely reduced and completely oxidised cytochrome *bc*$_1$, we have identified redox-linked protonation state changes of titratable residues in the complex. The calculations are based on the crystal structures of cytochrome *bc*$_1$ from yeast (4,5). Conformational flexibility of the CoQ oxidation site as observed in the crystal structures has been considered in the calculations.

All residues undergoing redox-linked protonation state changes are located in the CoQ oxidation site, the CoQ reduction site, or at the *c*$_1$ haem group of the complex. Our results are in agreement with Fourier transform infrared (FTIR) spectroscopy experiments and will help to interpret and extend these data. Analysis of the individual redox-linked protonation state changes helps to evaluate the often conflicting mechanistic models of CoQ oxidation in cytochrome *bc*$_1$ complex. We discuss our results in relation to the more recently proposed gating models of CoQ oxidation that may explain how harmful bypass reactions are prevented. A modified model of proton uptake to the CoQ reduction site will be presented.

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P1.1.38. CYTOCHROME bc COMPLEX OF RB. CAPSULATUS: PROTONS THAT ARE LIBERATED UPON UBIQUINOL OXIDATION STAY TEMPORARILY TRAPPED IN THE CATALYTIC POCKET

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Cytochrome bc_1 complex (*bc*) oxidizes a ubiquinol molecule to ubiquinone by a unique "bifurcated" reaction where the two released electrons go to different acceptors: one is accepted by the iron-sulfur Rieske protein, and the other goes to cytochrome *b.* The reaction is accompanied by obligatory liberation of two protons. In membrane vesicles (chomatophores) from phototrophic bacterium *Rb. capsulatus,* the oxidation of ubiquinol by *bc* can be triggered by flash of light, and the partial steps of enzyme turnover can be traced. Still, the kinetic correlation between the electron and proton transfer reactions in *bc* has remained unclear because the flash-induced redox changes of cytochrome *b* are not seen in the absence of inhibitors - the oxidation of cytochrome *b* is faster than its reduction. We have found that Zn^{2+} ions retarded the oxidation of cytochrome *b* and made the kinetics of its reduction visible [2,3]. At < 100 mkM Zn^{2+}, the voltage generation by *bc* slowed somewhat but retained its full extent, so that enzyme remained functional. The cytochrome *b* was reduced at 1-2 ms, while the voltage generation by *bc* took about 10 ms. The kinetic mismatch (1-2 ms versus 7 ms) was observed even in response to weak flashes, when the *bc* could turn over not more than once [2].

Here we have checked, in the presence of Zn^{2+}, the kinetic correlation between the reduction of cytochrome *b* and proton release into the chromatophore lumen, as monitored by neutral red [4]. Even under single-turnover conditions, the proton release was slower than the reduction of cytochrome *b*. Apparently, the protons, which were liberated upon ubiquinol oxidation, remained transiently trapped in the quinol-oxidizing pocket. It is suggested that the proton release from *bc* is delayed because this thermodynamically favourable reaction is utilized to drive the rate-limiting electrogenic step of the *bc* turnover.

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P1.1.39. UBIQUINOL OXIDATION BY THE CYTOCHROME bc_1 COMPLEX OF RB. CAPSULATUS IN THE PRESENCE OF Zn IONS: DEPENDENCE ON TEMPERATURE AND ON DCCD

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In chomatophore vesicles of *Rb. capsulatus*, a flash of light triggers the oxidation of ubiquinol in the cytochrome bc_1complex (bc_1), at the interface between cytochrome *b* and the mobile FeS domain of the Rieske protein. The addition of Zn ions at < 100 mkM had no impact on the extent of voltage generation by bc_1 but likewise slows down three reactions, namely: (i) the oxidation of cytochrome *b*, allowing thus to resolve its otherwise elusive flash-induced redox changes (as monitored at 561-570 nm), (ii) the re-reduction of cytochrome *c* by ubiquinol via the FeS domain (as monitored at 552-570 nm), and (iii) the generation of transmembrane voltage (as monitored by electrochromism at 522 nm). Thereby the reduction of cytochrome *b* was found to be by order of magnitude faster than (i) the cytochrome *c* re-reduction by ubiquinol and (ii) the voltage generation [1, 2]. We tried to increase the kinetic mismatch between these reactions further by varying the temperature and by combining the Zn-treatment with DCCD (N,N,-dicyclohexylcarbodiimide), another weak inhibitor of bc_1. At 30ºC and 40ºC the cytochrome *b* reduction was 20 times faster than the re-reduction of cytochrome *c* and the voltage generation (at 50 mkM of Zn). The addition of DCCD at < 100 mkM has still no impact on the extent of voltage generation by bc_1 but affected the kinetics of reactions in bc_1 similarly to Zn. The effect of DCCD was weak when it was added alone but pronounced when DCCD was added over Zn. The addition of Zn over DCCD resulted in a slow retardation of voltage generation and cytochrome *c* reduction; the effect developed as slow as DCCD did bind. We conclude that the equilibrium between the sub-conformations of bc_1 is affected by temperature and that these sub-conformations have different affinity both to Zn and to DCCD. Apparently, the binding of Zn brought bc_1 into a conformation where the DCCD-targeted carboxyl(s) were more exposed.

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P1.1.40. A NOVEL STRONG COMPETITIVE INHIBITOR OF MITOCHONDRIAL NADH: UBIQUINONE REDUCTASE

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We recently discovered a very potent and specific inhibitor (NADH-OH) of mitochondrial complex I (NADH:ubiquinone reductase) [1] which appears to be a derivative of NADH. The inhibitor is spontaneously formed during aerobic incubation of the reduced dinucleotide under alkaline conditions or in anaerobiosis in the presence of H_2O_2, and Fe^{+2} or Cu^{+2}. The latter suggests that the mechanism of NADH-OH formation includes modification of NADH with hydroxyl radical ($OH^{\cdot}$). The molecular mass of NADH-OH estimated by ESI-MS (696 Daltons) and preliminary 1H NMR data strongly indicate that the inhibitor is derived from attachment of two oxygen atoms one to the adenine residue and the other to the nicotinamide residue of the inhibitor. The inhibitor is competitive with respect to NADH with a Ki of about 10^{-8} M. The inhibitor efficiently suppresses NADH-oxidase, NADH-artificial acceptor reductase, and NADH-quinone reductase reactions catalyzed by submitochondrial particles, as well as the reactions catalyzed by either isolated complex I or the three subunit flavoprotein fragment of complex. The structural similarity of NADH-OH and NADH, the high potency and specificity of the inhibitor with respect to Complex I and stimulation of the inhibitor formation by ROS raise the exciting possibility that it is a natural derivative of NADH that can modulate activity of Complex I in normal conditions and under periods of oxidative stress.

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P1.1.41. CYTOCHROME C OXIDASE INHIBITION BY ZINC IONS

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Zn^{2+} ions inhibition of cytochrome *c* oxidase (COX) activity has been studied on mitochondrial and bacterial COX both in solubilized and liposome-reconstituted forms. Some works showed that Zn^{2+} ingibits COX in micromolar concentrations [1-5]. But the data published are quite discrepant. Effective concentrations of Zn^{2+} vary fundamentally and the side of a membrane, where the Zn^{2+}-binding site is located still is not determined. We find out that the effect of zinc on the solubilized COX develops in two time-scales. Initial rapid interaction of zinc with the site exposed to inner aqueous phase (corresponding to mitochondrial matrix) is fully reversed by EDTA and results in a partial inhibition of the enzyme activity (50-90%, depending on preparation) with an effective K_i of ca. 10^{-5} M. Presumably, zinc blocks the entrance of the D-proton channel. Rapid phase is followed by slow (tens of minutes-hours) zinc irreversible binding to COX which results in almost complete inhibition of the enzyme. The slow phase is characterized by high affinity of the inhibitor for the enzyme:full inhibition can be achieved upon incubation of the solubilized oxidase for 24 h with zinc concentration as low as 2 μM. A series of experiments was carried out in order to determine k_i of the slow phase. According to the data received it's value is less than 1 μM.

According to published data outside Zn^{2+} does not effect COX activity in proteoliposomes (COV) unless they are in the coupled state. In fact the effect of Zn^{2+} is not observed in uncoupled COV but reappears when proteoliposomes are supplied with alamethicin that makes the membrane permeable to low-molecular substances and allows Zn^{2+} to go inside. The slow interaction of zinc with the outer surface of liposome reconstituted cytochrome oxidase (similar to that described by Nicholls [1]) is observed only with the enzyme turning over or in the presence of weak reductants, whereas incubation of zinc with the fully oxidized proteoliposomes does not induce the inhibition. Inhibitory effect is highly enhanced if Zn^{2+} and weak reductans are preincubated with COV whereas incubation with zinc alone does not change COV's enzymatic activity. Thus cytochrome oxidase has at least two Zn^{2+} binding sites. One is located inside near D-channel and is responsible for main Zn^{2+} effects on solubilized COX. The second is somewhere outside and becomes accessible only in turning-over COV or in the presence of weak reductants.

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P1.1.42. COMPLEX I IN COLOR - SPECTROSCOPIC CHARACTERIZATION OF MICRO-CYTOCHROME C FUSION PROTEINS

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Complex I (or NADH:quinone oxidoreductase) is the least understood enzyme in the respiratory chain. However, the membrane protruding, hydrophilic domain that contain EPR-detectable iron-sulfur clusters is relatively better understood compared to the virtually unexplored membrane spanning domain that must harbour important parts of the proton pump but completely lack distinct spectroscopic features.

We have introduced covalently bound heme into the hydrophobic domain of *Escherichia coli* Complex I by constructing fusion proteins of membrane subunits to a 26 amino acid micro-cytochrome *c* domain, as described in [1].

The spectral characteristics of heme are to a high degree influenced by its immediate environment. Changes in the environment induce shifts in the absorption and emission spectra of the chromophore. The redox characteristic of a cytochrome is also largely dependent on to what degree the heme group is shielded by surrounding protein. We have compared the properties of the small micro-cytochrome c domain expressed alone with the fusion proteins expressed individually and within Complex I.

The investigation is performed on a high resolution, high sensitivity, single beam spectrophotometer originally designed for experiments on micro-crystals [2]. To facilitate electrochemical analyses of the fusion proteins and to determine midpoint potentials of the heme in different locations, the instrument has been equipped with an electrochemical cell constructed in-house, resulting in a system that has the ability to automatically record series of spectra at predetermined redox potentials. The cell features include computer controlled infusion of reducing and oxidizing agents, temperature control, magnetic stirring and an inert Argon atmosphere in a 500 µl cuvette with 10 mm light path. In addition, high definition spectra can be recorded at down to 4K by cooling the sample in a custom built He-cryostat [3] at well defined redox-potentials.

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P1.1.43. TEMPERATURE EFFECTS ON THE CONTROL AND CAPACITY OF MITOCHONDRIAL RESPIRATION IN PERMEABILIZED FIBERS OF THE MOUSE HEART

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Temperature is among the most important factors affecting metabolic rate. Although mammalian body temperature is ~37 °C, most respiratory studies on heart mitochondria are performed at 25 or 30 °C. In clinical states, heart may face hyperthermia (e.g. fever), or hypothermia (e.g. organ transplant preservation, topical cooling to limit ischemic damage, adjunctive therapy during minimally invasive surgery). Limited information is available on the control of mitochondrial respiratory capacity by temperature in the presence of various energy substrates. In the present study, therefore, temperature effects on mitochondrial respiration were investigated in permeabilized fibers from left ventricles of the mouse. High-resolution respirometers (OROBOROS Oxygraph-2k) were operated to measure mitochondrial respiratory capacities simultaneously at five temperatures (4, 25, 30, 37 and 40 °C) for each mouse left ventricle fiber preparation ($N \geq 4$). At 37 °C, the respiratory control ratio (malate+pyruvate; stimulation by ADP to State 3) was 5.7 ± 1.0 SD, declining to 2.1 ± 0.8 SD at 4 °C. Stimulation of respiration by cytochrome *c* was significant only at 25 °C. State 3 respiration was significantly higher with malate+pyruvate compared to malate+glutamate at 25 - 40 °C, but this was reversed at 4 °C. Parallel electron input into complexes I+II by addition of succinate to malate+pyruvate+glutamate increased State 3 respiratory flux 1.5- to 2.0-fold at all temperatures. Uncoupling by FCCP did not further stimulate respiration, indicating that the phosphorylation system was not limiting, in contrast to rat heart and human skeletal muscle. At 25 °C, respiratory capacity was reduced to 50 % of normothermic level, whereas cooling to 4 °C suppressed respiration to 2.6 %. COX activity was determined with ascorbate (2 mM) and TMPD (0.5 mM) after inhibition of complexes I, II and III. At 25 – 40 °C, COX activity was ~1.3-fold higher than respiration with parallel complex I+II electron input, and >3-fold at 4 °C, but these conditions yield an underestimate of the actual COX excess capacity. These results show that (1) malate+glutamate underestimates complex I capacity by 40 % at physiological temperature, and pyruvate should be added; (2) diagnosis of mitochondrial respiratory function can and should be performed at physiological temperature, using 0.7 mg wet

weight of muscle biopsy; and (3) metabolic shutdown of the heart is extensive at 4 °C but incomplete. The present data provide an important baseline for further studies of mouse heart metabolism, including various genetic models of mitochondrial diseases and dysfunction.

P1.1.44. THE BIOENERGETICS OF SELENATE RESPIRATION IN THAUERA SELENATIS

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Thauera selenatis is a bacterium capable of using the oxyanion selenate (SeO_4^{2-}) as a terminal electron acceptor in anaerobic respiration. *T. selenatis* reduces the soluble selenate initially to selenite (SeO_3^{2-}), and then to insoluble elemental Se. The periplasmic selenate reductase which catalyses the 2-electron reduction of selenate to selenite has been purified and characterised [1]. However, a number of fundamental questions remain unanswered with regard to the bioenergetics of selenate respiration, for example, how does selenate reduction generate a proton-motive force? Additionally, the pathway of electrons from the Q-pool to the selenate reductase has yet to be identified, and the mechanism by which selenite is reduced to elemental selenium is unclear. The transfer of electrons from the Q-pool to selenate reductase may be mediated by a membrane bound multi-haem quinol dehydrogenase of the NapC/NirT family [2], or transferred from a cytochrome bc_1 complex in the membrane to the selenate reductase via a soluble *c*-type cytochrome. Reduction of selenite to selenium may also occur in the periplasm, in a manner similar to nitrite reduction, although no specific selenite reductase has yet been identified. We propose a mechanism by which selenate reduction can generate a pmf and cycle electrons back into the Q-pool: when selenite is generated in the periplasm by the action of selenate reductase, it is transported into the cytoplasm via a selenite/selenide antiporter. An NADH dependent reductase in the cytoplasm similar to the assimilatory nitrite/sulfite reductases [3] could then catalyse the 6-electron reduction of selenite to selenide (Se^{2-}). Selenide is then transported back across the cytoplasmic membrane, where a putative selenide oxidoreductase functions to oxidise selenide to selenium and return 2 electrons to the Q-pool. An overview of recent work to elucidate the bioenergetic pathway of selenate respiration will be presented.

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P1.1.45. BIOENERGETIC EVALUATION OF ENVIRONMENTAL FACTORS IN THE RAPID ECOLOGICAL CROP TRIAL

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Introduction: Light and nutrient energy transformation are investigating at all levels of the plant organization from molecule up to biogeocenosis. Three living cell convertible "energy currency" (ATF, the H^+ potential and the Na^+ potential) is a mean of utilization of external energy sources in the performance of useful work [1]. Useful work of agricultural plants is greatest possible productivity.

Material and Methods: Potato plants (*Solanum tuberosum L.*) were grown in Northwest of Russia (58°26'N-73°30'N, 27°45'E-66°10'E). Experiments were conducted as the rapid ecological crop trial (RECT method) [2]. Plant productivity, soil bioenergetic potential and photosynthetic active radiation energy potential [3] were estimated.

Results and Conclusions: Influence of these characteristics on commodity productivity are determined as significant. Using agrophytocenosis energy constituents it was described 82,8-95,4 % of productivity variability. Productivity variability was depended on factors of each of soil mobile element (N_{total}, P_2O_5, K_2O) energy and photosynthetic active radiation energy as 2,8-23,0 % and 2,3-10,3 % correspondingly. The energy potential of these factors was acted on productivity with low positive significant correlation.

The evaluation of external energy sources is efficiently to study productivity process 1. for mathematical modelling at an assessment of agriculture systems, 2. for energy source economy in agriculture, 3. for comparison of different species energy utilization.

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P1.1.46. THE 'E-PATHWAY HYPOTHESIS' OF TRANSMEMBRANE ELECTRON TRANSFER ASSISTED BY TRANSMEMBRANE PROTON TRANSFER IN DIHAEM-CONTAINING QUINOL:FUMARATE REDUCTASES

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Experiments performed with inverted vesicles and proteoliposomes containing QFR demonstrated that the reaction catalysed by the dihaem-containing quinol:fumarate reductase (QFR) from *Wolinella succinogenes* is not directly associated with the generation of a transmembrane electrochemical proton potential (reviewed in [1]). However, the three-dimensional structure of this membrane protein complex, initially solved at 2.2 Å resolution [2], revealed locations of the active sites of fumarate reduction [3] and of menaquinol oxidation [4] that are oriented towards opposite sides of the membrane, thus indicating electrogenic catalysis. A hypothesis has been presented, the so-called "E-pathway hypothesis", which reconciles these apparently conflicting experimental observations [5]. Theoretical [6] and experimental [7, 8, 9] results support this hypothesis. New results of measurements on proteoliposomes containing either wild-type or variant QFR enzymes will be presented.

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P1.1.47. THE ROLE OF THE 11.5 KDA SUBUNIT OF MITOCHONDRIAL COMPLEX I IN ENZYME ACTIVITY

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The respiratory chain of the mitochondrial inner membrane includes proton-pumping complex I, which catalyses electron transfer from NADH to ubiquinone. Mammalian complex I exists in two slowly equilibrating forms: active (A) and de-actived (D). A and D forms differ in their catalytic and structural properties. Only the A form can catalyze the rapid rotenone-sensitive NADH-ubiquinone reductase reaction, whereas the D form is not able to catalyze this reaction, but it can catalyze the oxidation of NADH by artificial electron acceptors. These two forms have different sensitivities to SH-reagents and only the de-activated form is susceptible to the action of SH-inhibitors. It was discovered that complex I from *N. crassa* also exhibits active/de-active transitions, though with different characteristics (2). As revealed by studies with the fluorescent analogue of NEM, one of the bovine complex I subunits with a molecular mass of 15 kDa is specifically labelled in the D-form of the enzyme (3). Several subunits of bovine complex I are candidates for this labeling, such as IP15, IP13, B14.7 and B13. All of them have *N. crassa* homologues, but only the fungal homologues of the IP15 and IP13 have cisteine residues that can bind SH-reagents. The *N. crassa* 11.5 kDa polypeptide is homologous to the IP15 subunit of bovine complex I and contains four conserved cysteine residues. To elucidate the role of this protein in the active/de-active transition, we produced a nuo11.5 null-mutant (1) and a series of site-directed mutants, in which the individual cysteine residues were independently altered for serine residues. The assembly and catalytic properties of complex I, as well as the active/de-active transition process, have been studied in the mutants and results will be presented.

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P1.1.48. ATR-FTIR REDOX DIFFERENCE SPECTROSCOPY OF YARROWIA LIPOLYTICA AND BOVINE COMPLEX I

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Electrochemically-induced redox difference ATR-FTIR spectroscopy was used to investigate *Yarrowia lipolytica* and bovine complex I. The redox spectra show broad similarities with previously published data on *Escherichia coli* complex I (1). Comparisons of amide I/II changes that dominate the redox IR spectra of complex I with redox IR spectra of small model ferredoxins demonstrate that they arise primarily from characteristic structural changes local to the iron-sulfur centers rather than more global alterations. Bands arising from substrate ubiquinone were evident, as was a characteristic 1405 cm^{-1} band of the reduced form of the FMN cofactor. Other signals are likely to arise from perturbations or protonation changes of a carboxylic amino acid, histidine and, possibly, several other specific amino acids. Redox difference spectra of center N2, together with substrate ubiquinone, were isolated from those of the other iron-sulfur centers by selective redox potentiometry. Its redox-linked amide I/II changes were typical of other 4Fe-4S iron sulfur proteins. Features of the substrate ubiquinone associated with the center N2 spectrum were particularly clear, with firm assignments possible for bands from both oxidized and reduced forms and the data could be used to estimate stoichiometry and midpoint potential.

Comparable redox difference spectra could also obtained by perfusion with NADH/NAD^+ and these will be compared with electrochemically-induced redox difference spectra in order to address concerns of whether substrate reduction may induce additional physiologically-important changes.

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P1.1.49. THE SACCHAROMYCES CEREVISIAE MITOPROTEOME PLASTICITY IN RESPONSE TO RECOMBINANT ALTERNATIVE UBIQUINOL OXIDASE

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The energy-dissipating alternative oxydase (AOX) from *Hansenula anomala* was expressed in *Saccharomyces cerevisiae*. We have found that the recombinant product is properly addressed to the mitochondria where it was functional. A comparative analysis by two-dimensional differential in-gel electrophoresis (2D-DIGE) of mitochondrial protein patterns found in wild-type and recombinant AOX strains was performed. This analysis has shown that AOX expression affects energy-related enzymes in a very specific manner: 60 proteins exhibiting a significant difference in their abundance were identified and were implicated in major metabolic pathways such as Krebs cycle and amino-acid biosynthesis. At the level of the respiratory chain, we found a ~1.4-fold increase in the proton pumping complex III (ubiquinol-cytochrome c oxidoreductase), which competes with AOX for the reduced Q, as well as a ~1.7-fold increase in the succinate dehydrogenase that delivers electrons produced by the reduction of succinate to the Q pool. In addition, the NADH-cytochrome *c* reductase (MCR1) that transfers electrons from the externally-produced NADH directly to cytochrome *c* was ~1.4-fold greater in AOX^+ mitochondria. This increase in MCR1 could force electrons to pass through the cytochrome *c* oxidase, the other proton pumps of the yeast respiratory chain. Up-regulation of the complex III as well as of MCR1 would influence the electron partitioning at the level of the Q pool in favour of the cytochrome pathway in order to diminish the impact of recombinant AOX on oxidative phosphorylation and energy conservation. Surprisingly, this up-regulation of the respiratory-chain was associated with a down-regulation of the ATP synthase complex. This decrease in ATP synthase content would allow the establishment of a novel steady state between the rate of $\Delta\mu H^+$ building and the rate of $\Delta\mu H^+$ consumption, favourable to the ATP synthase to perform ATP synthesis

P1.1.50. THE NHAD ANTIPORTER FROM THE NQOB OPERON OF RHODOTHERMUS MARINUS COMPLEX I

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The NADH:menaquinone oxidoreductase (complex I) is present in the respiratory chain of the thermohalophilic bacterium *Rhodothermus marinus*. The genes encoding the *R. marinus* complex I subunits were recently sequenced, clustering in two operons [*nqo1* to *nqo7* (nqo_A) and *nqo10* to *nqo14* (nqo_B)] and two independent genes (*nqo8* and *nqo9*). Two genes encoding homologues of a NhaD Na^+/H^+ antiporter (NhaD) and of a pterin-4α-carbinolamine dehydratase (PCD) were identified within nqo_B, flanked by *nqo13* and *nqo14*. Moreover, RT-PCR experiments showed that *nhaD* and *pcd* are co-transcribed with the other complex I genes encoded by nqo_B. (1) A characterization of NhaD is presented and its possible relation with *R. marinus* complex I is discussed.

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P1.1.51. PROBING THE UBIQUINOL-BINDING SITE IN CYTOCHROME bd BY SITE-DIRECTED MUTAGENESIS

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Cytochrome *bd* is one of two terminal ubiquinol oxidases in *Escherichia coli*, and generates proton-motive force. In the periplasmic loop VI/VII (Q-loop) of subunit I, binding of monoclonal antibodies to ^{252}KLAAIEAEWET^{262} (1) and proteolytic cleavage with trypsin at Tyr290 or chymotrypsin at Arg298 (2, 3) suppressed ubiquinol oxidase activity. Photoaffinity labeling studies with azidoquinols indicated that Glu280 in Q loop could form a hydrogen bond(s) to the 2- and 3-methoxy groups on the quinone ring (4). Inhibitor binding studies indicate the close proximity of heme b_{558} to the quinol oxidation site (5). These findings suggest the presence of the ubiquinol oxidation site in Q loop, which transfers electrons to heme b_{558} bound to the periplasmic ends of helices V and VII. To probe the structure of the quinol oxidation site in Q loop, we substituted three conserved residues (Gln249, Lys252 and Glu257) in the N-terminal region and three glutamates (Glu278, Glu279, and Glu280) in the first internal repeat. We found that substitutions of Glu257 by Ala or Gln, and Glu279 and Glu280 by Gln severely reduced the *in vivo* and *in vitro* activity and the expression level of cytochrome *bd.* In contrast, Lys252 mutations reduced only the oxidase activity. Blue shifts in the 440- and 629.6-nm peaks of the reduced Lys252 mutants and in the 561-nm peak of the reduced Glu257 mutants indicate the proximity of Lys252 to the heme b_{595}-*d* binuclear center and Glu257 to heme b_{558}, respectively. Perturbations of reduced heme b_{558} upon binding of aurachin D support structural changes in the quinol-binding site of the mutants. Kinetic analysis of the ubiquinol-1 oxidation suggests that Lys252 and Glu257 are directly involved in the quinol oxidation, and that both Gln249 and Glu280 contribute to the substrate binding. In conclusion, mutational studies on the N-terminal region of Q-loop identified the key residues for the binding and oxidation of substrates by cytochrome *bd*-type quinol oxidase.

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P1.1.52. THE SMALLEST FUNCTIONAL UNIT OF COMPLEX I IS COMPOSED OF 11 PROTEIN SUBUNITS

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Respiratory chain Complex I is a complex enzyme that has evolved from the combination of smaller functional building blocks. NuoG resembling a Fe-only hydrogenase has combined with NuoE and F to form the NADH dehydrogenase module, NuoB and D, resembling a soluble NiFe hydrogenase, form together with NuoH, I and N a membrane-bound hydrogenase. NuoKLM finally, comprise the antiporter module [1, 2]. Some membrane-bound hydrogenases contain more than one antiporter-like polypeptide, but no hydrogenase containing NuoA has been found to date. In most bacteria the Complex I encoding genes are present as a gene cluster or operon, but there are exceptions. In at least one bacteria, *Aquifex aeolicus*, a gene cluster encoding 11 Complex I polypeptides is present with NuoEF and NuoG present in the purified Complex I but expressed from other locations on the chromosome [3]. Chloroplasts and cyanobacteria have an enzyme containing 11 standard Complex I proteins, but lack NuoE, F and G. It has been suggested that the NADH dehydrogenase module have been replaced with other proteins [1], and several studies have attempted to identify such alternative partners. A similar situation exists in some archea, such as *Methanosarcina mazei* and *Archeoglobus fulgidis* where FpoF seemingly replace NuoEFG and function as a F_{420} dehydrogenase module [4].

In this work we report a detailed bioinformatic survey of the distribution, properties and putative function of the 11 subunit Complex I and their different alternative modules. Taken together, such 11 subunit Complex I enzymes are much more common in nature than what was previously thought. We found that some other archea, like *Thermoplasma acidophilum*, contain a gene cluster encoding the 11 NuoABCDHIJKLMN equivalent polypeptides, but contain neither a FpoF nor NuoEFG encoding genes. The same situation is seen in other phyla such as green sulfur bacteria, black pigmented Gram-negative bacteria and Gram-positive bacteria from the low GC group. These enzymes are decisively not hydrogenases. Furthermore, they form several distinct subgroups that differ both from each other and from classical 14 subunit Complex I, revealing interesting aspects of the structural and functional evolution of the Complex I enzyme family.

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P1.1.53. TOXICITY OF CARBARYL ON HEPATIC MITOCHONDRIAL BIOENERGETICS – SELECTIVE INHIBITION OF THE MITOCHONDRIAL RESPIRATORY CHAIN

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Insecticides and other pesticides occupy an important position among the wide range of compounds used by humans in agricultural practices. Nevertheless, such compounds present a risk for human health and can afford collateral damage to other animal species.

Carbaryl, an insecticide belonging to the carbamate class, is generally considered a safe insecticide (1) although some safety issues remain to be explored (2) One such issue is mitochondrial toxicity. So far, no work has been done concerning the toxicity of carbaryl at the mitochondrial level. In this in mind, our objective was to test the effects of carbaryl on isolated liver mitochondria, a model already used with other insecticides and pesticides (3,4).

Mitochondria were isolated from the livers of Wistar rats and mitochondrial function was evaluated in the presence and absence of carbaryl, namely concerning parameters like mitochondrial respiration, membrane potential, membrane integrity and enzyme activity.

It was observed that carbaryl, in a range of concentrations between 0.2 and 1 mM has a depressive effect on mitochondrial respiration and on the generation of mitochondrial membrane potential, although membrane integrity was not affected. Succinate dehydrogenase and cytochrome c oxidase were both affect by carbaryl. Mitochondrial phosphorylation system was not significantly affected by carbaryl.

The results permit us to conclude that carbaryl inhibits mitochondrial respiration due to an effect on the mitochondrial respiratory chain and not due inhibition of phosphorylative systems. Comparison of results with data from other works involving pesticides and insecticides allow us to conclude that is carbaryl is mitochondrially toxic for higher concentrations.

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P1.1.54. OH RADICAL FORMATION FROM THE LYSOSOMAL ELECTRON CARRIERS

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Ubiquinone (UQ) is widely distributed in the living nature,suggesting the maintenance of homeostasis in biological systems.The isoprenic side chain which is linked to the bionergetic active benzoquinone ring ankers UQ in the lipid phase of biomembranes.Despite the high partition coefficient UQ is inhomogenously distributed in biomembranes suggesting a functional role in UQ-enriched biomembranes.The functional role of UQ in mitochondria was extensively studied since more than four decates.Less is known about the role of UQ in Golgi vesicles.Fred Crane suggested a role in electron transfer combined with proton translocation.In plasma membranes UQ is involved in electrn and proton transport.Nothing is known so far upon the role in lysosomes although lysosomes have the third highest UQ concentration among organelles.We suggest an accumulation through digested mitochondria is the reason why lysosomal UQ concentration is such high.We also suggest that this high UQ concentration has functional activities.The chemistry of UQ reveals a reversible addition of electrons and protons.Driving force are redox couples due to reductants and oxidants.The resulting UQ species differ in their charge and protonation state.If these species are produced in lipid-membranes they are distributed accross the whole span of the membrane.This is a prerequisite for linear electron transfer and unisotropic proton translocation.We isolated lysosomes and purified them by isopycnic centrifugation using an Iodixanol as a self-generating gradient.With this methode we enriched lysosomes by a factor of 13,4 with neglectable contaminations of microsomes and mitochondria.We found an NADH-oxidase in combination with FAD which reduces UQ stepwise until Ubiquinol is built.Acid hydrlyses require pH values down to 5.5.In ananlogy to mitochondria we suppose that UQ can also transport protons into lysosomes.This was prouved with TEMPAMINE.Tempamine is arrested in lysosomes due to protonation.We could titrate proton translocationusing higher NADH levels.In the absence of oxygen there was no proton translocation.According to the chemistry of UQ the reduction state was performed via Ubisemiquinone.Ubisemiquinone in lysosomes autoxidize since there is no stabilization,which gives rise to superoxide formation, and further due to the low pH forms rapidly hydrogeneperoxide and is transformed to OH radicals due to the presence of iron or ubisemiquinone oxygen.

P1.1.55. WHY IS PROTON-TRANSLOCATING TRANSHYDROGENASE A DIMER?

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Transhydrogenase couples the redox reaction between NADH and $NADP^+$ to proton pumping across a membrane. The enzyme has three components: dI, which binds NAD(H), and dIII, which binds NADP(H), protrude from the membrane. The dII component spans the membrane and contains the proton-translocation pathway. Transhydrogenase is a "dimer" of two dI-dII-dIII monomers although the polypeptide composition varies between species. The structure of a complex of dI and dIII (the "dI_2dIII_1 complex") is asymmetric, raising the question as to whether the intact enzyme is a functional dimer.

We are addressing this issue by making mutations at the monomer-monomer interface of the dI dimer of transhydrogenase from *Rhodospirillum rubrum*. The *R. rubrum* dI can be built into an intact transhydrogenase or into a dI_2dIII_1 complex by appropriate reconstitution. We have isolated a recombinant dI in which the invariant interface residue, Y146, is substituted with A. Unlike wild-type dI, which is a very tight dimer, gel filtration and analytical ultracentrifuge experiments indicate that dI.Y146A is in a monomer-dimer equilibrium with a (preliminary) K_d in the range of ~10 μM. Nevertheless, Trp fluorescence experiments show that the mutant protein (at ~1 μM) binds NADH with the same affinity as the wild-type protein. In steady state experiments the rate of hydride transfer (measured from "cyclic transhydrogenation") in complexes of dI.Y146A and dIII at 10 - 100 nM, is ~zero. However, the addition of dI.Y146A to wild-type complexes inhibits cyclic transhydrogenation. In stopped-flow experiments on complexes of dI.Y146A and dIII at ~100 μM, the first order rate constant for hydride transfer is ~200 s^{-1}, only 2-3 fold slower than in the wild-type dI_2dIII_1 complex.

These results suggest that dimeric dI.Y146A forms complexes with dIII that are moderately active in hydride transfer. However, monomeric dI.Y146A binds to dII to form inactive complexes.

P1.1.56. STRUCTURAL MEMBRANE PROTEOMICS ON BIOENERGETICALLY RELEVANT PROTEIN COMPLEXES FROM THE HYPERTHERMOPHILIC EUBACTERIUM AQUIFEX AEOLICUS

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Respiratory and photosynthetic proteins are the most abundant ones in native membranes. Therefore they can be isolated in sufficient quantities for structural studies. Hyperthermophilic organisms have a maximal growth temperature of up to 120˚C. Proteins from these organisms are considered to be more stable and more rigid than their mesophilic counterparts. Therefore, the chance to obtain stable, homogeneous and crystallizable membrane protein complexes is higher with complexes from (hyper)thermophilic organisms than from mesophilic ones. *Aquifex aeolicus* is such a hyperthermophilic eubacterium with known genome (Deckert, et al., 1998). We had purified and characterized several of the respiratory chain complexes from this organism, namely complex I (Peng, et al., 2003), complex V (F_1F_o-ATPase), and a supercomplex containing complexes III and IV. Crystallization attempts have led to crystals of the latter. Characterization of these multi-subunit complexes by mass spectrometry allowed to find out which of the subunit that are encoded by homologous genes, are actually present in the isolated complexes. Other bioenergetically relevant membrane protein complexes from *Aquifex aeolicus*, like cytochrome *bd* (a quinol oxidase), a sulfide-quinone oxidoreductase and several electron transfer protein complexes are also being isolated and/or characterized biochemically. These results provide hints on sulfur respiration and its adaptation in *Aquifex aeolicus*.

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P1.1.57. ATP SYNTHASE AS A SYSTEM FOR MOLECULAR FOLDING OF RECOMBINANT PROTEINS

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Enzyme ATP synthase is an advantageous object for molecular genetic manipulation including foreign protein fusion. It can also be used for engineering of recombinant proteins.

Mostly human proteins produced for pharmaceutical purposes require a refolding step *in vitro*, because recombinant polypeptides expressed in bacterial or yeast host systems are not able to get functionally right molecular structure. The human recombinant proteins need post- translational refolding to be biological active substances. To built accurately disulfide binding *in vitro* of purified recombinant protein is time-consuming and expensive procedure.

This study is a presentation how to use the ATP synthase as a system to achieve the correct molecular conformation of expressed recombinant protein which is folded *in vivo* in host cells.

P1.1.58. PATHWAYS OF NONCOUPLED AND UNCOUPLED RESPIRATION IN PLANT MITOCHONDRIA

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The term “free respiration” includes the pathways of non-coupled respiration (which is provided by some NAD(P)H dehydrogenases and alternative oxidase) and uncoupled respiration than uncoupling is mediated by free fatty acid cycling fascilated by uncoupling proteins or ATP/ADP antiporter [1].

It was shown in our experiments that free respiration has induced in the cases of plant adaptation. Flowering of *Arum orientalis* causes coordinated induction of alternative oxidase and rotenone-insensitive NADH dehydrogenases. It was established that mitochondria isolated from thermogenic tissues of this plant demonstrate significantly elevated levels of activities of two non-coupled NADH dehydrogenases oxidizing intramitochondrial and cytoplasmic NADH pools. It is postulated that operation of a completely non-coupled respiratory chain consisting of non-coupled NADH:quinone oxidoreductases and cyanide-resistant alternative quinoloxidase is the main mechanism of heat production in thermogenic plants [2].

Generally alternative oxidase is one of well-studied pathways of free oxidation, but its physiological significance in plants is still undo discussion. We have found that inhibition of alternative oxidase caused an increase in production of reactive oxygen species. We describe the influence of inhibitors of the cytochrome oxidase pathway and of the alternative oxidase on respiration rates and the production of reactive oxygen species in pea mitochondria. Using spin traps and epinephrine oxidation, it was shown that the hydrogen peroxide accumulation in pea mitochondria was due to a substantial increase in the rate of superoxide radical production.

Low temperature was effective inductor of uncoupling through ATP/ADP antiporter in potato mitocondria. Cold exposure of tubers *in vivo* for 48-96 h resulted in some uncoupling that could be completely reversed by BSA and partially by ADP, ATP, UDP, carboxyatractylate and atractylate. The recoupling effects of nucleotides were absent when the nucleotides were added after carboxyatractylate. This indicates that the cold-induced fatty acid-mediated uncoupling in potato tuber mitochondria is mostly due to the operation of ATP/ADP antiporter rather than uncoupling proteins.

So the non-coupled substrate oxidation mediated by components of the electron transport chain and uncoupled respiration are specific features of plant mitochondria. It is proposed that non-coupled oxidation could regulate the level of reduction of components of the electron transport chain, heat production and the rate of one-electron reduction of oxygen, thereby affecting the rate of formation of reactive oxygen species.

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P1.1.59. CALCULATION OF MIDPOINT REDOX POTENTIALS OF COFACTORS IN PHOTOSYSTEM I AND FERREDOXINS

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The midpoint redox potentials (E_m) of iron-sulfur (Fe_4S_4) clusters in two soluble ferredoxins from *Azotobacter vinelandii* and *Clostridium acidiurici*, and clusters F_X, F_A and F_B in photosystem I (PS I) were calculated using the crystal structure of ferredoxins and the trimeric PS I complex from *Synechococcus elongatus.* We used a phenomenological electrostatic approach based on the classical Poisson–Boltzmann equation. The influence of protein environment on the redox properties of the cofactors was analyzed using a model that considered protein and aqueous solution as homogeneous dielectric mediums with different dielectric permittivities. The effects of (*i*) permanent charges, (*ii*) charged amino acid residues and (*iii*) solvation energy changes on redox potentials of cofactors were described by a unified set of model parameters.

The presentation of microscopically heterogeneous proteins as a uniform dielectric medium with a single permittivity is still discussed. In particular, the permanent charges (mainly the partial atomic charges of backbone) have two effects on the protein electrostatics: (1) they create an intraprotein electric field, and (2) the appearance of electric field induces a shift in the equilibrium positions of permanent charges, which is a part of the total dielectric polarization of the protein. To overcome this inconsistency, Krishtalik et al. introduced in the theory two dielectric constants, the optical ε_o and the static ε_s ones [1].

Our calculations showed that the experimental E_m values of redox cofactors could be obtained with the accuracy of ~60 mV only by using two different dielectric permittivities, ε_o (about 2.5) for permanent charges preexisting in the crystal structure and ε_s (derived from photoelectric measurements) for the charges, which do not exist in the crystal structure (solvation energy changes caused by redox transitions of the cofactors and protonation of amino acid residues). We estimated the E_m values of Fe_4S_4 clusters in ferredoxins from *A. vinelandii* and *C. acidiurici* to be –610 and –435 mV, respectively. The E_m values calculated for F_X, F_A and F_B in PS I from *S. elongatus* were –710, –600 and –660 mV, respectively. All these values are within the accuracy of 60 mV to those obtained experimentally.

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P1.1.60. PROTON TRANSFER PATHWAYS IN NITRIC OXIDE REDUCTASE (NOR) AND cbb_3-TYPE OXIDASES

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The superfamily of heme-copper oxidases (HCuOs) is comprised of terminal oxidases reducing oxygen to water as the last step in the respiratory chain. The super-family also has bacterial nitric oxide reductase (NOR) as a divergent member. NOR catalyses the reduction of NO ($2NO+2e^-+2H^+ \rightarrow N_2O$) as part of the denitrification process. Among the conventional HCuOs, the *cbb_3-type* oxidases have been shown to be the HCuOs with the highest capability of reducing NO. TheNORs and *cbb_3*s share a high sequence similarity and are hence thought to be evolutionary closely related. The fact that they are involved in two different metabolic pathways, NOR in nitrogen respiration and *cbb_3* in oxygen respiration, but can catalyse the same reactions makes them interesting study objects. The mechanism of NO reduction is still unclear and we are currently comparing NO reduction characteristics of these two enzymes.

We have studied charge transfer reactions in NOR from *Paracoccus denitrificans* during NO and O_2 reduction using electrometrical measurements. Our data confirm NOR's exceptional electrically silent character among the heme-copper oxidases, i.e. there is no contribution to the generation of a membrane potential. This means that, in contrast to other HCuOs, NOR seems to accept electrons and protons from the same side of the membrane, the periplasm. Thus, NOR must provide a proton transfer pathway leading from the periplasm down to the catalytic site. Homology modelling in combination with biochemical studies on mutated potential proton channelling residues allows us to describe a hypothetical proton transfer pathway.

Cbb_3–type oxidases are conventional terminal oxidases that translocate protons across the membrane during the conversion of O_2 to H_2O. However, the paths of the chemical and pumped protons through the enzyme are unknown, since the *cbb_3* oxidases lack the conserved amino acids shown to play these roles in traditional HCuOs. Also, whether different proton transfer pathways are used during NO reduction is unknown. This question is addressed using homology modelling and a series of biochemical and biophysical techniques.

P1.1.61. DIFFERENTIAL STABILITY OF DIMERIC AND MONOMERIC CYTOCHROME C OXIDASE EXPOSED TO ELEVATED HYDROSTATIC PRESSURE

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Dimeric and monomeric bovine heart cytochrome *c* oxidase (CcO) respond very differently when exposed to elevated hydrostatic pressure. The activity of dimeric CcO is unaffected following exposure to 2-3 kbar of hydrostatic pressure for 2 hr while the activity of monomeric CcO significantly decreases in a time- and pressure-dependent manner. Pressure-induced inactivation is essentially irreversible since the original activity is not recovered even 9 hours after CcO is returned to normal atmospheric pressure. Inactivation correlates with changes in the enzyme's quaternary structure. Dimeric CcO is very stable and is structurally unaltered after 2 hr exposure to 3 kbar hydrostatic pressure. However, these same conditions trigger the selective and sequential dissociation of four subunits from monomeric CcO, i.e., dissociation of subunits VIa &VIb, followed by dissociation of subunits VIIa & III. Activity loss correlates with dissociation of subunits VIIa & III and a concomitant generation of a 9-subunit complex. This is the first demonstration that dimeric CcO is structurally and functionally important.

Enzyme depleted of subunits VIa & VIb is even more susceptible to pressure-induced damage than is the 13-subunit, monomeric enzyme. The 11-subunit complex is immediately inactivated and subunits III & VIa dissociate upon application of 3 kbar of pressure. We conclude that subunits VIa and VIb are critically important for maintaining the structural stability of CcO. Not only are they essential for CcO dimerization, but their removal significantly destabilizes the quaternary structure of the remaining CcO core. We conclude, as well, that factors stabilizing the association of these two critical subunits, e.g. the 1-2 cardiolipin bound near subunit VIa, are also essential for maintaining the structural stability of CcO.

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P1.1.62. CHANGES IN THE RESPIRATORY CHAIN ACTIVITY OF MITOCHONDRIA OF FROG OLFACTORY EPITHELIUM UNDER AN EXPOSURE TO ODORANTS

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INTRODUCTION: Changes in respiratory chain activity of mitochondria of frog olfactory epithelium under an exposure to odorants were shown previously. However, the kinetics of these reactions and mechanisms of effect of odorants in the exposure are still poorly studied. MATERIAL AND METHODS: Fluorescence of NADH and oxidized flavoproteids (OFP) in an isolated frog olfactory epithelium in responce to odorants was recorded. RESULTS: Duration of NADH and OFP reactions, and front slope stepness of NADH reactions on amyl alcohol (AA) were more expressed than those on ammonia. After inhibition of an initial domain of the respiratory chain of olfactory epithelium mitochondria by rotenone, NADH and OFP response on ammonia reduced in amplitude. Front slope stepness of NADH reaction and its duration diminished. In inhibiting succinate dehydrogenase (SDG) by malonate, NADH and OFP reactions on AA reduced essentially in amplitude. Front slope stepness of NADH reaction diminished. CONCLUSION: Pronounced duration of NADH and OFP reactions, and large front slope stepness of NADH reactions caused by AA are supposed to be stipulated by participation of the intracellular signal system (cAMP) and Ca^{2+}, whereas ammonia penetrates into mitochondria of olfactory cells and treats them directly. Presumably rotenone and ammonia interact competitively with mitochondria and SDG is of importance in signal transduction from AA.

P1.1.63. SUBSTRATE-BINDING SITES ON AN EXTREMOPHILE-TYPE (SOXB-TYPE) CYTOCHROME C OXIDASE STUDIED BY SITE-DIRECTED MUTAGENESIS

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Heme-copper oxidases can be classified into three subfamilies; SoxM-type, SoxB-type and FixN-type (cytochrome cbb_3) based on the amino-acid sequence and the subunit composition. All mitochondrial oxidases and many bacterial ones are SoxM-type, while SoxB-type oxidases have been found in prokaryotes inhabiting in severe environments. The latter enzymes fundamentally conserve the structure of the redox active center and proton-pumping activity as the former ones, however, largely differ from them in structures of the proton-conducting pathway and the substrate-binding site, and also in substrate-docking properties. Cytochrome bo_3 is a SoxB-type cytochrome *c* oxidase isolated from a thermophilic Bacillus and has been suggested to interact with the intrinsic substrate cytochrome *c*-551 mainly through hydrophobic interaction (1-3). This is in contrast to SoxM-type oxidases, in which electrostatic bonds are the main factor in the enzyme-substrate docking process; between the acidic patch of the enzyme mainly on its subunit II and cytochrome *c* the basic substrate protein. In our previous report, the above suggestion has been confirmed by using site-directed mutants of cytochrome *c*-551 (4). Here, we have constructed a mutant-enzyme expression system for *Bacillus* bo_3 itself and have engineered several mutations on its subunit II; D49N, E64Q, E66Q, E68Q, E84Q, D99N, E116Q and E139Q. The analyses of their enzyme activity suggest that there are two substrate-binding sites on the subunit; one is located closely to Cu_A center and shows a high affinity to the substrate, while the other far from the center with lower affinity. In both cases, acidic residues contribute to the docking process but to a limited extent.

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P1.1.64. DOES THIOCYANATE INHIBIT GASTRIC JUICE SECRETION BY PROTONOPHORIC ACTIVITY?

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The gastric mucosa secretes a solution of hydrogen chloride (HCl) in the glandular lumen after stimulation by histamine, acetylcholine, or gastrin. Thiocyanate (SCN^-) is long known as a potent inhibitor of gastric acid secretion (1). For explanation, it has been proposed that SCN^- induces a backflux of H^+ from the acidic lumen of the stomack into the secreting cells (parietal cells), thereby decreasing the net secretion of HCl (2,3). This could suggests that SCN^- exhibits protonophoric activity at acidic pH. At pH < 4, the concentration of protonated SCN^- in the lumen was estimated to be sufficient for remarkable H^+ backflux (2), suggesting that protonophoric activity of SCN^- is not in contrast with acidic power of thiocyanic acid (pK_a = -1.28). Moreover, it has been demonstrated that SCN^- permeates black lipid "membranes" as anion and, much faster in the protonated form (2). The latter suggests that protonated SCN^- permeates biological membranes by passing protein-free hydrophobic regions. In general, SCN^- is transported by Cl^- channels across biological membranes. Even though SCN^- and Cl^- have similar physicochemical properties, in most Cl^- channels, SCN^- shows a higher permeability than Cl^- itself (4).

We examined protonophoric activity of SCN^- across membranes by swelling measurements using isolated rat liver mitochondria (RLM). RLM suspended in KSCN medium plus nigericin (a H^+/K^+-exchanger) swelled when the medium-pH was slightly acidic, indicating that SCN^- initiates a transfer of H^+ across the inner membrane. In contrast, in KCl medium plus nigericin, mitochondria did not swell.

To rationalize the protonophoric activity of thiocyanate, we considered the dehydration of SCN^- to be critical for transmembranal H^+ transfer. For modelling this process, the hydration energies of various hydrate clusters of SCN^- and Cl^- were were calculated by quantum chemical methods. The calculated hydration energies led to the conclusion that SCN^- is less efficiently solvated than Cl^-. Due to the more easy removal of the hydration shell of SCN^- relative to Cl^-, SCN^- is favoured in going across the membrane giving rise to the protonophoric activity.

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P1.1.65. 3D-ARCHITECTURE OF THE RESPIRATORY CHAIN SUPERCOMPLEX $I_1III_2IV_1$ FROM BOVINE HEART MITOCHONDRIA

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In the inner mitochondrial membrane the respiratory chain complexes (complexes I-IV) generate an electrochemical proton gradient, which is utilised to synthesise most of the cellular ATP. According to an increasing number of biochemical studies these complexes are assembled into supercomplexes where a direct substrate channelling can take place and the individual complexes might stabilise each other in these multi-complex assemblies. Respiratory supercomplexes of different compositions have been found in bacteria, e.g. *Paraccocus denitrificans* (1), and in mitochondria from *Saccharomyces cerevisiae* (2), other fungi (3), higher plants (4) and mammals (2,5) by means of blue-native PAGE and gel filtration. However, little is known about the architecture of the proposed multi-complex assemblies. Only a projection map of a supercomplex consisting of complex I and dimeric III (III_2) from plant mitochondria has been published (6) so far.

Here, we report electron microscopic proof of the two respiratory chain supercomplexes I_1III_2 and $I_1III_2IV_1$, also known as supercomplex a and b, respectively, from bovine heart mitochondria. They are the two major supercomplexes in bovine and human mitochondria. After purification and demonstration of enzymatic activity, their structures in projection were determined by electron microscopy and single particle image analysis. A difference map between the supercomplexes I_1III_2 and $I_1III_2IV_1$ closely fits the X-ray structure of monomeric complex IV and shows where it is located in the assembly. By comparing different views of supercomplex $I_1III_2IV_1$, two models of the the location and mutual arrangement of complex I and the complex III dimer were proposed.
A negative stain 3D map of supercomplex $I_1III_2IV_1$ was created by electronmicroscopic random conical tilt studies and single particle analysis. With this 3D map the positions and orientations of all complexes in the supercomplex were determined and one of the two models could be verified.

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P1.1.66. THE INTRAPROTEIN PROTON TRANSFER IN THE D-CHANNEL MUTANTS OF RHODOBACTER SPHAEROIDES CYTOCHROME C OXIDASE

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In addition to the protons delivered to the oxygen-reducing center and consumed in water formation, cytochrome *c* oxidase pumps 1 H^+ per electron across the membrane against the proton gradient. The detailed mechanism of the redox-coupled proton transport in cytochrome *c* oxidase remains to be resolved. It is believed that the input protonic D-channel is involved in delivery of protons coupled to transfer of the 3-rd and 4-th electrons during the catalytic cycle. The partial electrogenic steps associated with the one-electron F->O transition (4-th electron in the catalytic cycle) have been identified in the mutant forms of *Rhodobacter sphaeroides* cytochrome oxidase with amino acid substitutions in the D-channel. The single-residue replacements in the D-channel such as N139D or D132N result in decoupling of molecular oxygen reduction and transmembrane proton translocation. Under steady-state conditions, these cytochrome oxidase mutants incorporated in liposomes retain ability to reduce molecular oxygen to the water, but the outward proton release is lost. Time-resolved studies of the single electron F->O transition show that the N139D substitution leads to disappearance of one of the two electrogenic protonic phases, observed in wild type cytochrome oxidase (Siletsky S.A. et al. 2004). The vanished electrogenic phase is linked to proton transfer towards the outer membrane surface, while the retained phase reflects protonation of the reduced oxygen atom in the binuclear center from the internal water volume. A comparison between the electrogenic phases in the N139D and D132N mutants will also be presented. This work was supported by Russian Fund for Basic Research (Grant 06-04-48608).

Siletsky S.A., Pawate A.S., Weiss K., Gennis R.B., Konstantinov A.A. Transmembrane Charge Separation during the Ferryl-oxo -> Oxidized Transition in a Nonpumping Mutant of Cytochrome *c* Oxidase. *J. Biol. Chem.*, 2004, v. 279(50), p. 52558-52565.

P1.1.67. Na^+-PUMPING ATP SYNTHASE COMPOSED OF PROPIONIGENIUM FO AND THE THERMOPHILIC BACILLUS F1

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In a rotory motor F_oF_1-ATP synthase, F_o works as a proton motor in general, but a few species of bacteria such as *Propionigenium modestum* has a sodium motor, in which F_o *c*-subunits have affinity to Na^+ instead of H^+. We constructed recombinant plasmids having the *P. modestum* genes for F_o subunits with *uncI* at the 5'-end, and genes for F_1 of thermophilic Bacillus PS3 at the 3'-end. Some of the plasmids produced functional hybrid F_oF_1 (PF_oTF_1) in *E.coli* cells. The purified PF_oTF_1, showing 8 different subunits including oligomeric *c*-subunits (about 60 kDa, a ring with 11mer)) upon SDS-PAGE, was purified and reconstituted into proteoliposomes and their energy-transducing properties were measured.

In the absence of Na^+, the enzyme pumped H^+ instead of Na^+ upon ATP hydrolysis. In the presence of Na^+, it pumped Na^+ electrogenetically, which were followed by fluorescence increases of Sodium Green (S6900) for Na^+ conc. in liposomal lumen, and 8-anilinonaphthalene 1-sulfonate for membrane potential. The K_m for Na^+ was estimated to be about 0.8 mM (ATP hydrolysis assay) or 1 mM (Na^+-dependence of membrane potential formation). The enzyme synthesized ATP when a certain amount of electrochemical Na^+ gradient was applied. These data, indicating the presence of totally-functional hybrid enzyme, show that the interaction of TF_1 stalk (ge) and PF_o *c*-ring is functional, and the very C-terminus of TF_1 *b*-subunit is necessary to interact with TF_1, since recombination point of two genes was designed at the coding region tether part of PF_o*b*-subunit.

P1.1.68. TEMPERATURE DEPENDENCE OF ELECTRON AND PROTON TRANSFER IN CYTOCHROME C OXIDASES

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The reduction of molecular oxygen to water leads to the built-up of a protonmotive force under the activity of the cytochrome *c* oxidase. During one catalytic turnover, four protons are pumped over the membrane barrier in addition to the ones needed for water formation. We followed the time course of potential built-up after electron injection into the oxidised enzyme with the black lipid membrane technique. Proteoliposomes containing the oxidase are adsorbed to an underlying lipid bilayer leading to capacitive coupling and the recording of electrogenic events in an external circuit. Charge movements catalysed by the oxidase can be initiated by rapid electron injection from the excited state of an organometallic ruthenium complex. The electron enters the enzyme via the binuclear copper centre and is transferred further to a heme *a* moiety in the middle of the membrane dielectric. The charge movement is compensated by proton uptake from the opposite site (Ruitenberg et al., *PNAS* (2000) 97, 4632-4636). This leads to two electrogenic events with distinct relaxation times.

Here, we report the temperature dependence of the electron and proton transfer processes in the range from 5° to 40° C in oxidases from a thermophile, the ba_3-oxidase from *Thermus thermophilus*, and a mesophilic bacterium, the aa_3-oxidase from *Paracoccus denitrificans*. While we observe a low activation energy of about 15 kJ/mol for the electron transfer, the proton transfer rate depends stronger on temperature leading to an activation energy of 40 kJ/mol for this process in both oxidases. For the bacterial enzyme, conditions are varied to investigate the effect of a point mutation in one of the proton pathways (K354M), inhibition by cyanide and a possible kinetic isotope effect. The results are discussed and compared to available literature data (Farver et al., *Biophys. J.* (2006) *accepted*; Brzezinski, *Biochemistry* (1996) 35, 5611-5615).

P1.1.69. INTERACTION OF THIAMINE WITH HEMOPROTEIN OXOFERRYL FORMS

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It is known that highly oxidized forms of hemoglobin, myoglobin, cytochrome c and other hemoproteins play important role in destructive processes during oxidative stress. During methemoglobin interaction with hydrogen peroxide or organic peroxides oxoferryl form of hemoglobin is formed. Hemoglobin oxoferryl form oxidizes nitric oxide (NO) to nitrite, decreases NO level and, possibly, can cause endothelium dysfunction.

We showed that incubation of methemoglobin with glycosylated amino acids yielded hemoglobin oxoferryl form which in turn generates nitrosyl radicals. Nitrosyl radicals cause nitration of proteins tyrosinyl residues leading to the protein activity inhibition. We showed thiamine and its phosphoric ethers to degrade hemoglobin oxoferryl form.

Acceleration of hemoglobin oxoferryl form conversion to methemoglobin after thiamine addition was observed. This conversion was accompanied by thiamine oxidation and resulted in formation of fluorescent products.

Growth of thiamine concentration in solution was accompanied by increase of fluorescence intensity and consequently the yield of fluorescent products. Thiochrome and oxodihydrothiochrome were identified among thiamine oxidation products using spectral fluorescent and chromatography methods. At excess of thiamine in solution thiochrome was the main product of thiamine oxidation by hemoglobin ferryl form. Oxodihydrothiochrome was also detected when lower concentrations of thiamine in incubation mixture were used.

Interaction of oxoferryl form with both thiochrome and oxodihydrothiochrome resulted in formation of non-fluorescent products.

Thiamine is oxidized to thiochrome by nitrosyl radicals which are formed due to interaction of nitrite with ferryl form of hemoglobin. Thiochrome, metabolite of thiamine, is 10 times more effective scavenger of nitrosyl radicals than thiamine.

It is suggested that thiamine and its metabolites eliminate toxic oxoferrylic forms of hemoproteins, inhibit nitration of tyrosine and tyrosinyl residues of proteins by nitrosyl radicals.

P1.1.70. FEATURES OF PEA MITOCHONDRIA OPERATION DEPENDING ON COLD STRESS INTENSITY

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A range of mitochondrial responses to cold stress, such as some changes in functional activity of main cytochrome and alternative respiratory pathways, protein complement and ultrastructure, has been observed in various plant species. The reaction appears to be related to species, its tolerance, severity of cold temperatures and length of exposure. However, the investigative strategy very often lay in comparison of species differing in cold- and freezing-tolerance and effect of only one cold treatment has been studied usually. The aim of our work was to investigate the features of functional activity of plant mitochondria under various cold conditions, such as hardening ($+7^0$C, 7days), "mild" ($+2^0$C, 7days) and "severe" (-7^0C, 1.5h) stress by the example of pea seedlings (*Pisum sativum* L. cv. Marat).

Our results showed that all cold treatments inhibited main cytochrome pathway operation and caused about 20% equal decrease in coupling state of the mitochondria oxidizing malate in the presence of glutamate, however the phosphorylative rates depended on stress intensity. Thus, during cold hardening of pea seedlings no significant change in phosphorylative oxidation rate occurred but nonphosphorylative one increased slightly leading to the uncoupling effect. While "mild" stress increased both rates, low negative temperature strongly inhibited substrate oxidation. Inhibitor analysis using inhibitors of first (rotenone) and forth (KCN) complexes of mitochondrial ETC and also AOX inhibitors and activators revealed that the hardening did not evoke significant real AOX activation, whereas "mild" treatment caused about double AOX activation and "severe" cold shock slightly inhibited it. The using of AOX activators allowed to determine potential activity of the enzyme and revealed that all cold treatments led to its 3-4 fold increasing in comparison with the AOX activity in control mitochondria. Analysis of real and potential AOX activity seems to suppose less cold sensitivity of AOX than enzymes of TCA cycle and/or carriers transporting substrates. Western blotting studies did not reveal significant cold-related changes in AOX content. Positive low temperatures did not change level of rotenone-insensitive respiration, which was about 20% from state 3 oxidative rate, and negative temperatures inhibited internal NAD(P)H DHs operation. At the protein level, all cold treatments decreased the amount of the enzyme. Thus, oxidative capacity of pea seedlings mitochondria is cold stress intensity-dependent, but the

uncoupling under various cold treatments is common stress-reaction for the organelles. It seems that under cold conditions the fine tuning of the alternative enzymes operation at the activity level takes place.

P1.1.71. VIBRATIONAL MOTIONS IN PHOTOACTIVE BIOMOLECULES SUCH AS ALL-TRANS RETINAL AND WILD TYPE BACTERIORHODOPSIN: AN INFRARED EMISSION SPECTROSCOPIC STUDY

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The mechanism of the primary events in bacteriorhodopsin (bR) has been the subject of intense study for over decades but is still only partially understood. Key problems include the understanding of the charge redistribution along the chromophore and elucidating the retinideledyne chromophore structure in the primary photoproduct to determine the mechanism of the energy storage that drive later events.

Infrared emission Fourier Transform Spectroscopy (IREFTS) is developed to measure the vibrational excitation in biomolecules during the recent years [1-4]. The instrumental principles underlying IREFTS together with IREFTS applications in structural studies are discussed.
The methodology is applied to *in situ* characterized retinideledyne chromophore of bR in the vibrational excited states at room temperature [1-2, 4].

Restoration of significant light-induced charge redistribution along the retinal polyene initiates IR-emisison response of chromophore. Complementary to resonance Raman (RR) and IR absorption spectra IREFTS results provide the first detailed view of vibrational degrees of freedom of photoexcited retinideledyne chromophore. Comparison of data observed with the chromophore structures of ground-state reveals that the excited state events are coupled with more vibrational motions than those observed by RR experiments. It was shown, the IR-emission transitions involve states where a vibrational quantum number change is $\Delta = \pm 1$ as well as the states in which at least one combination band (IR active or Raman active) take parts. The IREFTS spectra contain a more rich information about the composition of the eigenstates, as compare the basic spectroscopy, because of the strong selection rules involved in the Raman or IR spectroscopy, in most cases, the spectrum provide information about a single contribution to the eigenstate, i.e. the contribution from the IR-active or Raman-active transition, respectively.

Emission spectra of *all-trans* retinal like bR [2] were highly reproducible when they were recorded under the same experimental conditions. However, changes of illumination conditions were significantly influence the spectral behavior. We assume that the extremely high-sensitivity of vibrational emission spectra is result the complex nature of the states involved in IR-transitions. One would expect that even relatively small changes in coordination geometry would have a significant effect on the infrared spectra. By using of approach presented, it appears possible, that the IR spectral data needed for precise vibrational assighment of the bR's structure can be available.

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P1.1.72. EXPLORING THE ENVIRONMENT OF IRON-SULFUR CLUSTERS IN COMPLEX I FROM YARROWIA LIPOLYTICA

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Complex I (NADH:ubiquinone oxidoreductase) is a huge multisubunit protein which pumps protons across the mitochondrial inner membrane to generate a protonmotive force by a still unknown mechanism. Mitochondrial complex I harbors one FMN and eight iron-sulfur clusters. Apart from their role in "wiring" electrons from FMN to ubiquinone, little is known about the functional interaction of the iron-sulfur clusters with complex I proteins.

In order to learn more about the iron-sulfur clusters of complex I, we have introduced a number of point mutations in complex I from the yeast *Yarrowia lipolytica*. Mutations were generated (i) in the NUAM/ 75 kDa subunit harboring iron-sulfur clusters N1, N4 and N5, (ii) in the NUCM/ 49 kDa which is close to iron-sulfur N2 and (iii) in the NUHM/ 24 kDa subunit assumed to coordinate an EPR invisible iron-sulfur cluster that corresponds to bovine cluster N1a. The effects of the point mutations on the EPR signals of iron-sulfur clusters as well as effects on complex I assembly, stability, catalytic activity and on the affinity of the enzyme to substrates and inhibitors are discussed.

P1.1.73. CHARACTERIZATION OF THE 5' REGION OF HUMAN COQ2, A GENE CAUSING PRIMARY COQ10 DEFICIENCY

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Ubiquinone, coenzyme Q(10) or CoQ10, is a lipid-soluble component of the mitochondrial respiratory chain (RC) where it transfers reducing equivalents from complex I and complex II to complex III. CoQ10 biosynthesis is still poor characterized in humans and consists of multiple enzymatic reactions. Primary CoQ10 deficiency causes a form of mitochondrial encephalomyopathy which is inherited as autosomal recessive trait, and in some patients it is associated to mutations in the *COQ2* gene, encoding for Para-Hydroxybenzoate-Polyprenyl Transferase (Quinzii C. *et al.* 2006).

Human *COQ2* cDNA has been isolated from a human muscle and liver cDNA library (Forsgren *et al*. 2004) and encodes for a protein located in the mitochondrial inner membrane. The reported human sequence shows four different in frame ATG codons in the 5' region of the gene. However by aligning the human protein with other mammalian COQ2 proteins, we found that the N-terminus of the reported sequence did not show any not homology with other mammalian COQ2 proteins, moreover there are no GENBANK ESTs corresponding to the reported 5' region of the gene.

We therefore characterized the 5' region of *COQ2* by 5'-RACE and found different transcripts in human fibroblasts, but the most represented one (over 75% of total COQ2 mRNA) included only the fourth ATG. We then amplified cDNA using a fixed primer within exon 2 of the gene, distal to the predicted targeting sequence, and several primers located upstream of the fourth ATG. We none of the detected PCR products included the first two reported ATG. To confirm the physiological relevance of these shorter mRNAs, we cloned the PCR fragments in frame with GFP, and we demonstrated that the resulting fluorescent fusion protein is targeted to mitochondria.

These data show that the functional human COQ2 mRNA is shorter than what previuosly was thought. We believe the reported COQ2 transcript represents a very rare mRNA, with little physiological significance. These findings are crucial to perform correct mutation screening in CoQ10 deficient patients.

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P1.1.74. INTRA-DOMAIN AND INTER-DOMAIN MOTIONS IN PROTON-TRANSLOCATING TRANSHYDROGENASE

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Transhydrogenase is found in the inner mitochondrial membrane of mammalian cells and in the cytoplasmic membrane of bacteria. The enzyme couples the transfer of hydride-ion equivalents between NAD(H) and NADP(H) to the translocation of protons across the membrane:

$$NADH + NADP^+ + H^+_p \leftrightarrow NAD^+ + NADPH + H^+_n$$

The enzyme has three components, dI, dII and dIII. The dI component, which binds NAD(H), and the dIII component, which binds NADP(H), protrude from the membrane. The dII component, which houses the proton-translocation pathway, spans the membrane. During turnover, movements of the nicotinamide ring of bound NADH gate the hydride-transfer reaction to ensure efficient coupling to proton translocation[1].

The dI component has two domains, dI.1 and dI.2. Relative rotation of dI.1 against dI.2 appears to drive the NADH gating movements. The dI.1 domain contains a unique Trp residue (Trp72) with distinctive photochemical properties that indicate a particularly rigid local environment[2]. It was suggested that this rigidity might be important in the dI.1-dI.2 rotation. The hypothesis has been tested by introducing conservative mutations into the core of dI.1 and comparing the effects on Trp72 photochemistry with the effects on the kinetics of enzyme turnover.

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P1.1.75. ELECTROGENICITY DUE TO QUINONE-IRON COMPLEX TURNOVER

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An electrometrical technique was used to investigate proton-coupled electron transfer reaction between the primary plastoquinone acceptor Q_A^- and oxidized non-heme iron Fe^{3+} on the acceptor side of spinach photosystem II core particles incorporated into phospholipid vesicles. The sign of the transmembrane electric potential difference ($\Delta\Psi$) (negative charging of the proteoliposome interior) suggest that the quinone-iron complex is facing inside of the membrane. The oxidation of non-heme iron was achieved by potassium ferricyanide entrapped into proteoliposomes. Besides the fast unresolvable kinetic phase (τ ~0.1 μs) of the $\Delta\Psi$ generation related to electron transfer between the redox-active tyrosine Y_Z of the D1 polypeptide and Q_A, an additional phase in the submillisecond time domain (τ ~0.1 ms at 23˚C, pH 7.0) and maximum amplitude ~ 20% of the amplitude of the fast phase was observed under the first flash. The additional phase was absent under the second and subsequent laser flashes as well as upon the first laser flash but in the presence of DCMU, an inhibitor of electron transfer between Q_A and the secondary plastoquinone Q_B molecules. Measurements of kinetic H/D isotope exchange effect as well as the temperature dependence of submillisecond electrogenic phase suggest that the latter is most probably attributed to the vectorial transfer of the proton from the reaction center surface to certain residue(s) in the vicinity of the non-heme iron. The role of the non-heme iron in cyclic electron transfer in photosystem II complex is discussed.

P1.1.76. PROBING THE LOCAL STRUCTURE AND DYNAMICS OF FE SITE IN CYTOCHROME C EMBEDDED IN A DRY TREHALOSE MATRIX: AN X-RAY ABSORPTION SPECTROSCOPY STUDY

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Trehalose, a non-reducing disaccharide of glucose, has a peculiar efficacy in the preservation of isolated proteins, membranes and tissues (1). A number of experimental techniques, sensitive to atomic motions, and molecular dynamics simulations (MD) have shown that, in heme proteins embedded in trehalose matrices, the large-scale internal motions, which in glycerol-water start above ~ 180 K, are strongly inhibited. Such inhibition increases when the water content is decreased. Furthermore, FTIR spectroscopy and MD results enabled to infer the existence of water molecules at the protein interface involved in hydrogen bond networks, which anchor the protein to the surrounding water-trehalose matrix and couple the internal degrees of freedom of the protein to those of the external water-sugar matrix (2 and references therein).

This tight dynamical and structural coupling has been investigated in samples of reduced horse heart cytochrome (cyt) c, in water glycerol solution and embedded in a trehalose matrix, which was led to extreme drought, but still contained traces of residual water. We performed Fe K-edge X-ray absorption spectroscopy measurements at liquid nitrogen and at room temperature. Since the EXAFS function is damped by an exponential term, which contains the mean square relative displacement, information on thermal fluctuations and static disorder can be obtained. Our measurements evidenced that, while lowering the temperature from 300 K to 77 K does not significatively alter the EXAFS signal both in the water-glycerol solution and in the trehalose-water matrix, large differences are observed between the spectra in the two different environments. In particular the damping of the EXAFS function is dramatically decreased in the trehalose-water matrix as compared to the water-glycerol mixture. This indicates that the trehalose-water matrix drastically alters the protein energy landscape, most likely hindering the protein internal dynamics and promoting only some conformational substates at the level of local structure.

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P1.1.77. CHARACTERISATION OF THE REDOX BEHAVIOUR OF THE cbb_3 OXYGEN REDUCTASE FROM BRADYRHIZOBIUM JAPONICUM

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Bradyrhizobium japonicum, a diazotrophic Gram-negative soil bacterium has a c*bb*$_3$ oxygen reductase encoded by the f*ix*NOQP operon, expressed in symbiosis conditions. This oxygen reductase belongs to the C group of haem-copper oxygen reductases superfamily (Pereira, M.M. *et al* (2001) *BBA* 1505, 185-208). In this study, we purified and further characterized this oxygen reductase carrying a histidine-tagged FixN (Arslan, E. *et al* (2000) *FEBS Letters* 470, 7-10) from *B. japonicum* cells grown in microaerophilic conditions. Different spectroscopic techniques, namely UV-Visible, EPR and Resonance Raman, as well as anaerobic potentiometric titrations at different pH values, followed by visible spectroscopy, were used to study the 5 redox centres present in this complex.

P1.1.78. CHARACTERIZATION OF NAD(P)H DEHYDROGENASES OF THE MITOCHONDRIAL RESPIRATORY CHAIN FROM NEUROSPORA

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The proton-pumping NADH dehydrogenase or complex I is a major entry point of electrons into the mitochondrial respiratory chain. It catalyses electron transfer from NADH to ubiquinone through a series of protein-bound prosthetic groups. Complex I deficiencies have been implicated in various mitochondrial diseases. Complex I from the filamentous fungus Neurospora crassa contains at least 39 polypeptide subunits of dual genetic origin, mostly conserved in mammals, suggesting that the enzyme is involved in other cellular processes beyond bioenergetics. Mutations in different subunits have been generated in the last years, including mutations of conserved amino acid residues of iron-sulphur proteins as found in human diseases, in order to reveal the role of the proteins in complex I assembly and function. Complex I is likely regulated by transitions between active (A) and de-activated (D) forms and one of the proteins involved in this phenomenon has been identified. In addition to complex I and depending on the organism, several non-proton-pumping alternative NAD(P)H dehydrogenases may also be present in the inner mitochondrial membrane. The fungus N. crassa contains four alternative NAD(P)H dehydrogenases: the main external NAD(P)H dehydrogenase, an external calcium-dependent NADPH dehydrogenase, a third external enzyme and the single internal NADH dehydrogenase. These proteins appear to have both alternative and complementary functions. Overall, mitochondrial respiratory chain NAD(P)H dehydrogenases have important roles in fungal development.

P1.1.79. EFFECT OF CALCIUM ON THE HEME A REDUCTION IN MAMMALIAN CYTOCHROME C OXIDASE

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Subunit I of cytochrome c oxidase (COX) from mitochondria and many bacteria contains a cation binding site located near heme *a* and facing the P outer aqueous phase. In the bacterial COX it is occupied by tightly bound Ca^{2+}[1,2], whereas the mitochondrial oxidase can bind reversibly Ca^{2+} or Na^{+}. Until now the role of this center in the enzyme remained obscure. Our previous studies of the effect of sodium concentrations on dissociation constant (Kd) of Ca^{2+} complex with bovine COX revealed its significant dependence on the redox state of heme *a* [3]. To clarify the situation we have studied effect of Ca^{2+} and Na^{+} on the heme *a* reduction following 605 nm absorbance band at different redox potentials imposed by ferro/ferricyanide ratio. Media were also supplied by small amount of cytochrome *c* to facilitate enzyme interaction with iron hexacyanide. At 5mM ferrocyanide and 0.1mM ferricyanide (ca Eh 320 mV) COX depletion of calcium by chelator (0.1mM EGTA) or its substitution by sodium resulted in partial heme *a* oxidation. Accordingly addition of Ca^{2+} to calcium depleted enzyme increased heme *a* reduction. The effect was not observed with Mg^{2+} or K^{+} ions. More detailed oxidative and reductive titrations of bovine COX by ferro/ferricyanide pair have been carried out within range of redox potentials 240-480 mV. In different cases the media contained 0.2mM $CaCl_2$ /0.1mM EGTA, 0.1mM EGTA or 50mM NaCl/0.1mM EGTA. At low redox potential region (240-340mV) titration curves of Ca^{2+}-loaded COX showed much higher levels of heme *a* reduction confirming previous data that Ca^{2+} binding to COX stabilizes its reduced state by rising heme *a* midpoint potential. Such observation might be the first indication of the possible functional role of Ca/Na binding site for cytochrome oxidase. It should be emphathised that no effect on redox titration was observed when Ca^{2+} was replaced by Mg^{2+}. Also no effect has been found on the oxidative and reductive titration curves upon addition of Ca^{2+} or chelators to the bacterial COX of wild type from *Rhodobacter sphaeroides* in which Ca^{2+} is bound tightly and not removed by complexons [2]. Bacterial COX was kindly provided by Prof.R.Gennis from University of Illinois at Urbana-Champain.

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P1.1.80. THE ROLE OF CONSERVED RESIDUES IN THE MITOCHONDRIAL QUINOL: CYTOCHROME C OXIDOREDUCTASE FOR SUBSTRATE BINDING, CATALYSIS AND PROTON TRANSFER

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The quinol:cytochrome *c* oxidoreductase (QCR) is a major component of the respiratory chain. The enzyme catalyses electron transfer from ubiquinol to cytochrome *c* coupled to a vectorial proton transport across the membrane, in which the enzyme resides. The role of several residues in substrate binding and electron and proton transfer [1,2] was challenged by a combination of site-directed mutagenesis, enzymatic assays and EPR and FTIR techniques. Mutations in the cytochrome *b* gene were generated by biolistic transformation.

Residues E272, Y132 and F129 of cytochrome *b* located at centre P were shown to be important for QCR catalysis. Charge and side chain length at position 272 are supposedly important for efficient formation of the enzyme substrate complex, for controlled electron bifurcation and for prevention of superoxide formation at centre P [3,4].

At centre N, variants (Lange et al., 2001) in the substrate and lipid binding sites were generated. Disturbed electron transfer at both active sites and increased superoxide production at centre P could be observed for all centre N variants indicating a long-range interaction in the complex [4].

The obtained results provide new information of residues critical for stabilisation of substrate and controlling electron short circuit reactions within the complex.

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P1.1.81. THE USE OF Zn^{2+} IONS TO PROBE THE PROTON-TRANSLOCATION REACTIONS OF TRANSHYDROGENASE

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Transhydrogenase is found in the inner mitochondrial membrane of mammalian cells and in the cytoplasmic membrane of bacteria. The enzyme couples the transfer of hydride-ion equivalents between NAD(H) and NADP(H) to the translocation of protons across the membrane:

$NADH + NADP^+ + H^+_p \leftrightarrow NAD^+ + NADPH + H^+_n$

In recent years the redox reaction has been studied in some detail, and there is a crystal structure of the part of transhydrogenase that houses the hydride transfer site[1]. However, information on the mechanism of proton translocation through the membrane-spanning region is somewhat limited.

Zn^{2+} and Cd^{2+} are known to block proton translocation pathways in a number of proteins (for refs, see[2]). Recently, it was shown that Zn^{2+} and Cd^{2+} inhibit transhydrogenase[2]. The site of inhibition was located at the NADP(H) binding/release steps; these metal ions did not affect the hydride-transfer reaction or NAD(H) binding and release. The implication is that proton translocation in transhydrogenase is associated with NADP(H) binding/release. These studies are being extended to investigate the effects of Zn^{2+}, Cd^{2+} and other transition metal ions on the partial reactions of intact transhydrogenase and its protein components.

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P1.1.82. SITE-DIRECTED MUTAGENESIS STUDY OF NADH-UBIQUINONE OXIDOREDUCTASE (NDH-1) FROM RHODOBACTER CAPSULATUS: A ROLE OF THE HIGHLY CONSERVED 51ASN-VAL-VAL-GLY-ALA-PHE56 SEQUENCE IN LOOP 1 OF THE NUOH (ND1) SUBUNIT IN $\Delta\mu_H^+$ -DEPENDENT ELECTRON TRANSFER

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NADH-ubiquinone oxidoreductase (complex I/NDH-1) catalyzes the oxidation of NADH with ubiquinone, coupled to proton translocation across the inner mitochondrial membrane or bacterial cytoplasmic membrane to generate the electrochemical proton gradient, $\Delta\mu_H^+$ (*1*). The redox reactions take place via redox cofactors, FMN and iron-sulfur clusters, located in the hydrophilic part and protons are pumped through the hydrophobic membrane part. However, the mechanism of coupling between the redox reaction and the proton translocation is unknown. Among seven essential membrane subunits (ND1-6, 4L), ND1/NuoH subunit is the most highly conserved subunit but its function remains unknown. It has been pointed out that the predicted Loop 1 of this subunit is one of the most conserved polar segments and contains a short peptide sequence (^{51}Asn-Val-Val-Gly-Ala-Phe^{56} for *Rhodobacter capsulatus*), which is well-conserved in complex I/NDH-1 but completely absent in homologous subunits of the energy-transducing [NiFe] hydrogenases (*2*). In order to investigate a role of this 6-amino acid (6AA) segment, we individually substituted Asn-51, Val-52, Val-53, and Phe-56 of the *R. capsulatus* NuoH subunit by Ala, or the 6AA segment was deleted. The *R. capsulatus nuoH*-urf6 deletion mutant strain was complemented *in trans* with the mutated *nuoH* genes and the transconjugants were characterized in terms of their growth phenotype, enzyme assembly, electron transfer activity, proton pumping activity, and inhibitor sensitivity. The results showed that mutations in this segment significantly affected growth phenotypes and enzymatic properties. The 6AA segment deletion mutant was unable to grow in both respiratory and photosynthetic conditions. The mutant enzyme assembled in the chromatophore membrane showed virtually no Q reductase activity, indicating that the 6AA segment is essential for Q-mediated

electron transfer. Among Ala mutants constructed, N51A and V53A mutants could barely grow in photosynthetic conditions (Res^+/PS^-) and the enzymes exhibited 50-60 % of the NADH-Q reductase activity of wild type. All Ala mutants constructed showed the same inhibitor sensitivities (e.g. piericidin A and rotenone) as wild type under uncoupled conditions. However, when $\Delta\mu_H^+$ was built across the membranes, V53A and F56A mutants became highly resistant to inhibitors tested. These results indicate that the 6AA segment of the NuoH/ND1 subunit plays an important role in $\Delta\mu_H^+$-dependent electron transfer, which most likely involves conformational changes.

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P1.1.83. A NEW CLASS OF BACTERIAL MEMBRANE-BOUND OXIDOREDUCTASES

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The filamentous anoxygenic phototrophic bacterium *Chloroflexus aurantiacus* does not contain any cytochrome bc_1-like complexes, which makes it unique among photosynthetic organisms. How then does it complete the cycle of light-driven electron transfer and how does it interact with other membrane-bound electron transfer complexes such as cytochrome oxidase? Yet, the bacterium contains two similar multi-subunit complexes with two cytochrome *c* subunits. One of them is expressed in anaerobic phototrophic conditions, while the other under dark aerobic ones. In the published genome of the bacterium we found operons encoding the complexes. Searching homologues for these operons in genomic databases we found that they belong to a previously unknown class of oxidoreductases. This class apparently originated from the broadly distributed three-subunit molybdopterin oxidoreductases by acquiring additional subunits, namely two heme *c*-bearing subunits, one additional integral membrane subunit, and an unknown subunit with no homologues outside the class. In published genomes, such operons coexist with the operons of oxidases, which form a separate homologous cluster among Cu-heme terminal cytochrome and quinol oxidases. The oxidases of this cluster contain a motif in the amino acid sequence of the second subunit characteristic for the enzymes interacting with periplasmic mobile electron carriers, not with quinols. Yet, the bacteria containing the operons of these pairs of enzymes, like *Chl. aurantiacus*, do not contain operons of bc_1-complexes. Thus, in addition to the widely distributed team of cyt bc_1-complex and cytochrome oxidase in respiratory chains and the team of cyt bc_1 or cyt b_6f complex and reaction centers in photosynthetic chains, other teams work in respiratory chains of certain sets of bacteria and in the photosynthetic cyclic chains of *Chloroflexi*. The previously unknown cytochrome *c* complex derived from a membrane molybdopterin oxidoreductase must work as oxidoreductases transferring electrons from quinol to periplasmic mobile e-carriers. The assumption implies transformation of the catalytic center previously interacting with chemical compounds into one interacting with a globular protein. Indeed, we found the absence of the motifs characteristic for binding of molybdopterin and the adjacent Fe-S cluster in

the derived large subunit in comparison to the parental type.

P1.1.84. FUNCTIONAL IMPLICATIONS FROM NOVEL STRUCTURAL FEATURES IN COMPLEX I FROM YARROWIA LIPOLYTICA

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Complex I is the largest and least understood enzyme of the respiratory chain. An extensive electron microsocopic structural analysis of single particles uncovered a number of novel features in a 3 D reconstruction of complex I from *Yarrowia lipolytica*.

Using monoclonal antibodies (Zickermann et al., 2003) and a subcomplex lacking the FP fragment individual subunits can be assigned to distinct subdomains within the peripheral arm.

The membrane arm is curved and viewed from the cytoplasmic side a groove in the direction of the long axis can be seen. On the matrix side the membrane arm shows two major protrusions. From here a highly variable structure originates that forms a second connection between the two arms.

The emerging architecture of complex I has major implications for the understanding of the catalytic mechanism as it seems to rule out a direct coupling between redox chemistry and proton translocation. Instead a growing body of evidence suggests a conformationally driven proton pumping mechanism and it is tempting to speculate that the flexible linker between membrane and peripheral arm is involved in this process.

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Poster session 1.2. Light-driven energy transducers

P1.2.1. THE CRYSTAL STRUCTURE OF PLANT PHOTOSYSTEM I – TOWARDS ATOMIC RESOLUTION

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Plant photosystem I (PSI) was purified from pea (*Pissum sativum*) leaves, crystallized and solved using heavy-atom derivatives to 4.4 A resolution. The complex structure revealed 16 different subunits, 45 transmembrane helices, 167 chlorophylls, two quinones and three iron-sulfur clusters. The most significant change in the plant PSI is the presence of four light harvesting complexes (LHCI) containing each 12 to 13 chlorophyll molecules and several additional chlorophyll that function in the connections among the LHC units and the LHC complex with the reaction center. The interaction between the reaction center and the peripheral antenna of PSI (LHCI) as well as the interactions among the LHCI monomers are flexible. Plant PSI is essentially the first membrane super complex to be solved by x-ray crystallography. This achievement required numerous technical adjustments to preserve the integrity of the supercomplex during the purification and crystallization. Large amounts of highly purified PSI were required to get enough crystals amenable for structural determination by X-ray crystallography. The stability of PSI was analyzed by sucrose gradient centrifugation and gel electrophoresis. We observed that the super complex was stable for few weeks in the crystallization solution. The pure and homogeneous preparation of PSI allows for relatively tight crystal packing, which holds promise for obtaining atomic resolution in the future.

P1.2.2. INTERACTION OF THE CAROTENOID ANTENNA WITH THE RETINAL IN XANTHORHODOPSIN: pH DEPENDENT TRANSITIONS AND CHIRALITY OF BOUND SALINIXANTHIN

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The cell membrane of the halophilic eubacterium *Salinibacter ruber* contains a retinal protein-carotenoid complex, xanthorhodopsin (XR), which functions as a light-driven proton pump. It exhibits substantial homology to bacteriorhodopsin (BR) of archaea and proteorhodopsin (PR) of marine bacteria, but differs from them in that it acquired a light-harvesting antenna. The antenna is the carotenoid salinixanthin bound to XR that transfers about 40% of absorbed quanta to the retinal [1]. In this study we examined the pH dependent properties of this proton pump. We show that the pK_a of the retinal Schiff base is as high as in BR. It deprotonation at pH 12.5, and the subsequent alkaline denaturation of the pigment, results in major changes in the carotenoid antenna bands which exhibit the same broadening and decrease of extinction as upon hydrolysis of the Schiff base with hydroxylamine. The pK_a of the counterion of XR is about 6 (compared to 2.6 in BR, and 7.5 in PR), as one can judge from a small red shift of the retinal chromophore band (ca. 5 nm) and disappearance of the M intermediate as the pH is lowered. Protonation of the counterion is accompanied by a minor (0.5 nm) blue shift of the carotenoid antenna absorption bands. Further insight into specific interaction of carotenoid antenna with the retinal protein was obtained with CD measurements in the visible. We found that the cell membrane exhibits a CD spectrum with sharp positive and negative bands, related to the structured absorption spectrum of salinixanthin. Solubilization of the membranes in detergent (dodecyl maltoside) does not affect the shape of the spectrum substantially, but incubation with hydroxylamine which hydrolyses the retinal-Schiff base of XR, or elevation the pH above 12.5, eliminates all CD bands in the visible. This and other data indicate that free salinixanthin in the membranes has no intrinsic optical activity. Salinixanthin acquires optical activity only upon binding to xanthorhodopsin, from its asymmetric conformation and/or environment, and probably from exciton interaction with the retinal chromophore. The CD spectrum of the antenna chromophore is sensitive to protonation of the counterion to the Schiff base, more so than its absorption spectrum. CD appears to be a useful tool for studying interaction of the carotenoid antenna with the retinal proton pump.

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P1.2.3. THE STRUCTURE OF LIGAND-MODIFIED IRON-SULFUR CLUSTER F_X IN PHOTOSYSTEM I AS DETERMINED BY EXAFS

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Photosystem I (PSI) is a transmembraneal multisubunit complex that mediates light-induced electron transfer from plactocyanine to ferredoxin. The electron transfer proceeds from an excited chlorophyll *a* dimer (P700) through a chlorophyll *a* (A_0), a phylloquinone (A_1) and a [4Fe-4S] iron-sulfur cluster F_X, all located on the core subunits PsaA and PsaB, to iron-sulfur clusters F_A and F_B, located on subunit PsaC. Structure function relation in the native and F_X cluster which has four cysteine ligands and the cysteine to serine mutant (*psaB*-C565S/D566E) were studied by X-ray absorption spectroscopy and real time optical spectroscopy. In order to measure the structure of F_X subunit PsaC that binds clusters $F_{A/B}$ clusters was removed by interruption of the *psaC2* gene of PS I in the cyanobacterium *Synechocystis* sp PCC 6803. PsaC-deficient mutant cells grew under heterotrophic conditions and assembled the core subunits of PS I in which light induced electron transfer from P700 to A_1 occurred. The photo-reduction of F_X was largely inhibited. X-ray absorption fine structure analysis of the iron resolved a [4Fe-4S] cluster in the native PsaC-deficient PS I. Each iron hade 4 sulfur in the first shell and 3 irons in the second shell with an average Fe-S and Fe-Fe distances of 2.27 Å and 2.69 Å, respectively. The high quality of the collected data and the precision of the analysis is indicated by the Debye-Waller factor of 0.007 $Å^2$ and the S_0^2 of 0.9. There was an alteration in the structure of F_X in the PsaC-deficient PS I of the C565S/D566E mutant. In the serine mutant, one of the irons of the [4Fe-4S] cluster was ligated to three oxygen atoms. On the average each iron was coordinated to three sulfur atoms with Fe-S average bond distance of 2.2 Å and to one oxygen atoms with Fe-O distance of 1.9 Å in the mixed first shell. Three iron atoms with Fe-Fe average bond distance of 2.6 Å in the second coordination shell. The effect of structural changes on the electron transfer to the modified cluster will be discussed.

P1.2.4. ELECTRON AND PROTON TRANSFER EVENTS IN BACTERIAL REACTION CENTERS: INVESTIGATION OF THE ROLE OF THE IRON LIGANDS

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Bacterial reaction center proteins convert light excitation energy into chemical free energy. This is achieved through a series of coupled electron-proton transfer reactions which lead after the successive absorptions of two photons to the formation of the doubly reduced and doubly protonated secondary quinone acceptor, Q_BH_2. Q_B and Q_A, the first quinone acceptor, are situated on the cytoplasmic side of the protein. In a symmetrical position between these two acceptors lies a non heme Fe atom. The electron transfer between Q_A and Q_B is kinetically limited by protein rearrangements to which the iron environment might be associated. The iron atom *per se* does not participate to the interquinone electron transfer[1]. However, it is of possibility that the close (four His and a Glu) or further environment do participate to this "gating" process, in conjunction to protonation events. To track these phenomena we have genetically modified some of the iron ligands. We report the kinetics of the first and second electron transfer reactions, the proton uptake stoichiometries as well as the energetic effects of the mutations. Our data are consistent with the hypothesis that the structural motif composed by the iron and its close ligand environment is part of the interquinone electrostatic connection spread out on the cytoplasmic side of the protein. The delocalisation of the uptaken protons within hydrogen bond networks might constitute the limiting but unavoidable step for electron transfer between the quinones.

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P1.2.5. KINETICS OF CHARGE RECOMBINATION AND DISTRIBUTION OF THE UBIQUINONE POOL IN REACTION CENTER – LIGHT HARVESTING COMPLEXES PURIFIED FROM RHODOBACTER SPHAEROIDES

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We have recently shown that in the reaction center – light harvesting complex 1 (RC-LH1) purified from the photosynthetic bacterium *Rhodobacter sphaeroides*, at pH=7.8, flash-induced $P^+Q_B^-$ recombines with an average rate constant ($\langle k \rangle \approx 0.3\ s^{-1}$), significantly smaller than that measured in RC deprived of the LH1 ($\langle k \rangle \approx 1\ s^{-1}$). Since the kinetics of $P^+Q_A^-$ recombination is unaffected by the presence of the antenna, the $P^+Q_B^-$ state appears to be energetically stabilized in core complexes (1). The pH dependence of the $P^+Q_B^-$ recombination kinetics has been analyzed in dimeric and monomeric forms of RC-LH1 and compared with that observed in RC deprived of the antenna. At $6.5 < pH < 8.5$ recombination is essentially pH independent and significantly slower in all forms of core complexes as compared to LH1-deprived RCs. At increasing pH values, however, where the recombination rate increases, this stabilization effect decreases progressively and vanishes at $pH > 10.5$, indicating the involvement of protonatable groups. The recombination kinetics, moreover, becomes progressively distributed at $pH > 9$. The width of the rate constant distribution ($\sigma \cong 0.3\ s^{-1}$ at $pH < 9.0$) increases by more than one order of magnitude at pH 11.0, suggesting a variety of conformations, possibly differing in the protonation state. The observed pH dependence of σ could be explained when assuming that such conformations interconvert at a rate which is comparable to the rate of charge recombination at physiological pH values but is considerably lower at high pH values. Under this condition the conformational heterogeneity becomes therefore observable. A similar behaviour was observed in chromatophores of *Rhodobacter capsulatus* FJ2, a c_2- and c_y-minus strain, in which the kinetics of $P^+Q_B^-$ recombination could be accurately studied by avoiding any interference due to exogenous electron donors/acceptors.

The lipid complement of the examined RC-LH1 complexes, determined by Inductively Coupled Plasma Emission Spectroscopy of phosphorous, ranges between 200 and 400 phospolipid molecules per RC. A large ubiquinone (UQ) pool, varying from 15 to 30 UQ molecules per RC was systematically found to be associated with the core complexes. When similar determinations are

performed in chromatophores, it appears that the effective UQ concentration in the lipid phase of core complexes is at least three times higher than the average UQ concentration in the intact membrane. This finding argues strongly in favour of an *in vivo* heterogeneity in the distribution of the quinone pool within the chromatophore bilayer.

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P1.2.6. ROLE OF NAD(P)H:QUINONE OXIDOREDUCTASE ENCODED BY DRGA GENE IN REGULATION OF LIGHT-INDUCED ELECTRON TRANSPORT THROUGH PHOTOSYSTEM I IN CYANOBACTERIUM SYNECHOCYSTIS 6803

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The kinetic of $P700^+$ rereduction after its photooxidation was monitored in wild type (WT) and DrgA mutant of *Synechocystis* 6803 deficient in soluble NAD(P)H:quinone oxidoreductase (NQR) using the method of EPR spectroscopy. Low rate of Photosystem I reaction center ($P700^+$) rereduction in darkness after its oxidation with white light in the absence of Photosystem II activity, and low rate of $P700^+$ rereduction by electrons derived from exogenous glucose have been observed in DrgA-mutant. The mutation in *drgA* gene did not affect the NDH-1-dependent, rotenone-inhibited electron transfer from NADPH to $P700^+$ in thylakoid membranes of cyanobacteria, and did not affect the ability of *Synechocystis* 6803 cells to oxidize exogenous glucose under heterotrophic conditions. Low level of NADPH-dehydrogenase activity was observed both in NDH-1 and NQR deficient mutants indicating that NQR encoded by *drgA* gene, as well as NDH-1, participates in NADPH oxidation.

Comparative characterization of photosynthetic properties of mutants deficient in individual components of photosynthetic or respiratory electron transport chains indicate that in the absence of Photosystem II activity, mainly NADPH-dehydrogenases NDH-1 and NQR, and in less extent succinate-dehydrogenase and NADPH-dehydrogenase NDH-2, are important for the transfer of electrons on plastoquinone pool in thylakoid membrane of cyanobacteria. In *Synechocystis* 6803 triple mutant lacking of cytochrome oxidises CtaI and CtaII, and quinol oxidase Cyd, the rate of $P700^+$ rereduction was increased due to high level of plasoquinone pool reduction. The results obtained may indicate that NQR encoded by *drgA* gene, as well as NDH-1, participates in regulation of electron transport from NADPH to plastoquinone pool in thylakoid membranes of *Synechocystis* 6803.

Besides the ability to perform the bioactivation of nitroaromatic inhibitors, NQR encoded by *drgA* protects cyanobacterial cells from menadione-induced oxidative stress by two-electron reduction of menadione to menadiol. These properties of NQR indicate the probable functional

homology of *Synechocystis* 6803 NQR with mammalian and plant NAD(P)H:quinone oxidoreductases (DT-diaphorases).

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P1.2.7. MOLECULAR HYDROGEN PRODUCTION BY RHODOBACTER SPHEROIDES: PHENOMENON, REDOX POTENTIAL AND PROTON-COUPLED PROCESSES

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Molecular hydrogen is produced by *Escherichia coli* under mixed-acid fermentation. It has been proposed that the proton F_0F_1-ATPase is associated with membrane formate-hydrogenlyase (FHL) composed of formate dehydrogenase (Fdh-H) and hydrogenase 4 (Hyd-4), which is responsible for H_2 production at alkaline pH [1-2]. In photosynthetic bacteria *(Rhodobacter*) H_2 is also produced. Change in H_2 production can be observed upon transition from aerobiosis to anaerobic photosynthesis [3] depending on light intensity [4]. The proteins, which could be redox regulators of a photosystem formation in this bacterium, have been identified and characterized using their over-expression in *E. coli* [5]. But in both cases with *E. coli* and *R. spheroides* the appropriate mechanisms are not known well. Redirection of metabolic pathways, gas sparkling and maintaining of low partial pressure of hydrogen, efficient product removal and the others integrating fermentative processes with photosynthesis are some of the ways that have been attempted to improve H_2 productivity [2].

In this respect, environment physico-chemical conditions (pH, redox potential (E_h), light intensity and etc) and effects of different carbon and nitrogen sources, oxidizers and reducers on bacterial growth and H_2 production are being studied with *R. spheroides* strain A-10 isolated from Arzni spring waters in Armenia (in comparison with *E. coli*).

R. spheroides was shown to grow well under anaerobic conditions and light (1500 lx) using succinate and lysine as sources of carbon at pH 6.8-7.0. During the growth, decrease in E_h to negative values (-385 mV) and alkalization of the medium (from 7.0 to 8.5) both were observed, and H_2 production was determined. However at the presence of glucose H_2 production was not observed.

H_2 production was suppressed with *N,N'*-dicyclohexylcarbodiimide and sodium azide as well as with Cu^{2+} ions. A role of proton-coupled transport and membrane-associated hydrogenases will be investigated.

This may evaluate novel ways in regulation of H_2 production by bacteria to improve H_2 biotechnology with future application in the energy industry using effective bacterial constructs and enzymes.

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P1.2.8. ASSESSMENT TO THE HIGH TEMPERATURE EFFECT ON CHESTNUT SEEDLINGS GROWTH

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Previous works have been demonstrated that chestnut growth is significantly affected under high temperatures, over than 30ºC (Gomes-Laranjo *et. al.*, 2005*a*). The aim of this work was to analyse the effect of the temperature increase from 25 to 35ºC. For this purpose, seedlings from Judia cultivar with 90's days (1st period) old growth under 25ºC, were submitted during 60 days to 35ºC (2nd period). According gas exchanges, in control plants, optimal temperature (T_{100}) decreased from 1st(32,5ºC) to 2ndperiod (30ºC), being T_{100} for 35ºC's plants group, 32,5ºC. This plants also showed a significant photosynthesis reduction (33%) in relation to those from control (8.5 $\mu molCO_2.m^{-2}.s^{-1}$) but a significant increase in Chla/b ratio was detected (3.15 to 3.37). Concerning to membrane potential (2nd period), in control seedlings maximal value was obtained at 19-20ºC (as reported previously by Gomes Laranjo *et al.*, 2005*b*), being lower than 20% over 30ºC, instead in 35ºC's plants, this reduction occurred over 33ºC and maximal value shifted to 21-22ºC. Although, increased in temperature did not induced inhibition at the level of PSII, namely in O_2 evolution which is preserved even at 43ºC, neither in PSI activity. The activities of superoxide dismutase and catalase were investigated in plants growth at 25 and 35 ºC. SOD activity was significantly stimulated at 35ºC (14%), however CAT activity was not significantly stimulated.

In relation to the lipid composition, significant decrease was observed in the α-linolenic acid content between 25 and 35ºC's seedlings, respectively 48 to 36% and an increase in the saturated fatty acids was detected, with a variation in palmitic acid (2nd most representative) from 12 to 16%.

Gomes-Laranjo, J.C.E., P. Salgado, H.W. Wong Fong Sang, R. Kraayenhof and J.M.G. Torres-Pereira (2005). Isolation of chestnut chloroplasts. Membrane potentials of chestnut and spinach thylakoids. Photosynthetica 43 (2): 237-246.

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P1.2.9. ANALYSIS OF CHARGE RECOMBINATION KINETICS IN CYANOBACTERIAL PHOTOSYSTEM I WITH SITE-SPECIFIC SUBSTITUTIONS IN THE VICINITY OF THE PHYLLOQUINONE MOLECULES

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The question of whether one or both branches of the nearly symmetrical branches of cofactors in Photosystem (PS) I participate in charge recombination between the reduced iron-sulfur acceptors F_A/F_B and the oxidized chlorophyll dimer $P700^+$ is under debate.

To investigate this issue, we measured electron transfer in PS I from the cyanobacterium *Synechocystis* sp. PCC 6803 using mutants with site-specific substitutions of Trp and Ser residues in the vicinity of the phylloquinones Q_K-A and Q_K-B- in the PsaA and PsaB. These substitutions involved changing the Trp residues, which are π-stacked with the headgroups of the phylloquinones (W697F on PsaA and W677F on PsaB) and Ser residues, which are within 3.1 to 3.2 Å of the carbonyl oxygen on the phylloquinones (S692C on PsaA and S672C on PsaB). The charge recombination reactions were studied over the temperature range 300 K to 5 K.

In addition to employing the traditional multi-exponential analysis of the observed kinetic heterogeneity, we used the maximum entropy method (MEM), which derives a distribution of lifetimes that may indicate a distribution of different conformational states in the protein complex. This approach minimizes the number of closely spaced kinetic components while preserving the most significant features of the spectrum. The modified program (GRAPH-MEM), which includes not only the MEM analysis but additional algorithms, was developed in our group. This program provides the opportunity to discriminate between exponential and non-exponential kinetic components.

The kinetic analysis of charge recombination reactions in PS I complexes from the wild type and four above-mentioned PsaA and PsaB mutants revealed significant differences in their kinetics as well as in their temperature dependencies. We consider these results to reflect the participation of two active brunches in charge recombination, but with different efficiencies in cyanobacterial PS I.

P1.2.10. THE ELECTROGENESIS OF NEUROSPORA RHODOPSIN PHOTOCYCLE

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The *Neurospora* Rhodopsin (NR) is an eukaryotic analog of bacteriorhodopsin (BR) from *Neurospora crassa* (Beiszke at al, 1999). The absorption spectrum of NR (530 nm) is blue shifted relative to BR from *Halobacterium salinarium*. The photocycle of NR was first studied in (Brown at al, 2001, Furutani at al, 2004). It was shown that NR releases proton under illumination and forms blue-shifted intermediate similar to M intermediate of BR and then after conversion into red-shifted intermediate (similar to O intermediate of BR) relaxes to ground state. In the present work pH dependences of NR's membranes washed in 0.5% DM was studied. Two pK values 4.8 and 8.6 were found. The former one corresponds to small absorption peak shift, the last one corresponds to retinal release. To investigate the photocycle of NR, we measured the photoinduced absorption changes of NR membranes from 380 to 660 nm at different pH, ionic strength and concentration of NaN_3. All measurements were done by the logarithmic sweep in the range from 1 μs to 40 sec. The apparent differential maximum of M intermediates was found at 390 and maximum of O intermediate was at 590 nm at pH=7.4, t=22°C, 150mM NaCl. Global fitting of absorption kinetics gave maximum probability to 8 exponents which are cover range from microseconds to seconds. Only one exponent in the M intermediate decay (τ = 45ms) shows NaN_3 dependency. The NR containing proteoliposomes were adsorbed on the teflon film and charge transfer kinetic were measured by the direct electrical method (Drachev at al, 1978). The teflon film with high resistance (discharge constant ~20 sec) allows to get kinetics up to second range. The net charge transport during NR photocycle was shown. Surprisingly the shape of NR electrical response is qualitatively different from that in the BR. In the middle cycle time (0.1 – 100 ms) the voltage has sign opposite to the final one. But the most striking feature of NR electrogenesis is directionality reversing of net charge transport on pH with pK=6.2. The exponential analysis reveals net-charge transfer components which are in line with absorption kinetics data. Like absorption spectra the exponential analysis of electrogenesis reveals only one azide-dependent exponent with (τ = 40ms).

This work was partly supported by the RFBR (grant No. 03-04-49153) and ISTC (grant No. 866).

P1.2.11. RELATIONS BETWEEN STRUCTURE AND BIOLOGICAL AFFECTIVITY FOR Q_B SITE INHIBITORS OF BACTERIAL PHOTOSYNTHETIC REACTION CENTERS

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Many herbicides used in agriculture (e.g. triazine compounds: atrazine, terbutryne, promatryne, ametryne, etc.) and some antibiotics (e.g. stigmatellin) bind to a specific site of the reaction center protein blocking the photosynthetic electron transport[1]. Crystal structures showed that all these inhibitors bind at the secondary quinone (Q_B) site albeit in slightly different ways[2]. It has already been demonstrated that different herbicide molecules have different binding affinities[1] (characterized by us as inhibitor constants, K_i, and binding enthalpy values, DH_{bind}). The action of inhibitors depends fundamentally on the chemical nature: (i) of the inhibitor molecule, (ii) of the quinone/inhibitor binding site, and (iii) of the protein environment.

In our investigations we determined K_i and DH_{bind} for different inhibitors available in our lab. Molecular structures were optimized and their intramolecular charge distributions (*Mullikan charges*) were determined by *ab initio* computer calculations. Experimental and modeled data were compared to those from crystal structures.

We can state that herbicide affectivity (K_i) as well as geometry of binding to the protein both depend basically on steric (molecular geometry) and electrostatic effects (charge distribution). For steric reasons apolar bulky groups on the N-7 nitrogen atom of the molecule (like t-butyl in terbutryne) are preferable, while they are not recommended on N-8 (like i-propyl in promatryne). For electrostatic reasons a $-SCH_3$ group on the C-1 carbon is preferable since it is less electrophylic, like in terbutryne and contrary to the case of terbumeton (where the same position is instead occupied by a $-OCH_3$). Then in terbutryne the N-4,7,8 nitrogens maintain a larger electron density so that more effective hydrogen bonds may be formed between the inhibitor and the surrounding amino acids of the protein.

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P1.2.12. PS II BIOENERGETICS OF BARLEY SEEDLINGS GROWING UNDER ABIOTIC STRESSES

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Photosynthetic activity of two barley cultivars Arabi Aswad and Arabi Abiad growing under different abiotic stresses (temperature, light, salinity, heavy metals, and nutrient deficiency) was studied by the application of quantitative analysis of the fluorescence transient (JIP-test). This was done to explain the stepwise flow of energy through PS II at the reaction centre (RC) level (ABS/RC, TR0/RC, ET0/RC and DI0/RC) as well as the level of a PS II cross-section (CS) (ABS/CS0, TR0/CS0, ET0/CS0 and DI0/CS0). The obtained results showed different values of extracted and technical fluorescence parameters, quantum efficiencies, specific and phenomenological fluxes for each cultivar.

This work confirms that, JIP test is a vital tool to provide monitoring of PSII reaction to different abiotic stresses which could allow identifying stress kind that plant experience in certain environmental growth conditions.

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P1.2.13. STUDY OF THE SPECTRAL BEHAVOIR OF THE CROWNED RETINALS AND BACTERIORHODOPSIN ANALOGS

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Three retinal analogs, with normal (**2,3**) and 11,12-didehydropolyenic chain (**1**) and monobenzo-15-crown-5 (**1,2**) and monobenzo-18-crown-6 (**3**) an ionophoric moiety in stead of trimethylcyclohexenic ring were synthesized as described in [1,2]. Further these crowned retinal analogs **(1-3)** were tested in recombination with bacterioopsin (**BO**) from apomembranes *H. salinarum.* It was found that the formation of pigments **ABR 1-3** takes place already within 1-2 hours. It should be noted that λ_{max} of **ABR 1-3** is located within the wavelength range typical of pigments, in which trimethylcyclohexene ring of the retinal is replaced by other aromatic rings (460-530 nm) [3]. We have studied the photochemical properties of the crowned **ABR 1-3.** The exposure of **ABR** samples to laser light flashes caused reversible bleaching of the main absorption band of the pigments (absorption changes induced by laser flash (λ = 532 nm, $t_{1/2}$ = 15 ns) in a suspension of membrane samples at 15-21°C). The photocycles of the pigments include an M-like intermediate. The quantum efficiency of the crowned pigments photocycle was about ~3,5-4 % for **ABR 1** 22-23% for **ABR 2-3,** that found for the control **BR**, respectively. This estimate was obtained from analysis of the photoinduced response amplitude in the main absorption band as normalized to the absorption value in this band: $\Delta D^{ABR}/D^{ABR}$ and $\Delta D^{570}/D^{570}$ (control BR). The measurements were done at equal protein concentration or equal absorption level at the actinic light wavelength. According to the relaxation kinetics of the M-like intermediate, the photocycle rate of the crowned **ABR** was lower than that in the control **BR**, especially significantly for **ABR 1**.

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P1.2.14. BICARBONATE REQUIREMENT FOR THE WATER-OXIDIZING COMPLEX OF PHOTOSYSTEM II

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It is known that bicarbonate (BC) facilitates electron transfer between the primary and secondary electron acceptors, Q_A and Q_B, in formate-inhibited PS II; the non-heme Fe between Q_A and Q_B plays an essential role in BC binding. Strong evidence for BC requirement within the water-oxidizing complex (WOC) (both O_2-evolving and assembling from apo-WOC and Mn(II)), of PSII has been presented recently (reviewed in [1]). The following explanations for the involvement of BC in the events within the WOC are considered: **1)** BC facilitates re-assembly of the WOC from apo-WOC and Mn(II); **2)** BC is an integral component of the WOC essential for its function and stability; it may serve as a direct ligand to the inorganic core of the WOC modifying the redox properties of Mn; **3)** BC is an easily accessible base (with an appropriate pK) involved in the removal of protons released during water oxidation.

Comparative studies of electrochemical and functional properties of complexes of Mn(II) and Mn(III) with BC and other carboxylates (formate and acetate) show that the unique capability of BC to initiate the assembly of the tetramanganese cluster of the WOC from Mn(II) and apo-WOC-PSII [2-4] can be attributed to formation of electroneutral, easily oxidizable Mn/BC-complexes that serve as building blocks for the WOC [5-7]. It is suggested that due to this property BC might have been critical to the evolution of the first O_2-evolving cyanobacteria from a non-oxygenic bacterial precursor in the Archean period (>2.2 BYA) [6,7]. (*Supported by the HFSP, grant RGPO 029 and the program PCB RAS).*

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P1.2.15. PROTEIN DYNAMICS CONTROL OF ELECTRON TRANSFER IN REACTION CENTERS FROM RPS. VIRIDIS

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Transformation of light energy to chemical potential in reaction centers of photosynthetic bacteria is associated with directional electron transfer across the membrane as well as protein relaxation, i.e., reorganization of the protein globule caused by the electron transfer. As was shown in Refs. [1,2], a change in the relaxation rate probably caused by a change in the state of protonation of some groups can change the electron transfer rate by a few orders of magnitude. If the electron transfer depends on the medium relaxation, one should be able to extract information on the medium dynamics from experimental ET kinetics. This idea was realized in works from Nienhaus' [3] and Kotelnikov's [1,2] groups. Here, the most direct method is developed based on the Ovchinnikova-Helman-Sumi-Marcus model [4], in which the diffusion-reaction equation is used to describe the reaction in a slowly relaxing medium. The ET rate constant at a given conformational state is expressed in terms of static equilibrium parameters as prescribed by the current ET theories, and the dynamical properties of the medium are characterized by a single parameter τ, the relaxation time of dipole reorientation. Experiments were performed on reaction centers from *Rps. viridis*, as described in [1], from 153 to 295 K. The static parameters of the model were determined from the measured kinetic curves at low and high temperatures, where the ET kinetics are independent of τ. At intermediate temperatures, the lower and upper bounds on τ were determined by minimizing the standard deviations of the calculated kinetics from the measured ones. The slope of the linear fit to the plot of log τ versus $1/T$ gives the activation energy of 0.4-0.6 eV for reorientation dipole relaxation. This value can be associated with breaking H-bonds in the protein matrix. This work was supported by the Russian Foundation for Basic Research and the U.S. Civilian Research and Development Foundation.

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P1.2.16. PROTON TRANSFER COUPLED TO THE UBIQUINONE REDUCTION IN THE REACTION CENTER OF BLASTOCHLORIS VIRIDIS

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The photosynthetic reaction center (RC) of purple bacteria utilises the energy of light to reduce an ubiquinone QB to ubiquinol. QB is reduced in two steps following absorption of two light quanta. This reaction is accompanied by electrogenic proton transfer from membrane surface to QB-site [1]. In the functionally best-studied RC of *Rb.sphaeroides*, the transfer of the second electron and the first proton to semiquinone were thermodynamically coupled [2] and proceed with the same rate [3,4]. In RCs of *Bl viridis*, however, second electron transfer was much faster (<100 mks) [5] than electrogenic proton transfer in response to second flash (~400 mks [6]). As opposed to the results obtained for *Rb.sphaeroides* [3,4], no electrogenic proton uptake after the first flash was reported [6].

To clarify these discrepancies, we used capacitive voltametry to study proton transfer in *Bl.viridis* RCs incorporated in liposomes. The voltage generation was detected both after the first and second flash (~6 and ~14% of the primary charge separation, respectively, pH7.5). The proton uptake kinetics upon the first flash resembled that in *Rb.sphaeroides*, with a fast, almost temperature-independent component of ~100 mks (7°C, pH7.5) and a slower component with stronger temperature dependence (Ea~50kJ/mol). Voltage kinetics in response to second flash featured a very fast component with a time constant of ~6 mks and a slower component with time constant of ~450 mks (20°C, pH7.5). The latter component, apparently, corresponded to that reported previously for *Bl.viridis* [6]. The revealed faster component, which is absent in the case of *Rb.sphaeroides*, might account for the electron-coupled transfer of the first proton to the semiquinone. The small relative amplitude of the faster component (~1% of the primary charge separation in RC) might indicate that proton transfer to the semiquinone proceeds from a nearby residue, which is then reprotonated on a slower time scale.

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P1.2.17. THE BINDING BEHAVIOR OF COENZYME Q TO THE PHOTOSYNTHETIC REACTION CENTER

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The photosynthetic reaction center is a transmembane protein, that converts light energy into the chemical energy of reduced coenzyme Q. Light absorption induces the stepwise transfer of two electrons and two protons to a coenzyme Q molecule bound in the Q_B site of the protein. The reaction product (the quinol QH_2) is released to the coenzyme Q pool of the membrane. During the light-induced reaction several intermediate protonation and redox states of coenzyme Q ($Q^{\cdot-}$, $QH^{\cdot}$ and QH^-) are formed. Two alternative positions of coenzyme Q in the Q_B site (distal or proximal to the non-heme iron of the photosynthetic reaction center) have been observed in crystal structures (1).

In the presented study, we have investigated the binding behavior of the educt (the quinone Q) and the first intermediate (the semiquinone $Q^{\cdot-}$) of coenzyme Q reduction to the two possible binding positions in the Q_B site of the photosynthetic reaction center. The protein and its environment are described by a continuum electrostatic model for which the Poisson-Bolzmann equation can be solved. Resulting protonation and binding state energies have been sampled by the Metropolis Monte Carlo algorithm. We thereby obtained binding probabilities in dependence of pH and coenzyme Q concentration (2).

A central aspect of our study is to identify residues which are involved in the binding of coenzyme Q. Coupling of the binding process and of the (de)protonation of certain protonatable residues was therefore evaluated based on a correlation quantity. It is known, that AspL213, a residue located in the Q_B binding site, plays a dual role in the light-induced reaction (3-4). AspL213 is involved in the proton transfer to coenzyme Q and stabilizes a proton in the environment of coenzyme Q. Based on our calculations, we suggest a third role for AspL213: protonation of AspL213 is a prerequisite for the binding of the first reaction intermediate (the semiquinone $Q^{\cdot-}$).

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P1.2.18. BLUE LIGHT-INDUCED FLUORESCENCE QUENCHING OF PHYCOBILISOMES IN THE CYANOBACTERIUM *SYNECHOCYSTIS* SP. PCC 6803 IN THE ABSENCE OF THE IsiA PROTEIN

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We have found that irradiation of a PSII-deficient mutant of the cyanobacterium *Synechocystis* sp. PCC 6803 with intense blue light causes a ~40% decrease of phycobilisome fluorescence induced by carotenoids [1]. This may involve the water-soluble OCP (orange carotenoid-binding protein; Slr1963) (D. Kirilovsky, unpublished observations). A similar phenomenon was demonstrated in cyanobacteria under iron starvation, and was suggested to involve the chlorophyll-binding protein IsiA [2, 3].

The possible role of IsiA in blue light-induced fluorescence quenching was studied in cyanobacterial strains lacking *isiA* or PSII (*psbB* and *psbDIC*). In conditions of iron deficiency, in wild type and in the PSII-less strain but not in the strain lacking IsiA, the chlorophyll absorption band shifted to 676 nm indicating the formation of IsiA complexes under these conditions. However, in all strains investigated here blue light-induced fluorescence quenching was observed, indicating that IsiA is not required for this quenching in cyanobacteria. The difference between the emission spectra before and after blue light-induced quenching peaked at 662 nm (due to phycobilisomes) and depended on neither the presence of iron nor the excitation wavelength (440 or 580 nm). Blue-light-induced quenching was particularly significant in strains lacking PSII or under conditions of iron deficiency, suggesting that the quenched fluorescence originated from phycobilisomes that did not transfer energy effectively to chlorophylls. Our results indicate that IsiA is not necessary for blue light-induced fluorescence quenching in cyanobacteria, and we interpret this blue light-induced quenching to be due to a carotenoid-induced quenching of phycobilisome (presumably allophycocyanin) fluorescence.

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P1.2.19. RECONSTITUTING THE ELECTRON TRANSFER CASCADE FROM PHOTOSYSTEM-I TO FERREDOXIN-NADP$^+$-REDUCTASE AND NITRITE REDUCTASE: KINETICS, ENERGETICS AND CATALYSIS.

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In oxygen-evolving photosynthetic organisms, photosystem-I drives the photoreduction of ferredoxin, a small acidic soluble enzyme which is involved in many metabolic pathways in chloroplasts and cyanobacteria. Electron transfer cascades were studied by flash-absorption spectroscopy in reconstituted systems involving photosystem-I, ferredoxin, ferredoxin-NADP$^+$-reductase (FNR) or nitrite reductase (NiR), either in the absence of the substrate (NADP$^+$ or nitrite) or in its presence(1). FNR catalyzes the two-electron reduction of NADP$^+$ into NADPH whereas NiR catalyzes the six-electron reduction of nitrite into ammonia. All proteins were obtained from the cyanobacterium *Synechocystis* PCC 6803 except NiR which was purified from spinach leaves.

The reported experiments have allowed us to obtain detailed knowledge on the kinetics, energetics and catalytic activities of FNR/NiR by taking advantage of the sensitivity of flash-absorption spectroscopy at microsecond time resolution and of the possibility of controlling precisely the number of redox equivalents delivered to the ferredoxin partner via the amount of photosystem-I.

Some of the results are briefly mentionned here:

- the dissociation rate of reduced ferredoxin from photosystem-I was derived both with wild type enzymes (800-2000 s^{-1}) and with mutants of either photosystem-I and ferredoxin. Two different ferredoxin residues were thus shown to be involved in the redox dependence of the dissociation process.
- the midpoint redox potentials for single reduction of FNR/NiR were deduced using variants of ferredoxin of known redox potentials.
- the second-order rate constants for single reduction of FNR/NiR were measured in the presence or absence of the substrate.
- rates of reoxidation of reduced ferredoxin were measured during multiple turnover of the ferredoxin partner: Initial rates of 340 electrons/s.FNR and 170 electrons/s.NiR were determined for the reoxidation of 3.0 μM reduced ferredoxin.

- the stromal extrinsic subunit PsaE of PSI is probably not involved in $NADP^+$ photoreduction in cyanobacteria, contrary to previous hypotheses.

It is anticipated that the present approach can be extended to the study of various mutants and of other soluble partners of ferredoxin.

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P1.2.20. THE INTERACTION BETWEEN PHOTOSYSTEM 1 AND CYTOCHROME b_6f COMPLEX IN HYBRID PROTEOLIPOSOMES STUDIED BY EPR METHOD

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The interaction between photosystem I (PS I) and cytochrome (cyt) b_6f complex from cyanobacterium *Synechocystis sp* PCC 6803 in hybrid proteoliposomes was studied by the electron paramagnetic resonance method using Varian E-4 X-band spectrometer. It was previously shown that the primary donor P700 in PS I and cyt *f* are predominantly localized near the outer surface of the proteoliposomal membrane, which made it possible to study interaction between these two protein complexes in this model system. Hybrid proteoliposomes were placed in standard quartz cuvette positioned in the resonator of the EPR spectrometer and illuminated with a sub-saturating white light from a 100-W tungsten lamp. We used chemically reduced decyl-plastoquinol ($DPQH_2$) as a donor to cyt b_6f complex, cyt c_6 as an electron carrier between cyt b_6f complex and PS I, and metylviologen (MV) as an acceptor for PS I. Illumination of proteoliposomes with white light caused the appearance of the EPR signal characterized by the linewidth ΔHpp = 8 G and g = 2.0025. This signal was typical of the EPR signal from $P700^+$.

The kinetics of $P700^+$ dark reduction was characterized by two components (in the time ranges of seconds and minutes). In the presence of cyt c_6 the contribution of the faster component was ~50% ($t_{1/2}$ ~1.5 s). Addition of MV and $DPQH_2$ considerably increased the contribution of the fast component (~ 80%) and accelerated the kinetics of $P700^+$ reduction ($t_{1/2}$ ~ 0.5 s). Stigmatellin, the inhibitor of cyt b_6f complex quinol-oxidizing site Q_o, slowed down the faster component by a factor of 3 ($t_{1/2}$ ~ 1.5 s). The fast component of $P700^+$ dark reduction with $t_{1/2}$ ~ 0.5 s was attributed to reduction of photooxidized $P700^+$ by cyt b_6f complex. These data indicate the effective interaction between PS I and cyt b_6f complexes in the presence of cyt c_6 and $DPQH_2$ in hybrid proteoliposomes.

Poster session 1.3. ATP-synthase/ATPase

P1.3.1. OLIGOMERIZATION AND INHIBITORY POWER OF THE REGULATORY PEPTIDES OF SACCHAROMYCES CEREVISIAE ATP SYNTHASE

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Mitochondrial ATP synthases are regulated by an inhibitory peptide (IF1) that binds to a catalytic interface in the absence of protonmotive force and the presence of MgATP. In bovine species, the inhibitory form of IF1 is thought to be dimeric and favoured by low pH whereas high pH favours the formation of a non-inhibitory tetramer [1]. bIF1 (*B. taurus*) is 84-residues long and yIF1 (*S. cerevisiae*) is 63 residues-long, the difference in size being mainly due to a shorter C-terminal part in yIF1. *S. cerevisiae* also contains a second inhibitory peptide homologous to yIF1 and called STF1, the role of which is unknown. yIF1 and STF1 are thought to exist under monomeric and dimeric forms, but the relationship between their oligomeric state and their inhibitory power is uncertain [2].

To know the relationship between the oligomeric state of yIF1 and its inhibitory power, we have engineered redox-dependent artificial dimers of this peptide by mutating into cysteins selected residues (E33C, Q31C/E33C, L54C), the bovine homologous of which face together or not in the published structure of tetrameric bIF1 [3]. $CuCl_2$-mediated crosslinking occurred in a few seconds regardless the position of the mutated residue(s). yIF1 dimers crosslinked in the C-terminal part by 54C-54C could still rapidly inhibit ATPase activity of isolated F_1-ATPase, SMP or alamethicin-treated mitochondria, whereas yIF1 dimers or oligomers crosslinked by 31C, 33C or both residues were no more inhibitory. In the absence of $CuCl_2$, the degree of crosslinking was found dependent of the position of the mutation. The double mutant Q31C/E33C was practically 100 % crosslinked, but the additional mutation E21A, which affects the pH-dependency of IF1-inhibition [4], significantly decreased the degree of crosslinking. Our data suggest that the natural interface of dimerization of yIF1 corresponds to the the inter-dimers interface of tetrameric bIF1. The functional study of 54C-54C dimer, that mimicks the natural bIF1 dimer, also proves that such a dimer does inhibit ATPase activity, even in the matricial environment.

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P1.3.2. FUNCTIONAL ROLE OF SUBUNIT E OF F0F1ATPSYNTHASE IN BOVINE HEART MITOCHONDRIA

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Subunit e of the F0 sector is an integral inner membrane protein located in the peripheral stalk of F0F1ATPsynthase, the function and stoichiometry of which are not clearly defined. It spans the inner membrane via a single segment located at the N-terminal, while the C-terminal protrudes into the intermembrane space both in bovine and yeast (1,2). Some clues to its possible function and importance are emerging from work carried out with the yeast enzyme, where it has been demonstrated that subunit e, together with subunit g and b of F0 sector, supports the formation of F0F1ATPsynthase dimers and oligomers, which play a critical role in cristae morphology (2). In mammals, a bioinformatic analysis suggests that subunit e is a multifunctional cell regulator involved in cell signalling (3). To begin to elucidate the functional role of e subunit in F0F1ATPsynthase of bovine heart mitochondria, its stoichiometry within the enzyme complex and its possible involvement in enzyme dimers/oligomers formation, which has been already followed by us with gel analysis (4), has been analyzed by a proteomic approach. Mitoplasts from bovine heart mitochondria were treated with trypsin to selectively degrade subunit e, or with chemical cross-linkers to obtain e-e homodimers. Treated mitoplasts were solubilized by Triton X-100 and subjected to 1D BN-PAGE. Bands corresponding to ATPsynthase monomer, dimer and oligomers were excised and submitted to iterative SDS PAGE followed by LC/ESI-MS analysis of subunit e and its cross-linking products. The results indicate that e subunit, differently to yeast, remains bound to all the oligomeric forms of ATPsynthase in apparent stoichiometric amounts. On the other hand, subunit e appears to promote the stabilization of the enzyme supramolecular forms. In fact, the selective complete degradation of e subunit by trypsinization alters the ratio in favour of the monomeric state of ATPsynthase and EEDQ-mediated formation of e-e homodimers stabilizes the ATPsynthase oligomers. We suggest that, as in yeast, subunit e could form, together with still unknown subunits, the interface between two neighbouring F0 sectors in ATPsynthase dimer, which has been recently observed in bovine heart mitochondria by transmission electron microscopy (4).

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P1.3.3. A BIND-LOCK MECHANISM FOR THE INHIBITORY PEPTIDE OF MITOCHONDRIAL ATP SYNTHASE

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The mechanism of inhibition of yeast mitochondrial F_1-ATPase by its regulatory peptide IF1 was investigated by correlating the rate constant of IF1 binding (k_{on}), deduced from kinetic studies, with the catalytic sites occupancy [1-2]. Catalytic sites occupancy was probed by fluorescence quenching of β-Trp-345 replacing β-Tyr-345 [3], which allowed to determine three different dissociation constants for nucleotides. After preincubation with PP_i, which mimicks ATP-loading of non catalytic sites, these dissociation constants were K_{d1}=10±2 nM, K_{d2}=1±0.2 μM, K_{d3}=53±5 μM for MgADP, and K_{d1}<1 nM, K_{d2}=0.08±0.01 μM, K_{d3}=41.5±5 μM for MgATP at steady state. ATPase activity of the PP_i-treated β-Trp-345 enzyme could be well fitted with simple Michaelis-Menten kinetics with K_m = 32.5±5 μM, superimposed to a background activity (presumably single-site activity) that represented about 1% of the maximum catalytic turnover. The fit was still improved by adding a component with K_m=0.08 μM, the contribution of which to the total catalytic turnover was only 2%. This suggests that significant rates of ATP hydrolysis correlate to the filling of the third catalytic site. The rate of IF1 binding, low for MgATP <1 μM, dramatically increased with increasing concentration of MgATP to about 20 μM, and slightly decreased thereafter. k_{on} was half-maximal at 1 μM MgATP, that is 10 fold higher than K_{d2} (ATP) and 30-40 times lower than K_{d3} (ATP). The decrease of k_{on} at high MgATP concentration correlated with the filling of the third catalytic site. These data suggest that IF1 binding to F_1-ATPase comprises two steps, loose binding and locking [2]. Loose binding is determined by catalytic sites occupancy which modulates the accessibility of the enzyme for IF1. After freezing the non-catalytic sites with PP_i, k_{on} is practically zero with the nucleotide-free enzyme, about 2.5 10^6 M^{-1} s^{-1} with one or two occupied catalytic sites, and about 1.9 10^6 M^{-1} s^{-1} with three occupied catalytic sites. Locking is turnover-dependent and competes with IF1 release. This model explains why the rise of k_{on} does not match the filling of the catalytic sites. It is a first step towards understanding of the dynamics of IF1-mediated inhibition.

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P1.3.4. INTRINSIC UNCOUPLING IN THE ATP SYNTHASE OF E.COLI

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The H^+/ATP ratio in the catalysis of ATP synthase has generally been considered a fixed parameter. However, in the ATP synthase of the photosynthetic bacterium *Rhodobacter capsulatus*, we have recently shown that this ratio can significantly decrease during ATP hydrolysis when the concentration of either ADP or Pi is maintained at a low level (1). We have next focused our attention on the ATP synthase of *E.coli*, looking specifically for evidence of intrinsic uncoupling in this organism as well.

The hydrolysis activity of the purified, reconstituted *E.coli* enzyme has been shown to be strongly inhibited, in the presence of ADP, by Pi, with an apparent Kd in the order of 500 microM, and by ADP, in the microM range (2). We have reproduced these results measuring as a function of Pi and ADP the DCCD-sensitive ATP hydrolysis activity of *E.coli* internal membranes. In contrast to this monotonic inhibition, however, the proton pumping activity of the enzyme, as estimated under the same experimental conditions by the fluorescence quenching of the DeltapH-sensitive probe ACMA, showed a clearly biphasic progression, both for Pi, increasing from 0 up to approximately 200 microM, and for ADP, increasing from 0 to few microM. This result can only be explained if the occupancy of ADP and Pi binding sites shifts the enzyme from a (partially) uncoupled state to a normally coupled state.

We conclude that the phenomenon of "intrinsic uncoupling", first shown in the ATP synthase of *Rb. capsulatus*, also takes place in the *E.coli* enzyme, suggesting its likey occurrence in all Prokaryotes.

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P1.3.5. A MODEL STRUCTURE OF THE PROTON CHANNEL OF THE E.COLI ATP SYNTHASE

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The proton translocation pathway in the F_o-complex of the ATP synthase lies at the interface of helices IV and V of the subunit *a*, and the outer surface of the rotating cylindrical oligomer built of 10 copies of the subunit *c*. The structure of the *E.coli* subunit *c* oligomer was first modeled from the NMR structure of the subunit *c* monomer and the intersubunit cross-linking data [1], and further refined by homology modeling using an X-ray structure of the subunit *c* complex from *Ilyobacter tartaricus* [2] as a template. No high-resolution structure of subunit *a* is available.

We have calculated a model structure of the subunit *a* of the *E.coli* ATP synthase by simulated annealing from a set of long-range distance constraints derived from (i) intramolecular Cys-Cys cross-linking in the membrane, (ii) a pattern of silver inhibition of the ATPase in a large set of single-cysteine mutants of subunit *a*[3], and (iii) second-site suppressor mutations. Additional distance restraints were derived from NMR line broadening of the sidechain resonances of tryptophans 231,232,235 and 241 by spin labels introduced at various positions along helices IV and V in a chloroform-methanol-water solvent. The distance constraints derived from the intersubunit *a*-*c* Cys-Cys cross-linking in the membrane were used to model the *a*-*c* complex and calculate the complete structure of the proton channel of the ATP synthase.

In the model, the helices II-V of subunit *a* are packed tightly at the periplasmic side of the membrane surrounding a cavity, accessible to small ions, which extends at an angle about halfway through the membrane and may provide access for protons to the essential Asp61 residue of the subunit *c*. The exit channel to the cytoplasm is surrounded by helices IV and V of subunit *a* and the outer helices of two neighboring *c* subunits and is wider than the channel leading from the periplasm to Asp61. This work was supported by US Public Health Service Grant GM-23105 to RHF.

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P1.3.6. CRYSTAL STRUCTURE OF YEAST V-ATPASE SUBUNIT C REVEALS ITS STATOR FUNCTION

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Vacuolar H+-ATPase (V-ATPase) has a crucial role in the vacuolar system of eukaryotic cells. It provides most of the energy required for transport systems that utilize the proton motive force that is generated by ATP hydrolysis [1]. Some, but not all, of the V-ATPase subunits are homologous to those of F-ATPase and the non-homologous subunits determine the unique features of V-ATPase. We determined the crystal structure of V-ATPase subunit C (Vma5p), which does not show any homology with F-ATPase subunits, at 1.75A° resolution. The structural features suggest that subunit C functions as a flexible stator that holds together the catalytic and membrane sectors of the enzyme. A second crystal form that was solved at 2.9A° resolution supports the flexible nature of subunit C [2,3]. These structures help address recent reports about the ability of subunit C to bind actin and ADP and provide a framework for exploring the unique mechanistic features of V-ATPases [4].

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P1.3.7. STRUCTURE OF DIMERIC ATP SYNTHASE FROM MITOCHONDRIA

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Respiration in all cells depends upon synthesis of ATP by the ATP synthase complex, a rotary motor enzyme. Although the enzyme functions as a monomer, dimeric ATP synthase supercomplexes were found in yeasts, bovine heart, *Arabidopsis* and *Chlamydomonas*. However, so far knowledge on dimeric ATP synthase is limited due to the lack of structural data.

A dimeric ATP synthase supercomplex was first discovered for yeast mitochondria by BN / SDS PAGE [1]. The supercomplex includes dimer-specific subunits termed e, g and k. More recently a very stable ATP synthase supercomplex was described in the algae *Chlamydomonas* and *Polytomella* [2]. The supercomplex includes an additional 60 kDa protein termed "Mitochondrial ATP synthase associated protein" or MASAP. All these additional subunits are speculated to be involved in dimer formation. Here we report purification and structural characterization of the dimeric ATP synthase from green alga *Polytomella* and baker's yeast *Saccharomyces cerevisiae*. Structural analysis by electron microscopy and single particle analysis revealed that dimer formation is based on specific interaction of the F_0 parts. Remarkely, the F_1 headpieces are not at all in close proximity. The angle between the two F_0 parts is about 70° in *Polytomella* and 35° between "pseudo-dimers" or 90° in case of "real-dimers" in yeasts. This arrangement is considered to induce a strong local bending of the membrane.

Our data provide a direct clue for the role of dimerization of ATP synthase monomers. We propose that dimeric ATP synthase supercomplexes represent basic building blocks of ATP synthase oligomers and that formation of these structures is the driving force for cristae formation and overall mitochondrial morphology [3].

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P1.3.8. PROTON-DRIVEN C SUBUNIT ROTATION WITHIN THE F_O MOTOR OF A SINGLE ATP SYNTHASE

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ATP formation by membrane-embedded F_oF_1-ATP synthase requires conformational changes which are induced by the stepwise rotation of the γ and ε subunit in the enzyme. The opposite direction of rotation during ATP synthesis and hydrolysis was confirmed by single-molecule fluorescence resonance energy transfer (FRET) using specific labeling of these rotary subunits of the F_1 part and the stator subunits of the F_o part [Diez et al, Nat. Struct. Mol. Biol. 11:135 (2004); Zimmermann et al, EMBO J. 24:2053 (2005)]. Rotation of γ and ε is coupled to the rotation of the *c* subunits of the ion-driven F_o motor within the lipid membrane. As shown recently, ATP hydrolysis resulted in a three-stepped rotation of the *c*-ring of F_o from a thermophilic bacterium. However, ATP synthesis by the Na^+-translocating enzyme from *P. modestum* was associated with a multi-stepped rotation. To distinguish between the two mechanisms we have developed a single-molecule FRET assay that allowed for monitoring of the *c*-ring rotation in F_o in real time. We use EGFP as the FRET donor fused to subunit *a* [Düser et al, Biochim. Biophys. Acta-Bioenergetics 1658:108 S Suppl. (2004)] of the *Escherichia coli* enzyme, and a single rhodamine-labeled *c* subunit as the FRET acceptor. Our data indicate a stepwise movement of the *c*-ring during proton-driven ATP synthesis as well as ATP hydrolysis in contradiction to a quasi-continuous rotation.

P1.3.9. REGULATION OF F_OF_1 ATP SYNTHASE: SUBUNIT EPSILON C-TERMINAL DOMAIN IS INVOLVED IN INHIBITION BY ADP AND IN ACTIVATION BY PROTONMOTIVE FORCE.

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We investigated the regulatory role of subunit epsilon in inhibition of ATP hydrolysis by F_OF_1-ATP synthase from thermophilic bacterium *Bacillus PS3*. It was previously demonstrated in this organism that the C-terminal alpha-helical domain of subunit epsilon undergoes large-scale conformational transitions. In one conformation the two C-terminal helices form a hairpin structure close to F_O; in another conformation they are extended so that the C-terminus of epsilon can reach the N-terminus of subunit gamma inside F_1 [1]. The inhibitory effect of epsilon is observed in the extended, but not in the hairpin conformation.

In this study we report that the inhibitory effect of the C-terminal domain of subunit epsilon was markedly enhanced in the presence of ADP. We also demonstrate that the activation of ATP hydrolysis by protonmotive force is dependent on the C-terminal domain of subunit epsilon. The results support the recently proposed hypothesis that subunit epsilon might act as a "safety lock" preventing spontaneous re-activation under de-energized conditions [2].

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P1.3.10. MICROSECOND TIME SCALE ROTATION MEASUREMENTS OF SINGLE F_1ATPASE MOLECULES

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A novel method to detect rotation of the F_1ATPase γ subunit in a manner sensitive enough to achieve acquisition rates with a time resolution of 2.5 μs (equivalent to 400,000 fps) is reported. This is sufficient to resolve the rate at which the γ subunit travels from one dwell state to another (transition time). Rotation is detected via a gold nanorod attached to the rotating γ subunit of an immobilized single molecule of F_1ATPase. Variations in scattered light intensity from the nanorod allow precise measurement of changes in angular position of the rod below the diffraction limit of light. Using this approach, the transition time of *E. coli* F_1ATPase γ subunit rotation was determined to be 7.62±0.15 (s.d.) rad/ms. The average rate-limiting dwell time between rotation events observed at saturating MgATP concentrations was 8.03 ms, comparable to the observed MgATPase k_{cat} of 130 s^{-1} (7.7 ms). Histograms of scattered light intensity from MgATPase-dependent rotation as a function of polarization angle allowed the determination of nanorod orientation to the axis of rotation and plane of polarization. Based on this information, and the high temporal resolution of the rotation, the drag coefficient was determined that implied that the instantaneous torque generated by F_1 was 63.3±2.9 pN•nm. The maximum amount of work observed during a 120° transition was 132.5±6.0 pN•nm, which is significantly larger than the free energy of ATP hydrolysis (90 pN•nm) under physiological conditions. The instantaneous torque generated during MgITP hydrolysis was significantly lower suggesting that substrate affinity at the catalytic site contributes to the free energy used to generate γ subunit torque.

P1.3.11. THE E. COLI F_1F_O-ATPASE: EFFECT OF DIFFERENT RECONSTITUTION AND ASSAY PROCEDURES ON ATP SYNTHESIS ACTIVITY

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New procedures for obtaining isolated *E. coli* F_1F_o-ATPase and its reconstitution into liposomes are described. The procedures were fast (6-8 hours) and provided highly active F_1F_o (up to 70 μmol/min per mg of protein at 37°C and pH 8.0). The reconstitution procedure yielded well-coupled proteoliposomes capable of ATP synthesis at the expense of K^+/valinomycin diffusion potential and pH difference across the membrane. Electrical membrane potential induced by valinomycin was indispensable for ATP synthesis. Among lipids used for reconstitution, egg yolk phosphatidylcholine was the best one in terms of the initial rate of ATP synthesis and ATP yield.

Kinetics of ATP synthesis by proteoliposomes were registered using either automatic injection or the quench-flow technique. Both methods gave consistent results.

P1.3.12. QUANTUM DOTS AS ENERGY DONORS FOR FLUORESCENCE RESONANCE ENERGY TRANSFER IN A SINGLE MEMBRANE BOUND H^+-ATPSYNTHASE

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H+-ATPsynthases, F0F1, couple the transmembrane proton translocation with synthesis of ATP in bacteria, chloroplasts and mitochondria. The movement of the rotor subunits (γεc10) relative to the stator subunits (α3β3δab2) has been investigated with membrane integrated F0F1 from *E. coli* with single molecule spectroscopy. A fluorescence donor (tetramethylrhodamine) was covalently bound to the γ- or ε-subunit, a fluorescence acceptor (Cy5) was bound to the b-subunit. The efficiency of the fluorescence resonance energy transfer (FRET) was measured during ATPhydrolysis and ATPsynthesis with EF0F1. The measurements show a stepwise rotation of the γε-complex relative to the b-subunit, the direction during hydrolysis being opposite to that during ATPsynthesis (1,2). A problem in these investigations was the limited photophysical stability of the organic dyes. The light intensity of the exciting laser must be limited and this leads to a low number of detected fluorescence photons per time. Quantum dots are luminiscent CdSe-ZnS nanocrystals, which can be synthesized with a well defined size. Due to the quantum confinement of the charge carriers within the quantum dots, the photo emission spectra can be tuned to the required wavelength region by varying their size. We constructed a system, where a quantum dot with a hydrophilic shell was attached covalently to the b-subunit and an organic acceptor at the ε-subunit of membrane integrated EF0F1. This system showed ATPhydrolysis and ATPsynthesis activity and FRET changes were observed during catalysis in single molecule experiments.

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P1.3.13. FLAGELLAR MORTOR FROM THE THERMOPHILIC PS3

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Many species of bacteria swim by means of flagellar rotation. Energy for rotation comes from H^+ (or Na^+) electrochemical potential gradient. The bacterium flagellum is composed of three structurally defined parts: the basal body, the hook and the filament. MotA/MotB complex, stator of the flagellum motor, transfers proton from outside to across the membrane. The rotor of flagellar motor consisted of three proteins (switch complex) that regulate rotational direction. Flagellar biosynthesis starts assembly of MS-ring into the cytoplasmic membrane first, subsequently C-ring and export apparatus form to cytoplasm. The rod, the hook and the filament proteins are penetrated cytoplasmic membrane by export apparatus, and assemble distal end of filament where a capping complex helps its assembly. However, the molecular mechanisms of torque generation are not fully understood.

Thermophilic *Bacillus* PS3, a gram-positive bacterium used by this work, has two advantages; thermo-stability of components and relatively simple structure lacks an outer membrane and P-L ring located around the rod.

In order to analyze these mechanism and functions, *fla/che* operon (large flagellar gene cluster, >26ktp) was isolated from the PS3 genomic library, and the sequences of all 32 ORFS were determined. These deduced amino acid sequences correspond to basal body, hook, chemotaxis proteins, and flagellar biosynthesis protein were compared with those of another microorganisms. FliI has homologous region to other bacterial ATPase F1βs. C-terminus of PS3 FliY is homologous to E. coli FliN, but the N-terminus share homology with PS3 FliM. In this work, MS-ring (FliF), C-ring (FliM, FliY, FliG) and CheY, were over-expressed as CBD (chitin binding domain) or MBP (maltose binding protein)-tagged protein. To investigate FliM and CheY properties, we constructed MBP-FliM or MBP-Δ1-16 FliM fusion protein, since N-terminus region of FliM has described as phosphorylated CheY binding area for *E. coli*, and CheY D53, this residue essential for CheY phosphorylation. The result of these tests showed that FliM N-terminus and CheY 53D residue is important for interaction between FliM and CheY. The *hag* operon, encodes a flagellin, was also isolated from the PS3 genomic library, and the sequence was determined. PS3 flagellin over-expressed in *E. coli* as fused protein with MBP was cleaved with enterokinase, but it could not completely cleave. We assumed it cause by interaction between N-terminus region and C-terminus

region of flagellin with MBP-flagellin, as a result of the study using the deletion mutant proteins lack N-terminal, C-terminal, or both regions.

P1.3.14. A CRUCIAL CLUE TO UNDERSTAND THE MOLECULAR ROTATION OF C-SUBUNIT RING COUPLED WITH PROTON TRANSLOCATION THROUGH F(0) OF THERMOPHILIC BACILLUS PS3 ATP SYNTHASE

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ATP synthases catalyze ATP synthesis/hydrolysis coupled with a transmembrane proton translocation in bacteria, chloroplasts, and mitochondria. The enzyme is composed of two portions, a water-soluble F(1), which has the catalytic sites for ATP synthesis/hydrolysis, and a membrane-integrated F(0), which mediates proton translocation. The bacterial enzyme has the simplest subunit structures, $\alpha_3\beta_3\gamma_1\delta_1\varepsilon_1$ for F(1) and $a_1b_2c_{10\text{-}15}$ for F(0), respectively. Proton translocation through F(0) drives rotation of an oligomer ring of *c*-subunits, which in turn drives rotation of γε-subunits in F(1). In this decade, the driving force for the rotation of *c*-subunit ring was proposed to be generated by the conformational changes in TMH-2 of one *c*-subunit relative to the stationary TMH-4 of *a*-subunit due to its swivel motion for approximately 140 degree, revealed with the cross-linking studies and NMR structural analysis of *Escherichia coli c*-subunit.[1,2] Recently, molecular structures of *c*-subunit ring have been resolved by X-ray analysis of V-Type Na^+-ATPase crystal from *Enterococcus hirae*[3] and Na^+-ATPase crystal from *Ilyobacter tartaricus*[4]. The solution structures are tightly packed with surrounding residues, implies that the conformational changes may not occur during *c*-subunit ring rotation. In order to answer the question: *what is the driving force for the rotation of c-subunit ring*; in this study, the coupling of proton translocation and rotation in the F(0) of ATP synthase was investigated using a stable thermophilic *Bacillus* PS3 enzyme functionally expressed within *E. coli* cells. Some mutants with single or double cysteine replacements on TMHs of *c*-subunit ring were constructed based on the substantive structural information[3,4]. Double cysteine residues, which introduced into TMH-1 and TMH-2, respectively, were readily and efficiently cross-linked via disulfide bond formation without the addition of any oxidants such $CuCl_2$, while no cross-linked product was obtained whenever a single cysteine residue was introduced into TMH-1. Remarkably, the cross-linked products were active in both proton-pumping and ATPase. These results suggested that the swivel motion[2] in TMH-2 is not necessarily required for the F(0) function.

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P1.3.15. PROTON ATPASE IN REDOX SENSING BY BACTERIA

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The most of anaerobic bacteria are able to grow upon decrease in external oxidation-reduction potential (E_h) down to negative values [1-2].

Data on decrease in E_h and on lowering of external pH are presented during the growth of *Escherichia coli*, *Enterococcus hirae*, *Lactobacillus salivarius*, *L. lactis* and the other lacto-acid bacteria under anaerobic conditions upon the fermentation of sugars (glucose). These inter-related changes in E_h and pH during the bacterial growth could not be described by the theory of oxidation-reduction processes that deals usually with a pair of oxidized and reduced substances [3]. E_h is probably a factor determining bacterial anaerobic growth [4-5], when oxidizers and reducers can regulate the growth. Therefore, bacteria are assumed to have a redox taxis sensing E_h.

The effects of E_h on the transport of protons through the F_0F_1-ATPase and of the other substances across the membrane, for instance, accumulation of potassium ions via the Trk or Trk-like uptake system, and on activity of membrane-associated formate hydrogenlyase enzyme (in *E. coli*) as well as of the *N,N'*-dicyclohexylcarbodiimide-sensitive F_0F_1-ATPase have been shown. These processes were affected by oxidizers and reducers like ferricyanide (1 mM), Cu^{2+} ions (0.1 mM) and DL-dithiothreitol (3 mM), correspondingly. The changes in membrane proton conductance and proton-motive force, accessible thiol-groups number in membrane vesicles and disulphide-dithiol interchange in appropriate membrane proteins could be accounted for these effects.

A role of the F_0F_1-ATPase in redox sensing by bacteria under fermentation is proposed.

A regulation of bacterial metabolism by change in E_h is ordered in biotechnology, veterinary and medicine.

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P1.3.16. OVEREXPRESSION OF SUBUNIT C OF THE ATP SYNTHASE INCREASES TOTAL ATP SYNTHASE AMOUNT AND ACTIVITY IN BROWN-FAT MITOCHONDRIA

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Rodent brown adipose tissue mitochondria contain very low amounts of ATP synthase, relative to the respiratory chain components. In order to examine *in vivo* the role of the expression of P1 isoform of the c-Fo subunit in the biogenesis of ATP synthase, we made a transgenic mouse, overexpressing P1-c subunit isoform in brown adipose tissue under the promoter of the brown adipose tissue-specific protein UCP1. In the resulting UCP1p1 transgenic mice, levels of the transgenic, as well as total P1-c subunit mRNA, were significantly increased, as assessed by relative quantitative RT-PCR; mRNA levels of other F1Fo-ATPase subunits were unchanged. In isolated brown-fat mitochondria, protein levels of the total c-Fo subunit were increased in transgenic mice. Remarkably, this also led to an increase in protein levels of ATP synthase subunits that are part of the F1-ATPase complex. Oxygen consumption measurements in isolated brown-fat mitochondria confirmed an increased functional activity of the F1Fo-ATPase in transgenic mice compared to wild-type mice. We conclude that the levels of the c-Fo subunit P1 isoform are crucial for defining the final content of the ATP synthase in brown adipose tissue and suggest that the level of c-Fo subunit may be the determining factor for F1Fo-ATPase assembly in higher eukaryotes.

P1.3.17. NUCLEOTIDE BINDING PROPERTIES OF NONCATALYTIC SITES OF THIOL-MODULATED CHLOROPLAST ATP SYNTHASE

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The binding of ADP and ATP to noncatalytic sites of DTT modified chloroplast ATP synthase was studied. Selective binding of nucleotides to noncatalytic sites was attained by means of preliminary light incubation of thylakoid membranes in the presence of [^{14}C]ADP followed by its dissociation from the catalytic sites during dark ATP hydrolysis stimulated by bisulfite ions ("cold chase"). The binding to noncatalytic sites was preceded by dissociation of endogenous bound nucleotides. The step of nucleotide release from noncatalytic sites was found to be energy-dependent, whereas nucleotide binding to vacant noncatalytic sites did not require membrane energization. The equilibrium between free and bound nucleotides depended on their concentration and was attained during 2 – 5 min. The exposure of thylakoid membranes to 0.7 –12 μM nucleotide leads to binding of up to two noncatalytic sites of CF_0F_1 with a K_d value of about 1.5 μM. The sites differ in their specificity: one preferentially binds ADP, whereas the other is able to bind either ADP or ATP. Dependence of nucleotide incorporation on Mg^{2+} concentration was slight and changed insignificantly upon Mg^{2+} substitution by Ca^{2+}.

P1.3.18. ESSENTIAL Arg OF SUBUNIT A IN F_OF_1-ATP SYNTHASE PLAYS A KEY ROLE IN C-RING ROTATION BY PREVENTING THE FUTILE PROTON SHORTCUT

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In a rotary motor enzyme F_oF_1-ATP synthase (F_oF_1), the oligomer ring of F_oc subunits (*c*-ring) rotates relative to F_oa subunit as protons flow through putative channel(s) in F_oa and a carboxyl group in F_oc. Previous reports have indicated that a conserved Arg in F_oa controls the proton transfer at the F_oa/*c*-ring interface. In fact, thermophilic F_oF_1 with substitution of this Arg (*a*R169) to Glu, Ala, Val, Ile, Lys, Phe, or Trp lost proton-coupled ATP hydrolysis/synthesis activities, that is, no rotation occurred in these mutants. However, the mutants *a*R169E, *a*R169A and *a*R169V, but not other mutants, still mediated proton translocation down the proton gradient. This passive proton translocation was completely blocked by the second mutation (*c*E56Q) of F_oc. Then we generated a 'rotation-impossible' (c_{10}-*a*)F_oF_1 in which ten copies of F_oc in the *c*-ring [1] and F_oa were all genetically fused as a single polypeptide. This (c_{10}-*a*)F_oF_1 had a native-like structure because proton-coupled activities appeared upon cleavage of c_{10}/*a* linkage. We found that (c_{10}-*a*)F_oF_1 did not mediate passive proton translocation but (c_{10}-*a*)F_oF_1s with substitution *a*R169E or *a*R169A did so though slowly. Thus, it appears that the large, delocalized positively-charged side chain of the conserved Arg in F_oa ensures proton-coupled *c*-ring rotation by preventing futile proton shortcut.

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P1.3.19. STOCHASTIC HIGH-SPEED ROTATION OF ESCHERICHIA COLI ATP SYNTHASE F_1 SECTOR AND ITS MUTATIONS IN THE ATP-BINDING REGION

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The γ subunit of the ATP synthase F_1 sector rotates at the center of the $\alpha_3\beta_3$ hexamer during ATP hydrolysis. A gold bead (40 ~ 200 nm diameter) was attached to the γ subunit of *E. coli* F_1, and then its ATP hydrolysis-dependent rotation was studied. The rotation speeds were variable, showing stochastic fluctuation. The high-speed rates of 40-nm and 60-nm beads were essentially similar: 721 and 671 rps (revolutions per sec), respectively. The average rate of 60-nm beads was 381 rps, which is ~ 10-fold faster than that expected from the steady-state ATPase turnover number. These results indicate that the F_1 sector rotates much faster than expected from bulk ATPase activity, and that ~ 10 % of F_1 molecules are active on the m sec time scale. Furthermore, the real ATP turnover number (number of ATP molecules converted to ADP and phosphate per sec), as a single molecule, is variable during a short period. The ε subunit inhibited rotation and ATPase activity, whereas ε fused through its carboxyl-terminus to cytochrome b_{562} showed no effect. The ε subunit significantly increased the pausing time during rotation.

The γ subunit rotations of the F_1s with an amino acid residue substitution in the ATP-binding region were examined using 60-nm gold bead as a probe. Pauses longer than 10 m sec were frequently observed during rotations, and the average rotation rates were ~ 10-fold slower than that of wild type F_1. On the other hand, the rotation rate of revertant F_1 was almost the same as that of wild type. Interestingly, pauses similar to mutant F_1s were also observed with the revertant. These results indicate that the ATP-binding region plays an important role in driving smooth rotations.

Stochastic fluctuation of catalysis may be a general property of an enzyme, although its understanding requires combining studies of steady-state kinetics and single molecule observation.

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Stochastic high-speed rotation of *Escherichia coli* ATP synthase F_1 sector: The ε subunit-sensitive rotation. *J. Biol. Chem. In press*

P1.3.20. ATP SYNTHASE-REGULATION OF A COMPLEX MACHINE

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ATP synthase is the main enzyme producing ATP, the currency of energy in living cells, from ADP and inorganic phosphate [1]. The energy for this reaction is derived from a proton gradient across the bio-membrane in which ATP synthase is located. Energy conversion is done in two steps: first the electrochemical energy of the proton gradient is converted into mechanical energy of subunit rotation, subsequently this mechanical energy is converted into chemical energy of ATP. The enzyme can reversibly be separated into the membrane-bound Fo part which can conduct protons across the membrane and the hydrophilic F1 part, which houses the nucleotide binding sites and, when separated from Fo, can hydrolyze ATP.

Using the polystyrene-bead method to detect rotation of the gamma subunit of F1 [2] we have investigated how regulation of the enzyme's activity influenced the rotational movement. For this purpose we engineered thermostable F1 sensitive to redox-regulation and to the phytotoxin tentoxin [3]. We succeeded in observation of the enzyme's rotational movement in different modes [4,5].

In addition to this work on F1, we will discuss on-going projects using GFP-ATP synthase fusion proteins. These constructs may help to detect elastic energy transfer within ATP synthase during the ATP synthesis reaction.

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P1.3.21. EFFECT OF THE gammaM23-K MUTATION ON ATP SYNTHASE ACTIVATION BY THE PROTONMOTIVE FORCE AND ON COUPLING ATP HYDROLYSIS TO PROTON TRANSLOCATION

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The single-site mutation M23-K in the gamma-subunit of the *E.coli* ATP synthase has been reported to perturb the energetic coupling between F_1 and F_O and to increase the transition state activation energy for ATP hydrolysis (1). We have introduced the homologous mutation gamma-M23-K in the ATP synthase of *Rhodobacter capsulatus* in order to study its phenotype by taking advantage of the photosynthetic system.

In our hands, the most striking phenotypic difference to the wild-type complex was found in the ATP hydrolysis activation by the protonmotive force. The higher ATP hydrolysis rate is best observed when the activating protonmotive force is dissipated by addition of uncouplers. At low or zero protonmotive force, though, this activated state decays and can be measured only for a limited time. We have found that in the mutated enzyme, the half-life time of the light-activated state, measured under the above conditions, is reduced from 13 to 4 s. Moreover, after a burst of ATP synthesis triggered by a train of flashes, while the wild-type rapidly hydrolyzes the newly synthesized ATP, the mutant does not, indicating that stabilization of the inactive state is even higher at low ATP concentration.

The efficiency of proton coupling could be modified, both in the wild type (2) and mutated strain according to the experimental conditions. While a large difference in efficiency could be detected in the M23K mutated strain as compared to the wild type under certain conditions, in other cases the difference was much more limited. A detailed analysis of the two phenotypes will be presented and discussed in relation to the modulation of proton pumping activity and the possible differences produced by the mutation.

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P1.3.22. STRUCTURAL ANALYSIS OF THE COUPLING SUBUNIT F OF THE ARCHAEAL A_1A_O ATP SYNTHASE FROM METHANOSARCINA MAZEI GO1 IN SOLUTION

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Methanogenic, halophilic and thermoacidophilic archaea synthesize Adenosintriphosphat (ATP) by means of ion gradient driven phosphorylation. The key enzyme is the A_1A_O ATPsynthase (A_3:B_3:C:D:E:F:H:I:K_x), composed of a water-soluble, catalytic sector, A_1, and an integral membrane subcomplex, A_O, which functions in ion conduction. Like the F_1F_O ATPase, A_1A_O ATPase catalyzes ATP synthesis and –hydrolysis (1). Here we describe the production of subunit F of the A_1A_O ATPsynthase from the archaeon *Methanosarcina mazei* Gö1 in *Escherichia coli* and the purification to homogeneity. The secondary structure of the protein was analyzed by circular dichroism spectroscopy, showing that F comprises 43% α-helix and 33% β-sheet content. The molecular mass of this subunit, determined by gel filtration analysis and small angle X-ray scattering (SAXS), was approximately 15 ± 2 kDa, indicating a high hydration level of the protein in solution. With a radius of gyration and a maximum size of 2.03 ± 0.02 nm and 7.5 ± 0.2 nm, respectively, subunit F is rather elongated. Using two independent *ab initio* approaches the first low-resolution shape of the protein was determined, composed of two domains, a main globular part with a length of about 4.5 nm, and a hook-like domain of about 3.0 nm in length. The overall shape is similar to that of the atomic model of the homolog ε subunit of the related F_1F_O ATPsynthase from *Escherichia coli*. Moreover, the SAXS pattern, computed from the model of ε, yields a fair agreement with the experimental scattering from subunit F.

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P1.3.23. H^+-ATP SYNTHASE DIMERS IN THE CHLOROPLAST OF CHLAMYDOMONAS REINHARDTII

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H^+-ATP synthases play a major role in energy conversion of living cells. Increasing numbers of studies show that ATP synthases exist not only as individual complexes but also as stoichiometric supramolecular aggregates. Native electrophoresis is the method of choice to demonstrate dimerization or oligomerization of ATP synthases [1]. While mitochondrial dimers have been described in several biochemical and structural investigations, dimers of other ATP synthases are much less characterized [2]. The proposal of dimerization being a unique feature of the mitochondrion was challenged by our identification of chloroplast ATP synthase dimers in the green alga *Chlamydomonas reinhardtii* [3].

After demonstrating the existence of dimers the current challenge is to enlighten their physiological relevance. For mitochondria, a possible function is the involvement in cristae formation, as supported by single particle electron microscopy of isolated dimers [4]. In contrast, the role of the chloroplast dimer still remains enigmatic.

We demonstrate that chloroplast ATP synthase dimer dissociates into monomers upon incubation with vanadate or phosphate but not by incubation with molybdate, while the mitochondrial dimer is completely unaffected. This suggests a distinct dimerization mechanism for mitochondrial and chloroplast ATP synthases. Since vanadate and phosphate bind to the active sites, contact sites located on the hydrophilic CF_1 part are suggested for the chloroplast ATP synthase dimer. As the degree of dimerization varies with phosphate concentration, dimerization might be a response to low phosphate concentrations and might be of physiological relevance.

To prove physiological roles of dimerization, the response of *Chlamydomonas reinhardtii* to altered culture conditions is investigated. For quantification, an isotope labelling technique with ^{15}N in combination with MALDI-MS is applied. Besides a variation of the assembly of photosystems and light harvesting complexes, the dimer to monomer ratio of chloroplast ATP synthase is altered by the growth conditions.

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P1.3.24. ROLE OF PROPIONIGENIUM MODESTUM uncI GENE IN F_OF_1-ATP SYNTHASE: uncI IS A MOLECULAR CHAPERON THAT ASSISTS C11-RING ASSEMBLY

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The *unc*I gene, a first gene of the F_oF_1-ATP synthase (F_oF_1) operon, encodes a ~100-residue membrane protein that is not a subunit of functional F_oF_1. Although *unc*I is conserved among many bacteria, its function has remained elusive since last 20 years. When we constructed heterologous expression systems for a chimeric F_oF_1 in which F_1 (soluble sector, $\alpha_3\beta_3\gamma\delta\varepsilon$) is from thermophilic *Bacillus* PS3 and F_o (membraneous sector, ab_2+c_{11}-ring) from *Propionigenium modestum*, the expressed enzyme was functional only if intact *unc*I gene was included. SDS-PAGE analysis revealed that stable c_{11}-ring was not formed without *unc*I gene even if monomeric F_oc-subunit was contained in the F_oF_1-complex. Simultaneous expression of a plasmid containing *unc*I gene complemented *unc*I-less F_oF_1 operon to form functional F_oF_1. A plasmid containing genes for F_oc and *unc*I produced stable c_{11}-ring but a plasmid containing a gene for F_oc did not. Furthermore, direct interaction between UncI and F_oc was demonstrated. These results suggest that UncI is a molecular chaperon that assists F_oc to assemble into c_{11}-ring in the membrane.

P1.3.25. SUBCOMPLEXES AND SUPERCOMPLEXES OF MITOCHONDRIAL ATP SYNTHASE

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Earlier electron microscopic analyses of mitochondria from the ciliated protozoan *Paramecium multimicronucleatum* suggest that ATP synthase can oligomerize into helical twin rows that seem to be formed from dimeric ATP synthase building blocks (1). More recently, yeast mutant stains lacking dimer specific subunits of the ATP synthase demonstrated disturbed cristae formation (2,3). We used the mild detergent digitonin and clear native PAGE (CN-PAGE) to retain labile supramolecular assemblies of the ATP synthase. The estimation of native masses of the oligomeric states of the ATP synthase suggests the presence of only even numbered ATP synthase oligomers. The oligomycin-sensitive ATPase activity of all oligomeric forms was found comparable to that of monomeric ATP synthase suggesting that oligomerization/monomerization dynamics are not directly involved in regulating ATP synthase activity.

We used rho zero cells not containing mtDNA to search for the largest ATP synthase intermediates assembled in the absence of mitochondrially encoded subunits. We identified three subcomplexes of ATP synthase: (i) F_1 catalytic domain, (ii) F_1-domain with bound IF_1 inhibitor protein, and (iii) F_1-c subcomplex, containing also a ring of F_O-subunits c. All three F_1-forms exist in monomeric state. Two subcomplexes containing bound IF_1 exhibited considerably reduced ATPase activity as compared to the third one lacking IF_1. Therefore we postulate that inhibition and induction of dimerization of F_1-subcomplexes by IF_1 are independent events. Similarly, F_1-subcomplexes accumulated in mitochondria of patients with specific mitochondrial disorders. We present a simple electrophoretic assay as a straightforward approach to differentiate between various types of mitochondrial biosynthesis disorders and therefore is also useful to guide molecular genetic diagnostics in the field of mitochondrial neuromuscular disorders.

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P1.3.26. ROTATION AND STRUCTURE OF PROKARYOTIC ATP-ASE/SYNTHASE

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The prokaryotic V-type ATPase/synthases (prokaryotic V-ATPases) have simpler subunit compositions than eukaryotic V-ATPases, and thus are useful subjects for studying chemical, physical and structural properties of V-ATPase. Here our recent studies on the structure/function relationships in the V-ATPase from the eubacterium *Thermus thermophilus*.

Using the single-molecule technique, it was established that the V-ATPase is a rotary motor (1). At low ATP concentrations, the rotor subunit rotated stepwise, pausing every 120˚. The dwell time between steps revealed that V_1 consumes one ATP per 120˚ step. Noticeably, the angles for both ATP cleavage and binding were apparently the same in V_1. This is in sharp contrast with F_1, which cleaves ATP at 80˚ posterior to the binding of ATP. Thus, the mechanochemical cycle of V_1 has marked differences to that of F_1(2).

Next, the structures of central stalk subunit V_o-d and F are described. This funnel-shape subunit V_o-d appears to cap the proteolipid ring in the V_o domain in order to accommodate the V_1 DF shaft. This structure seems essential for the regulatory reversible association/dissociation of the V_1 and the V_o domains (3). The crystal structure of subunit F was determined to 2.2 Å resolution. The subunit reveals unexpected structural similarity to the response regulator proteins that include the *Escherichia coli* chemotaxis response regulator CheY. The structure was successfully placed into the low-resolution EM structure of the prokaryotic holo-V-ATPase at a location indicated by the results of cross-linking experiments. The crystal structure, together with the single molecule analysis using fluorescence resonance energy transfer (FRET), showed that the subunit F exhibits two conformations, a 'retracted' form in the absence and an 'extended' form in the presence of ATP. Our results postulated that the subunit F is a regulatory subunit in the V-ATPase (4).

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Poster session 1.4. Porters and some other subjects

P1.4.1. ENDOGENOUS RESPIRATION SUBSTRATES LEVELS IN SACCHAROMYCES CEREVISIAE CELLS

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Specific features of changes in levels of endogenous respiration substrates in *Saccharomyces cerevisiae* cells at low temperature have been studied. The pool of endogenous substrates of cells changes significantly during incubation at 0°C under aerobic conditions. The rate of respiration of *S. cerevisiae* cells in the absence of exogenous substrates decreased exponentially with a half-period of about 5 h when measured at 30°C. Addition of pyruvate to freshly prepared cells stimulated cell respiration three-fivefold. But under these conditions, exogenous L-malate poorly (19-45%) stimulated cell respiration. Addition of malonate activated 1.3-1.7-fold their endogenous respiration due to displacement by malonate of oxaloacetate from the complex with succinate dehydrogenase. The respiration rate during incubation in the presence of exogenous acetate rapidly decreased, as well as in the presence of glucose and pyruvate. This finding was consistent with the decrease in the mitochondrial level of oxaloacetate, which is necessary for condensation with acetyl-CoA generated in this case. The decrease in the rate of endogenous respiration during the aerobic incubation at 0ºC was accompanied by a significant increase in the L-malate level (~threefold for 24 h). And the concentration of L-malate increased not only in the cells, but also in the culture medium (after 12 h of the cell growth - by 21.6 ± 7.6 μM). Addition of the saturating concentration of succinate (20 mM) to the freshly separated cells only slightly stimulated their respiration. Thus, 1.4 h after the cell isolation, succinate only activated the respiration by 13%. However, with the increase in the malate level in the cells and decrease in the oxaloacetate concentration in the mitochondria *in situ*, the stimulating effect of exogenous succinate significantly increased. Thus, 13,6 h after the cell isolation, the rate of their respiration was increased 3.7-fold on the addition of succinate. The increase in the L-malate level in the cells and the concurrent decrease in the oxaloacetate level in the mitochondria should be associated with deceleration at 0ºC of the transport of endogenous respiration substrates from the cytosol into mitochondria. This deceleration is likely to be caused by a high Arrenius activation energy specific for transporters. The results suggests that plasma membrane of *S. cerevisiae* has to contain a dicarboxylate transporter. It seems that the physiological

function of this transporter in this yeast could be not utilization of dicarboxylates, but removal of excess malate from the cell. This viewpoint correlates with the data on L-malate secretion during the cell growth.

P1.4.2. IDENTIFICATION AND CHARACTERIZATION OF EXCRETORY CARRIERS IN C. GLUTAMICUM

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Corynebacterium glutamicum is a Gram-positive, non pathogenic bacterium used in a variety of fermentative processes mainly for the production of amino acids. Besides anabolic and catabolic pathways transport of substrates contributes significantly to the energetic status of a cell. Compared with other bacteria, *C. glutamicum* has an unusually high number of genes encoding putative transporter proteins. Being exposed to stress conditions or to high abundance of particular compounds, *C. glutamicum* excretes compatible solutes and metabolic end products but also abundant intermediates of anabolic pathways so preferring loosing energy-rich substrates to their toxic accumulation. Therefore uptake and excretion processes must be controlled tightly in order to avoid futile cycles of metabolic fluxes.

In spite of the fact that for *C. glutamicum* the excretion of many substrates is known, only three export systems have been identified and published yet. Instead of chasing one particular protein we use a different approach for identification of new carriers. First, experimental procedures to monitor compound excretion by the wild type of *C. glutamicum* are established. This includes the variation of nutrient supply, feeding with dipeptides or the application of different stress conditions like anaerobiosis. Thereby we could clearly show the excretion of amino acids as well as a variety of carbonic acids which was followed by HPLC, GC or GC-MS. Second, after the biochemical characterization of export functions regarding kinetic and energetic parameters as well as the inducibility, mutant libraries are used to identify carrier proteins for particular interesting substrates. The excretion profile of these mutants is assessed under conditions that were identified before.

By using this strategy the BrnFE system was identified as major methionine export system and the presence of a second exporter was shown. The identification of further carriers will be discussed. Finally we aim to discover transporters for particular biotechnologically relevant substrates and their role in metabolic network regulation.

P1.4.3. AN OLIGOMER FORMATION AND CONFORMATIONAL CHANGE OF NHAA FROM HELICOBACTER PYLORI STUDIED BY FRET

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Although dynamic conformational changes are expected for action of transporters, detecting methods have not been established. We tested FRET (fluorescence resonance energy transfer) for such method to study an oligomer formation and conformational change of Na^+/H^+ antiporter from *Helicobacter pylori* (HPNhaA). The HPNhaA-Green Fluorescence Protein (GFP) variant fusion at the carboxy-terminus of NhaA expressed in *nhaA* defective *E. coli* cells exhibited the wild-type level of antiporter activity in the everted membrane vesicles. Fluorescence intensity peak was shifted to 528 nm (Emission peak of Venus, YFP variant) from CFP emission peak (477 and 496 nm) in the membranes from *E. coli* coexpressing HPNhaA-CFP together with HPNhaA-Venus upon excitation of CFP. Treatment of the membranes with Proteinase K caused decrease of the Venus fluorescence with concomitant increase of CFP fluorescence. These results indicated that FRET between CFP and Venus occurred, suggesting that HPNhaA-GFP varinats are closely associated and form an oligomer. Co-precipitation of HPNhaA tagged by GFP with the FLAG-tagged form in the same *E. coli* cells confirmed the physical interaction between HPNhaA monomers. Addition of Li^+ to the membranes caused significant decrease of FRET regardless of the presence or absence of H^+ gradient across the membranes. However, NaCl caused much weaker effect. This Li^+ effect was not observed clearly for vesicles prepared from the Asp141 to Asn mutant which is defective in Li^+ binding. These results demonstrated that the monomer interaction in the HPNhaA oligomer became looser upon Li^+ binding. By using HPNhaA fused to Venus at the NhaA carboxy-terminus and HPNhaA with insertion of CFP in the loop 8, effect of pH on conformational change within a monomer NhaA was analyzed by FRET. The apparent FRET intensity increased upon increase of pH, suggesting that interaction of both GFP variants become closer. On the other hand, pH effect on FRET between the GFP variants fused to the C-terminus in the oligomers was not much affected. These results suggested that Loop8 region of HPNhaA causes conformational change upon pH change, and may move towards the C-terminal region upon alkalinization rather than that the both of loop 8 and the C-terminal regions moved together.

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P1.4.4. MOLECULAR MODELING INSIGHT INTO THE K_{ATP} CHANNEL OPENERS-MITOCHONDRIAL ADENINE NUCLEOTIDE TRANSLOCASE INTERACTION

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The adenine nucleotide translocase – one of the most abundant proteins of mitochondrial inner membrane catalyzes the transmembrane exchange between ATP generated inside mitochondria through oxidative phosphorylation and cytosolic ADP. Recently determined crystal structure of adenine nucleotide translocase has provided insight into the three-dimensional structure and mechanisms of mitochondrial inner membrane carriers. Based on the hypothesis about involvement of mitochondrial adenine nucleotide translocase in K^+ and H^+ transport to mitochondrial matrix we applied molecular modeling to elucidate the possible interactions between the adenine nucleotide translocase and its known (ADP, ATP, carboxyatractyloside) and putative ligands - K_{ATP} channel openers. Docking calculations indicate that K_{ATP} channel openers could bind in the specific location proximal to H4, H5 and H6 transmembrane helices within the cavity of adenine nucleotide translocase. The analysis of the predicted binding site suggests that K_{ATP} channel openers compounds could modulate the functions of adenine nucleotide translocase by selective interactions and hints at the mechanism of cardioprotection by K_{ATP} channel openers.

P1.4.5. PROOXIDANT-INDUCED LOW-CONDUCTANCE CHANNEL IN MITOCHONDRIA FROM THE YARROWIA LIPOLYTICA YEAST

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The Ca^{2+}-dependent, cyclosporin A-sensitive mitochondrial permeability transition (mPTP, pore) is considered to be one of the key events in the regulation of matrix [Ca^{2+}], pH, transmembrane potential, and volume, as well as in the transduction of apoptotic or necrotic signals in mammalian cells. In this work we used tightly-coupled mitochondria from the *Yarrowia lipolytica* yeast, possessing the fully competent respiratory chain with all three points of energy conservation and lacking a natural Ca^{2+} uptake pathway. *Y. lipolytica* mitochondria, when exposed to 300-500 μM Ca^{2+} in the presence of the Ca^{2+} ionophore ETH129 and varying [P_i], accumulated large amounts of Ca^{2+} with no classical mPTP induction. The absence of response in yeast mitochondria was not simply due to structural limitations, since large-amplitude swelling occurred when membrane pores unrelated to the mPTP, were formed. However, the joint action of prooxidants such as phenylarsine oxide, menadione and oxaloacetate caused a collapse of the membrane potential (DeltaPsi). The prooxidant-induced mitochondrial uncoupling was resistant to cyclosporine A (CsA) alone, spermine and Mg^{2+}, all of which are known to block the classical mPTP, while being totally preventing by water-soluble antioxidants (reduced glutathione and N-acetylcysteine) as well as low concentrations of ATP (in the presence of ologomycin). The protectory effect of ATP was totally abolished by Mg^{2+} and partially by atractyloside. Membrane depolarization was also partially restored by ADP, GTP, ITP, and CTP. Interestingly, in yeast mitochondria, like in brain mitochondria, the protectory effect of ADP was potentiated by CsA. Therefore, mitochondria from the *Y. lipolytica* yeast do have a CsA-sensitive, prooxidant-induced elevated permeability transition of the inner mitochondrial membrane, a low-conductance channel which conducts only H^+. Opening of the low-conductance channel requires SH-group oxidation. The permeability transition in yeast mitochondria it is not coupled with Ca^{2+} uptake and differently regulated compared to the mPTP of animal mitochondria.

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P1.4.6. TOPOLOGY OF RESIDUES AND CONFORMATIONAL CHANGE REQUIRED FOR THE pH DEPENDENT ANTIPORT ACTIVITY FOR H. PYLORI NHAA

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Na^+/H^+ antiporter of bacteria NhaA plays an important physiological role in Na^+, pH and osmotic regulations (1). We have been interested in the structural basis required for Na^+/H^+ antiport and pH dependency of NhaA from *H. pylori* (HP NhaA). *E. coli* NhaA (EC NhaA) shows high antiporter activity at alkaline pH range but very low at acidic and neutral pH range. We found that *H. pylori* NhaA (HP NhaA) has a high antiporter activity at neutral and acidic pH as well as at alkaline pH range (2). We have shown that the antiporter activity requires several residues in the trans-membrane domains (TM) 4, 5, 10 and 11 by studying alteration of activity caused by an amino acid replacement. These mutation sites including D141 (TM4), D172 (TM5) and K347 (TM10) are involved in ion transport (3). Mutation sites M138 (TM4) and K347 (TM10) are involved in the high activity at acidic pH range specific for HP NhaA. To show more precisely the function of residues in TM4, 5, 10 and 11, we surveyed functional alteration by Cysteine scanning mutagenesis. Topology of each residue was also analyzed by testing susceptibility of Cys to isotope labeled NEM. For TM4, Cys substitution of Thr-140, Asp-141 and Phe-144 alone caused extensive decrease of the antiporter activity and increase of K_M values of antiporter kinetic parameter (4). The carboxyl terminal half region of TM4 was susceptible to NEM binding, suggesting that this half region faces to water and comprises a part of channel pore. At the end of this putative pore Thr-140 and Asp-141 reside and possibly forms a part of ion binding site. Based on inhibitory effect of membrane impermeable AMS on NEM binding, we concluded that the putative water filled channel is open to the cytoplasmic side. The same analyses were applied for TM5, 10 and 11. Accordingly, here we propose function and topology of residues in TM4, 5, 10 and 11.

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P1.4.7. INHIBITORY ANALYSIS OF THE RAT LIVER MITOCHONDRIAL DICARBOXYLATE TRANSPORTER BY MEANS OF LIPOPHILIC DERIVATIVES OF ITS SUBSTRATES

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It has been established earlier that the active center channel of the dicarboxylate transporter in intact rat liver mitochondria has complicated topography. Probing of the active center by means of 2-monoalkylmalonates have shown that this channel consists of internal and external lipophilic areas devided by a polar zone [Sholtz K.F., Bondarenko D.I., Mamaev D.V. (1993) FEBS Lett., 327, 54 – 56]. Two substrate-binding sites model of the transporter have been supposed.

In order to check this model O–acyl–L-malates with the distance from anion groups to aliphatic chain for 0,23 nm longer in comparison with 2-monoalkylmalonates, have been used to study the topography of the active center of the transporter. Changes in the sound polar group not only prevented it´s binding but also showed transporter channel changing lipophility picture.

The α,ω-alkylendimalonates probing have not showed the second substrate-binding site in the active center in the channel polar zone (in 0,59 nm from the “malonate-binding” site) and in the area of the hypothetical “malate-binding” site (which is 0,23 nm distant from the “malonate-binding” site). According to these facts it is of great interest to solve the problem: what area of the transporter molecule binds a non-competitive with malate, but transporting phosphate. Differences in L-malate and D-malate substrate-binding sites affinity (K_i = 0,5 and 2,5 мМ, accordingly) is the evidence of L-malate hydroxyl group participation in binding of this substrate and of stereospecifity of the transporter substrate-binding site. At the same time O-acyl- derivatives of these substrates have an equal affinity. Probably, the hydroxyl group binding amino acid residue may move to the free area at the entrance of the transporter channel. Thus, the part of O-acyl-malate molecule (in the curved configuration) containing complex ether bound may be localized in this area.

P1.4.8. A mPTP-LIKE PORE IN YEAST MITOCHONDRIA

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The mitochondria permeability transition pore (mPTP) is a nonspecific, cyclosporin A-sensitive high-conductance channel, permeant to any molecules of 1.5 kDa, triggered by elevated matrix [Ca^{2+}], especially when this is accompanied by oxidative stress and depleted adenine nucleotide pools. Minimal molecular constituents of the regulated mPTP in mammals are believed to be the voltage-dependent anion channel, the adenine nucleotide translocator, and cyclophilin D. In vitro, mPTP opening causes membrane depolarization, massive swellingof mitochondria, repture of the outer membrane, and release of cytochrome *c* and other intermembrane apoptotic components. In vivo, the mPTP participates in the regulation of matrix Ca^{2+}, pH, transmembrane potential, and volume, its opening is a key event in a variety of toxic, hypoxic, and oxidative forms of cell injury, it has been has been widely considered as a mechanism implicated in both apoptotic and necrotic cell death. The presence of a mPTP-like pore in yeast mitochondria is still uncertain. Mitochondria from both the *Saccharomyces cerevisiae* yeast, accumulating Ca^{2+} via the Ca^{2+} transporter ETH129, and the *Endomyces magnusii* yeast, harboring the high-capacitive effectively regulated Ca^{2+} uniporter (Bazhenova et al. 1998) and a Ca^{2+}/nH^{+} antiporter for Ca^{2+} efflux (Deryabina et al. 2001), were are largely protected from Ca^{2+}-overloading (Jung et al., 1997; Kowaltowsky et al., 2000; Deryabina et al., 2004). It means that an ability of mitochondria to take Ca^{2+} up by an energy-dependent manner is not sufficient for mPTP opening. A Ca^{2+}-dependent PTP in permeabilized *S. cerevisiae* spheroplasts could be induced only by high (non-physiological) [Ca^{2+}] under oxidative stress conditions, PTP induction was accompanied by declined cell viability (Kowaltowsky et al., 2000). A Ca^{2+}-independent, CsA-insensitive pore did arise in *S. cerevisiae* mitochondria in response to ATP (Jung et al., 1997). We speculate that the absence of the regulated classical Ca^{2+}-dependent mPTP may be a general feature of yeast species. This could be due to a reduced concentration of [Ca^{2+}] in the matrix, high intramitochondrial concentration of inhibitory substances such as Mg^{2+}, decreased amounts of cyclophilin D located within the matrix, and strong antioxidant defence. It is also conceivable that yeast mitochondria may be endowed by other mechanisms underlying regulation of ionic homeostasis such as ATP-dependent K^{+}-channel and some others.

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Poster session 1.5. Mechanisms of energy coupling

P1.5.1. BACTERIAL MEMBRANE PROTON CONDUCTANCE: EFFECTS OF GROWTH CONDITIONS AND OSMOTIC STRESS

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Proton conductance (under gradient-less conditions without external source of energy) is a property of bacterial membrane that has a role in efficiency of H^+ circulation. This could correlate with energy-dependent H^+ efflux and be therefore important in coupling mechanisms.

The dependence of membrane H^+ conductance (C_m^{H+}) and energy-dependent H^+ efflux on growth pH was studied when cells were grown under anaerobic fermentation (*Escherichia coli* and *Enterococcus hirae*) and aerobic (*E. coli*) conditions. C_m^{H+} was determined by the acid-pulse technique described by Maloney [1]. The elevated H^+ conductance and lowered H^+ efflux were shown upon acidic pH and under anaerobic conditions, when bacteria were fermenting glucose. The changes in C_m^{H+} were correlated with the values of membrane potential as well as of external redox potentials determined. The low level of H^+ conductance and correlation of its change with H^+ efflux under fermentation could be an important property of the bacterial membrane, which is counted for efficiency of energy-transducing processes.

The *atp* mutants (*E. coli* FRAG115 and *E. hirae* MS116) with deprived or defective F_0F_1-ATPase had less C_m^{H+} independently of growth conditions. These results could indicate distinguishing pathways determining C_m^{H+} in cells grown under anaerobic conditions upon fermentation at different pH and an input of F_0F_1 in C_m^{H+}.

In addition, the effect of osmotic stress was demonstrated with *E. coli*, C_m^{H+} and H^+ efflux both were increased upon hyper-osmotic stress at slightly alkaline pH, those were inhibited by *N,N'*-dicyclohexylcarbodiimide and were less in *atp* mutant. This seems to be in favor with an idea that the F_0F_1-ATPase in *E. coli* is osmo-sensitive [2-3] confirming a role of F_0F_1 in osmotic adaptation of bacteria under fermentation conditions.

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P1.5.2. TWO-DIMENSIONAL AND THREE-DIMENSIONAL PROTON DIFFUSION ALONG THE BILAYER LIPID MEMBRANE

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Proton diffusion along the surface of planar bilayer lipid membrane was measured by means of acid/base injection with micropipette and recording of the kinetics of fluorescence changes of fluorescein-labeled lipid on the surface. In the absence of high pK binding sites lateral proton diffusion proceeded via bulk phase by means of buffer molecules as proton carriers (three-dimensional case) in the solution (D_b = 600 μm^2/s). Introduction of proton binding sites on the membrane surface led to two-dimensional proton diffusion on the membrane surface with D_s = 1100 μm^2/s. Analysis of the experimental data based on the phenomenological theory gave semi-quantitative description of the diffusion process and estimates of the parameters determining the kinetics of proton diffusion under different conditions. It was concluded that the major part of the proton surface pathway comprises by low pK binding sites or hydrogen-bonded water chains even in the presence of high pK binding sites necessary for retaining protons on the surface. The data obtained suggested the possibility of a regulation of proton transfer pathways between proton pumps in natural membrane systems.

P1.5.3. COUPLED FORMATE OXIDATION AND ATP SYNTHESIS IN FERMENTING ESCHERICHIA COLI

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In fermenting cells under anaerobic conditions, F_0F_1-ATPase might be an essential part of the H^+ efflux system associated with the formate hydrogen lyase reaction as previously reported [1]. If the formate hydrogen lyase reaction is coupled to the generation of a proton motive force, a functional F_0F_1-ATPase would be necessary for converting the energy into ATP synthesis. In the *atp*-mutants, the synthesis of formate hydrogen lyase complex might be down regulated: the membrane fraction of the E. coli *atp*-mutant AN 936 (with a defect in the *c*-subunit of F_0), grown under fermenting conditions, did not have any hydrogenase activity [2].

The correlation between the rate of ATP synthesis by F_0F_1 ATP-synthase and formate oxidation by formate hydrogen lyase (FHL) was obtained. This has been done in inverted membrane vesicles of anaerobically grown on glucose, in the absence of exterior electron acceptors (pH 6.5) *Escherichia coli* JW 136 mutant with double deletions *(Δhya/Δhyb*) of hydrogenase 1 and 2. ATP synthesis was suppressed by H^+-ATPase inhibitors N,N′-dicyclohexylcarbodiimide (DCCD) and sodium azide as well as by protonophore carbonyl cyanide-*m*-chlorophenyhydrazone (CCCP). Cooper ions inhibited formate-dependent hydrogenase and ATP-synthase activities but did not affect ATPase activity of vesicles. The maximal rate of ATP synthesis (0.83 μM/min·mg protein) stimulated by K^+ ions was determined when sodium formate, ADP and inorganic phosphate were applied simultaneously. Results confirm an assumption about a dual role of hydrogenase 3, formate hydrogen lyase subunit that is able to couple reduction of protons to H_2 and their translocation through a membrane with chemiosmotic synthesis of ATP.

Acknowledgment

Work supported by Armenian National Science and Education Foundation. We thank Prof. K.T. Shanmugam (USA) for providing *E. coli* strains.

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P1.5.4. PROPERTIES OF THE RIGID PAPP-REGION IN THE LINKER CONNECTING DOMAIN I AND DOMAIN II IN E. COLI TRANSHYDROGENASE

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The membrane bound transhydrogenase of *Escherichia coli* couples the redox reaction between NAD(H) and NADP(H) and the translocation of one proton over the cytoplasmic membrane of bacteria and the inner mitochondrial membrane in eukaryotes. One proton is translocated across the membrane as one hydride equivalent is transferred between the substratates. The enzyme participates in the bioenergetic processes of the cell and utilizes the electrochemical proton gradient, Δp, across the membrane to drive the formation of NADPH from NADH according to the reaction:

$H^+_{out} + NADH + NADP^+ \leftrightarrow H^+_{in} + NAD^+ + NADPH$

Out and in denotes to periplasmic space and cytosol in bacteria and intermembrane space and matrix in mitochondria. The NADPH generated is used to reduce glutathione (GSH) that eliminates oxidative stress, e.g. reactive oxygen species (ROS), and used in metabolic biosynthesis in bacteria.

A major difference between *E. coli* and *R. rubrum* transhydrogenase (TH) is that *ec* TH has 2 polypeptides while *rr* TH has 3 polypeptides. *Rr* TH has a split in the corresponding α-subunit of *ec* TH, resulting in a soluble domain I (dI). DI of *rr* has a significant higher affinity (10 000 times) for *ec* domain III (dIII), as compared to *ec* dI. The structural basis for this difference was investigated by comparing dI of the two species. The X-ray structure of *ec* dI, 2.0 Å [*Johansson et al* 2005], revealed a rigid highly conserved short peptide sequence starting at position αP369 (-PAPP-) in the α-linker (~30 amino acids) connecting dI with dII in *ec* TH that could be important in the conformational coupling as the so-called "hinge"-region, i.e. the β-linker connecting dII and dIII. The peptide bond of residue αP369 is in the *cis*-isomerization form in the crystal structure of *ec* dI, which is infrequent in known protein structures, and the characteristics of this property of the structure and/or function of the linker, was studied. Single cysteine mutants in the α-linker region were introduced, i.e. αP369C, αA370C, αP371C, αP372C, αQ374C, αP379C and αQ380C into a cysteine free construction of intact *ec* TH. The analysis of the data of the cysteine mutants revealed that especially the αP369C mutant showed strongly decreased reverse (25%) and cyclic (33%) activities, consistent with an important role of the α-linker in the conformational coupling between dI and the remainder of the protein.

P1.5.5. DISULFIDE-DITHIOL INTERCHANGE IN ENERGY TRANSFER FOR BACTERIAL MEMBRANE PROTEINS FUNCTIONING: EFFECTS OF COPPER IONS

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The hydrolysis of ATP by proton F_0F_1-ATPase is a main mechanism in maintaining proton-motive force, and an elevated accumulation of potassium ions has a distinguishing role in osmotic adaptation and enzymatic activity in bacteria. Arguments and results with fermenting *Escherichia coli* allow the hypothesis on associations of F_0F_1 with secondary transporters (potassium uptake Trk system) and/or key enzymes of anaerobic oxidation-reduction (formate hydrogenlyase) [1]. Associations are resulted from protein-protein interactions and formation of complexes within of which the energy could be transferred via a disulfide-dithiol interchange. The change in the number of accessible thiol-groups in the membrane by ATP that is inhibited by *N,N'*-dicyclohexylcarbodiimide (DCCD) points out an implementation in protein-protein interactions by a disulfide-dithiol interchange [2]. And oxidizers may affect this phenomenon.

Besides, copper ions (Cu^{2+}) promoting the bacterial growth in low concentration [3] are toxic in high concentration inducing membrane permeability [4]. The latter is suggested to result from the break of disulfides in redox proteins when Cu^{2+} are reduced on cell surface or by reducers. Therefore, Cu^{2+} are suggested to affect directly F_0F_1 or its association with the other mechanisms.

A significant (~1.7-fold) increase in the number of SH-groups in *E. coli* membrane vesicles with Cu^{2+} (0.1 mM) was shown. This was observed with wild-type cells and *atp*, *hyc(A-H)*, *hycE* and *hyfR* mutants. The elevating number of SH-groups by ATP was induced in wild-type cells in the presence of K^+ and *hycE* but not *atp*, *hyc(A-H)* and *hyfR* mutants and absent in the presence of Cu^{2+}. These were in addition to inhibitory effects of Cu^{2+} on acidification of the medium and the DCCD- and azide-sensitive proton-potassium exchange by intact cells. Moreover, H_2 production upon growth and under assays disappeared with Cu^{2+}.

The effects could be explained by the break of disulfides [4] inhibiting disulfide-dithiol interchange between the F_0F_1-ATPase and the other proteins.

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P1.5.6. PKC AND PMCA IN REGULATION OF THE Ca^{2+} EFFLUX IN RESPONSE TO GLUTAMATE EXPOSURE IN NEURONS

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In primary cultures of cerebellar and cortical neurons the long-term stimulation of glutamate receptors amplifies two early intracellular signals: free cytosolic Ca^{2+} and the translocation of protein kinase C (PKC) from cytosol to neuronal membranes. Both of these signals are sustained even after removal of glutamate from the incubation medium and mediate Ca^{2+}-dependent excitotoxic damage and apoptose of the neurons.

The aim of the study was to investigate the role of PKC functional state in regulation of the Ca^{2+} efflux and subsequent death in response to glutamate in neurons.

The primary cultures of cerebellar granule neurons were prepared from 7-day-old rats, cortical neurons from 1-day-old ones; cells were used on the 7^{th}-9^{th} day in vitro. The viability was estimated with the Hoechst 33342 and Ethidium bromide staining method and MTT test 24 hr after treatment with 100 μM glutamate for 1 hr in Mg^{2+}-free Gly-containing medium. To monitor changes in Ca^{2+} level the fluorescent Ca^{2+} indicator Fura-2 was used. Cells were exposed to sufficiently high concentration of glutamate (100 μM) for 40-60 min. It leaded to a delayed increase in intracellular Ca^{2+} level after the initial increase. We demonstrate that selective PKC inhibitor chelerythrine delays Ca^{2+} overload and decreases death in cerebellar and cortex cultures of rat neurons. Activator of PKC 12-myristate 13-acetate phorbol (PMA) facilitated Ca^{2+} influx in neurons. Down-regulation of PKC (in response to 24 hours PMA exposure) delayed Ca^{2+} overload both in cerebellar and cortical cultures. Ca^{2+} didn't recover when stimulus is removed. PMA-induced down-regulation of PKC decreased glutamate-elicited neurotoxicity.

These results support the view that PKC is involved in glutamate-induced destabilization of cytosolic ionized Ca^{2+} homeostasis and cell death.

P1.5.7. NON-OHMIC DEPENDENCE OF ROS GENERATION AND ITS CYCLING ACROSS MITOCHONDRIAL MEMBRANES MAY HAVE A REGULATORY ROLE IN BALANCING BETWEEN ENERGY TRANSDUCTION AND CELLULAR REDOX SIGNALLING

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Two models of 'Reactive oxygen cycle' (Liu Shusen,1996-1999) and 'Uncoupling double-loops of H^+ and $O_2^{\cdot-}$ cycling'(Nedergaard,2003) have been proposed to emphasize the functional role of superoxide or/and uncoupling proteins in H^+ leak and ROS cycling in mitochondria for balance between energy transduction and Redox signalling in various cellular processes. To test reliability of both models and elucidate the precise mechanisms of ROS cycling and uncoupling effect in mitochondria, here we show novel evidence that $O_2^{\cdot-}$ generated in rat liver mitochondria with no UCPs expression was able alone to be an endogenous protonophore to induce H^+ leak and heat production. By investigating the relationship between state 4 respiration H^+ leak production of ROS and heat in both euthyroid and hyperthyroid mitochondria, as well as the effects of exogenous SOD on these bioenergetic parameters, It was found that mitochondrial heat production is due to $O_2^{\cdot-}$ uncoupling effect in association with state 4 respiration; SOD could reduce H^+ cycling rate and the elevated heat in hyperthyroid mitochondria. Also, in rat muscle mitochondria, ROS and UCP3 expression were increased by strenuous exercise. By analysing state 4 respiration, uncoupling effects of superoxde and UCP3 as well as some other bioenergetic parameters it is concluded that in normal condition superoxide induced H^+ leak in mitochondria may be the major mechanism for diverting energy of proton motive force to keep lower level of ROS, as described in "ROS cycle" model; however, when mitochondrial ROS increase as in stress condition, the uncoupling role of superoxide not only directly exhibits in H^+ leak, but also through Redox-signalling indirectly to promote UCP3 expression (in muscle), and the coordinate interactions of both $O_2^{\cdot-}$ and UCP3 may induce higher rate of H^+ leak, leading to keep balance between formation of ATP, heat and ROS in mitochondria as described in Needergaard's model.

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P1.5.8. PHYSIOLOGICAL ROLE OF MITOCHONDRIAL FISSION AND FUSION

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Mitochondria control cell growth, division and cell death in response to environmental changes. These organelles form a highly dynamic reticulum, the structure of which is maintained by an equilibrium between fusion and fission processes. Little is known about the role of mitochondrial fusion and fission. We have developed cell lines in which the equilibrium between fission and fusion can be altered through tetracycline-inducible RNAi targeted to major components of the fusion or fission machinery. These cells represent interesting tools to study how fission and fusion alter the function of mitochondria and thereby cell life. I will present results that show that inhibiting mitochondrial fission alters cell metabolism and has an impact on cell cycle control.

P1.5.9. ADVANCES AND OPPORTUNITIES IN ANGSTROM LEVEL ELECTRON TUNNELING SIMULATIONS OF NATURAL PHOTOSYNTHETIC AND RESPIRATORY SYSTEMS AND DE NOVO DESIGNED CATALYTIC AND ENERGY CONVERTING PROTEINS

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From extensive analysis of lengthscales and energetic hierarchies we have found that natural selection of distances between redox cofactors goes a long way towards encompassing natural electron transfer protein functional design. Distances are selected short or long as required to direct or insulate promiscuously tunneling single electrons. Distances are very short to achieve high tunneling rates in light energy conversion or to promote the climbing of added energetic barriers at paired-electron catalytic centers. When large driving forces are available, as in photosynthesis, endergonic barriers to slow short-circuit electron transfer are also important. Besides distance, energetic barriers can be important to slow energy wasting reactions, such as short-circuit charge recombination in photosynthesis and shorts or proton-pumping bypass in respiration. Tunneling simulations that take advantage of Å level resolution of major photosynthetic and respiratory complexes provide clear illustrations of this simple engineering in native respiratory and photosynthetic chains. We anticipate that these simulations can be developed into quantitative frameworks not only for assessment of experimental results on tunneling chains and catalytic sites in individual complexes, but also for the exploration of the tolerances to perturbation and thresholds of failure in larger bioenergetic systems and organelles. This progress joins advances in the design and synthesis of the principal α-helix bundle motif of the membrane proteins. Key functional elements of photosynthesis and respiration are being reproduced in highly robust four α-helix bundle proteins, maquettes, both water-soluble (hydrophilic, HP) or partly water-soluble, partly lipophilic membrane-inserting (amphiphilic, AP) proteins. Examples of functional elements include simple light harvesting in metalloporphyrins and bacteriochlorophylls, electron tunneling between hemes, heme-glutamate coupling of electron and proton transfer, and formation of ferrous-heme A oxy state (compound A). These constructions suggest there is considerable future promise in protein

maquette designs of novel light-harvesting, electron tunneling and coupled proton transfer systems and ultimately of oxidoreductase catalysts.

P1.5.10. SITE-DIRECTED MUTAGENESIS OF THE METAL-ION TRANSPORTER DCT1

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Metal-ion transport by DCT1, a member of the NRAMP family, is driven by protons. The stoichiometry of proton to metal ion is variable and under optimal transport conditions more then ten protons are co-transported with a single metal ion. To better understand this phenomenon we used site-directed mutagenesis of DCT1 and analyzed the mutants by complementing of yeast SMF-null mutants and analyzing metal-ion uptake and electrophysiology with the transporters expressed in *Xenopus* oocytes. The mutation F227I resulted in an increase of up to 14-fold in the ratio between metal ions to protons transported. This observation suggests that low metal ion to proton transport of DCT1, resulted from a proton slippage, was not a necessity of the transport mechanism in which positively charged protons are driving two positive charges of the metal ion in the same direction. It supports the idea that the proton slippage has a physiological advantage and the proton slip was positively selected during the evolution of DCT1. The mutations G243A and F244V changed the specificity of DCT1 to various metal ions. These mutations resulted in a significant decrease in Co/Fe uptake activity ratio compared to the native DCT1. In addition, the mutation G243A almost abolish the proton slip current induced by Co^{2+} but did not affect the one induced by other metal ions tested. The mutation L238F shifted the pH value in which a symmetrical pre-steady-state current appears, from 6.5 to 7.5, suggesting that the mutation increased the transporter affinity to protons.

P1.5.11. STRUCTURE DETERMINATION OF A TRANSIENT COMPLEX BY NMR USING PARAMAGNETIC DISTANCE RESTRAINTS - THE COMPLEX OF THE SOLUBLE DOMAINS OF ESCHERICHIA COLI TRANSHYDROGENASE

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Determining structures of transiently interacting protein-protein complexes still represent a significant challenge in structural biology, despite their tremendous importance in all aspects of biology. Here, we present a methodology for determination of high-resolution structures of transient protein-protein complexes using NMR spectroscopy, on the basis of intermolecular paramagnetic relaxation enhancements. We have determined the structure of the complex between the soluble domains I and III of the membrane protein transhydrogenase from *Escherichia coli*. Spin labels were introduced in five different locations on domain I, and their effect on domain III were analyzed using NMR 2D ^{1}H, ^{15}N correlation spectra. Using rigid body minimization and molecular dynamics, the complex structure was determined utilizing the intermolecular paramagnetic relaxation enhancements and previously measured chemical shift mapping data.The resulting complex exhibits the same binding mode as seen in the crystal structure of the homologous complex from *Rhodospirillum rubrum*.

P1.5.12. ENERGY COUPLING IN NITROGENASE

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Nitrogenase (EC 1.18.6.1), an enzyme, catalyzing the reduction of N_2 to NH_3 at ambient temperature and pressure, consists of two metal-containing proteins, MoFe protein (Av1) and Fe protein (Av2). Av1, a 250-kDa heterotetramer $\alpha_2\beta_2$, contains two 8Fe-7S-clusters (P-clusters) and two Mo-7Fe-9S-homocitrate-clusters, FeMoco, the site of activation and reduction of the substrates. The first step of nitrogenase reaction is the electron transfer from an exogenous electron donor (dithionite or photodonor *in vitro*; flavodoxin *in vivo*) to Av2, a 64-kDa homodimer with single [4Fe-4S] cluster. The key step of nitrogenase reaction is an intermolecular electron transfer coupled with ATP hydrolysis from Av2 (E_m = -0,29 V) to Av1 against potential, with the formation of so called "super-reduced" state of FeMoco (E_m is about -0,6 V). ATP molecules bind to Av2 as the complex with Mg^{2+}. The overall stoichiometry of nitrogenase catalyzed reduction of nitrogen is described by the scheme: $N_2 + 8H^+ + 8e^- + 16MgATP \rightarrow 2NH_3 + H_2 + 16MgADP + 16P_i$. The kinetics of ATP hydrolysis steps in nitrogenase is similar to ATP hydrolysis in myosin. We have studied the kinetics of minimum turnover of nitrogenase, transfer of two electrons from a photodonor to Av2 and the kinetics of transfer of first and second electrons from Av2 to Av1 by kinetic laser spectroscopy [1]. We have analyzed [2] the structure of nitrogenase complex $Av1.(Av2)_2$, stabilized by MgADP-AlF_4^- [3]. It was seen, that ATP-binding site and whole metal-clusters of nitrogenase locate in one plane, and the distances between the nearest partners are equal about 14 A^o. So ATP-binding site in Av2, decreasing E_m of 4Fe-4S-cluster in Av2 up to -0,43 V, can not effect on E_m of P-cluster and FeMoco. It is known, that the β-subunit of Av1 contains a sequence of 74-95 amino acids, which is similar to other proteins, exhibiting ATPase activity, and answers for hydrolytic centre of ATPases [4] and direct ^{18}[O]-exchange between P_i and H_2O [5]. In such centre the phospho(P_i)-intermediate of ATPase reaction has tied. Namely this centre in Av1 has catalyzed such direct ^{18}[O]-exchange between P_i and H_2O [6]. As we have established [2], amino acids 74-95 in Av1 "wind round" P-cluster in Av1. So the phospho-intermediate of ATPase reaction of nitrogenase is in direct contact with P-cluster. Increasing its acceptor properties, this intermediate can assist in electron transfer from Av2 to P-cluster, transforming it into super-reduced state in the process of ATP hydrolysis, connected with conformation change. References: 1) Syrtsova L.A., Nadtochenko V.A., Denisov N.N., Timofeeva E.A., Shkondina N.I., Gak V.Yu., *Biochemistry (Moscow)*, 2000, **65**, 1353. 2) Syrtsova L.A., Tukhvatulin I.A. et al., *Russ. Chem.*

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P1.5.13. SOME CHARACTERISTICS OF FUNCTIONAL COUPLING BETWEEN NUCLEOSIDE DIPHOSPHATE KINASE OF THE OUTER MITOCHONDRIAL COMPARTMENT AND OXIDATIVE PHOSPHORYLATION

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It was earlier shown in our laboratory that in rat liver mitochondria all nucleoside diphosphate kinase (mNDPK) of the outer compartment is associated with the outer surface of the outer membrane, and that there are 2 – 3 fractions of mNDPK differing by tightness of membrane binding.

In the present study, conditions of mitochondria isolation were found under which less than 16% or more than 70% of mNDPK molecules was solubilized from mitochondria during five hours of storage on ice (mediums A and B, respectively).

In experiments with mitochondria isolated in medium A three systems operating as ADP donors for oxidative phosphorylation were analyzed. These systems employed mNDPK, yeast hexokinase (yHK), and yeast NDPK (yNDPK). The enzymes exhibited the same activity, but yHK and yNDPK could not bind to mitochondrial membranes. In all three systems, muscle creatine kinase (CK) was the external agent competing with the oxidative phosphorylation system for ADP. In the presence of increasing quantities of CK the mitochondrial respiration rate decreased. At large excess of CK activity over other kinase and oxidative phosphorylation activities the creatine kinase reaction reached a quasi-equilibrium state. Under these conditions equilibrium concentrations of all CK substrates were determined and K_{eq}^{app} of this reaction was calculated for the system with yHK. In samples containing active mNDPK the concentration of ATP, creatine, and phosphocreatine were determined and the quasi-equilibrium concentration of ADP was calculated using the K_{eq}^{app} value. At balance of quasi-equilibrium concentrations of ADP and ATP/ADP ratio the mitochondrial respiration rate with mNDPK was 21% of the respiration rate assayed in the absence of CK; in the systems with yHK and yNDPK it was 3 – 7%. It is concluded that during oxidative phosphorylation functional coupling appears between mNDPK and the oxidative phosphorylation system.

We also studied whether all mNDPK molecules participate in the functional coupling. Mitochondria isolated in mediums A and B were used. The initial mNDPK activity in both systems supported close to the maximal rate of oxidative phosphorylation. We found that in both systems the

mitochondrial respiration rate in the presence of CK excess did not change with time of mitochondria storage and was equal to ~ 20% of the respiration rate in the absence of CK. We concluded that only a small part of mNDPK molecules (most tightly membrane bound) participates in the coupling.

This work was partially supported by ALSAM Foundation (USA).

2. Physiology and pathology of mitochondria (*in vitro*, *ex vivo* and *in vivo* studies)

Poster session 2.1. Uncoupling

P2.1.1. CONTROL OF ATP/ADP IN PANCREATIC BETA CELLS: THE IMPORTANCE OF MITOCHONDRIAL PROTON LEAK

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Pancreatic beta cells respond to rising blood glucose concentrations by increasing their oxidative metabolism, which leads to an increased ATP/ADP ratio, closure of K_{ATP} channels, depolarisation of the plasma membrane potential, influx of calcium and the eventual secretion of insulin. Such a signalling mechanism implies that the ATP/ADP ratio is flexible in beta cells, which is in contrast to other cell types (e.g. muscle and liver) that maintain a stable ATP/ADP poise whilst respiring at widely varying rates. A comparative top-down metabolic control analysis of oxidative phosphorylation in mitochondria isolated from rat skeletal muscle and cultured beta cells reveals that this difference in flexibility is, at least partly, accounted for by mitochondrial peculiarities [Affourtit, C. and Brand, M. D. (2006), Biochem. J. 393, 151-159]. It appears that ATP/ADP is controlled more strongly (~7.5x) by proton leak in beta cells than in muscle. The comparably high importance of leak (relative to phosphorylation) in beta cell mitochondria is indicated furthermore by its relatively high control over membrane potential and respiratory activity. Modular-kinetic analysis demonstrates that the control differences are readily explained by a higher relative leak activity in beta cell mitochondria, which results in a comparatively high contribution of proton leak to the overall respiratory activity in this system.

Currently, we are performing a top-down control analysis of glucose metabolism in cultured beta cells to test predictions made from experiments with isolated mitochondria. Preliminary data suggest that proton leak activity is relatively high in intact beta cells too: the proportion of myxothiazol-sensitive oxygen uptake activity that is resistant to oligomycin (~75%) is nearly 7x higher than that observed in cultured myoblasts. The nature of this comparatively high leak activity

is presently unclear. Equally, it is not yet evident how and to what extent proton leak controls glucose metabolism. Results from studies to clarify these matters will be presented.

P2.1.2. EFFECT OF HIGH MEMBRANE POTENTIAL ON THE UCP- MEDIATED ELECTRIC CONDUCTANCE OF PLANAR BILAYER MEMBRANE

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Proton leak mediated by fatty acid cycling plays an important role in thermogenesis as assumed for UCP1 (1). The function of other proteins of the UCP subfamily is less understood. They may play a critical role in controlling of mitochondrial ROS production or be involved in the export of fatty acid peroxides from the inner to the outer leaflet of the mitochondrial membrane (2). These activities are inhibited by purine nucleotides (PN). The mystery is how UCPs operate *in vivo* despite the presence of millimolar concentrations of PN, sufficient to completely arrest the UCP functioning. We have shown previously (3) that PN inhibit the fatty acid mediated proton conductance by UCPs incorporated to the planar phospholipids membrane (BLM) at zero membrane potential. Now we studied the UCP-mediated electric conductance at membrane potentials above 170 mV. It is shown that the electrical current increases in the presence of UCP1 or UCP2 and long chain fatty acid. Without fatty acid, UCP1 and UCP2 were quite ineffective. The increase in total membrane conductance was highly non-ohmic at voltages above ca. 120 mV. Effect of high membrane potential on the PN inhibition was also studied. The data indicate that the variation in membrane potential may be a major regulatory factor for *in vivo* activities of UCPs.

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P2.1.3. ORIGINAL REGULATION OF ENERGY BALANCE IN LEAN LOU/C RATS

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Derived from the Wistar strain, the Lou/C rats display lighter body weight and reduced adiposity regardless of age. Although the leanness of the Lou/C rats is probably multifactorial, such capacity to remain lean implies a fine adjustment of energy balance and large capacities to dissipate excessive food energy as heat. The aim of this work was to investigate whether the thermogenic activity of brown adipose tissue (BAT), the thermogenic tissue of small rodents, was enhanced in Lou/C rats by comparison with Wistar rats.

Male Lou/C rats exhibited a higher energy expenditure near thermoneutrality (1.18 ± 0.07 vs 0.79 ± 0.03 LO_2/h/kg; $p<0.05$, indirect calorimetry) than Wistar rats of similar age (5 months) but their energy balance was equilibrated as assessed by parallel monitoring of food intake. The calorigenic response to a noradrenaline test injection (0.3 mg/kg) was unexpectedly lower in Lou/C rats (-44%) than in Wistar rats. This was associated with lower relative abundance of BAT uncoupling protein (UCP1) mRNA (-40%, RT-PCR) and protein (-45%, Western blot) in Lou/C than in Wistar rats. In order to relate the unexpected lack of BAT activation observed in Lou/C rats placed at thermoneutrality to substitution by other thermogenic processes, spontaneous activity was monitored. At 25°C, Lou/C rats exhibited a high level of spontaneous running activity amounting 10 km/day vs. a few hundred meters in Wistar rats. Although not activated at thermoneutrality, BAT thermogenesis was stimulated by prolonged cold exposure (4°C for 3 wks) as shown by marked hypertrophy, increases in tissue oxidative capacity, mitochondrial content, respiratory rate of isolated mitochondria, expression of UCP1 and proteins involved in lipid metabolism.

Altogether, these results suggest that the Lou/C rat can maintain its fat mass at a low level by finely adjusting energy expenditure and food intake. By keeping energy expenditure at a high level by non-BAT dependent mechanisms, partly involving spontaneous exercise, Lou/C rats could turn their metabolism toward fat oxidation thus reducing fat storage. This would allow the reduction of BAT sympathetic activity leading to BAT relative inactivation. By contrast, when energy demand is increased for thermoregulatory purpose, BAT stimulation can occur through the activation of thermogenic processes and lipid use. The Lou/C rat therefore represents an original

animal model of energy balance regulation which does not follow the classical model based on BAT thermogenic activity (Rothwell and Stock. *J. Nutr.* 1984).

P2.1.4. ALTERNATIVE OXIDASE AND UNCOUPLING PROTEIN EXPRESSION IN MAIZE AND WHEAT SEEDLINGS UNDER VARIED STRESSES

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It is known, that at influence of various stress factors in plant cells there are an accumulation of reactive oxygen species (ROS) damaging the cell. One of the major sources of ROS are mitochondria. In reply to stress the set of protective reactions connected with mitochondria is realized. This is results in the decrease of potential on an internal membrane of mitochondria and ceasing ROS production. The important role during regulation of ROS level attributes to alternative oxidase (AOX) and plant uncoupling mitochondrial protein (PUMP). These proteins reduce membrane potential by different ways. AOX creates the branch in electron transporting chain, resulting to that two of three points of coupling will not be involved, PUMP does not interfere with an potential production, but promotes its reduction carrying of fatty acids. Special interest represents a ratio of these two ways of membrane potential regulation at various plants as it is known, that for functioning PUMP in plants, free fatty acids are necessary, whereas activity of AOX is repressed with them. Whether it is not known also it is coordinated genes expression of AOX and PUMP at stresses.

The data obtained by multiplex RT-PCR have confirmed the assumption that in wheat seedlings the regulation of AOX and PUMP at a level of a transcription occurs is not coordinated. At the majority of stresses (dehydration, low temperature, and the oxidizing stress) was induced AOX gene. PUMP expression was increased only at oxidizing stress (H_2O_2 action) when there was an induction of both genes simultaneously. Whereas in maize the expression of AOX and PUMP occurred simultaneously, at all stresses where the similar reaction (dehydration, low temperature, the oxidizing stress) was observed. It is interesting, that neither at maize, nor at wheat there were no changes in a level AOX and PUMP expression at heat stress. Probably, these proteins do not take part in the plant answer on a high temperature. It should note that the rise of AOX or PUMP expression in relation to a base level at maize was always much higher, than at wheat. Probably, it is due to lower stability of maize seedlings to the used influences.

Results revealed, that AOX and PUMP expression can simultaneously grow in spite of the fact that their functional activity should occur in an antiphase. Which protein accepts the main

participation in regulation of ROS, probably, depends on species, stage of development and kind of stress.

P2.1.5. DISTINCTIONS BETWEEN STIMULATION BY CYTOCHROME C OF ASCORBATE (WITH TMPD) OXIDATION OR NADH OXIDATION VIA EXTERNAL PATHWAY IN LIVER MITOCHONDRIA IN SUCROSE HYPOTONIC MEDIA

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Stimulation by exogenous cytochrome *c* (cyt *c*) of ascorbate with TMPD (asc) oxidation or NADH oxidation via external pathway (which bypass the initial part of the main respiratory chain) was studied. The incubation medium contained antimycin, rotenone, Mops (pH 7.4), sucrose (at concentrations 290, 110, 10 and 0 mM) and either (a) asc. or (b) NADH.+$MgCl_2$. Maximum oxidation rate was calculated using methos of double inverse values, concentrations of either (a) TMPD or (b) cyt *c* were gradually increased. In medium with 290 mM or 110 mM sucrose (when respiratory control being decreased only on 22 %) maximum stimulation of asc oxidation (on 24 % and 70%-100 % correspondingly) was reached after addition of 2 – 3 μM cyt *c*; the rate of exogenous NADH oxidation was small. In hypotonic medium with 10 mM sucrose (respiratory control was absent) maximum stimulation of asc oxidation was higher, however it was achieved after additions of 4 – 6-μM or at higher concentrations of cyt *c*; the rate of NADH oxidation was essentially increased. Only for oxidation of NADH via external pathway but not for asc oxidation the mitochondrial concenration of free (not bound) cyt *c* should be high and outer mitochondrial membrane should be ruptured or should have such cyt *c*-permeable channel that cyt *c* can freely pass through it for reduction or oxidation. We propose that during stimulation of asc oxidation by cyt *c* in medium with 290 or 110 mM sucrose cyt *c* crosses the outer membrane with low rate through a channel induced by superoxide and radicals of ascorbate, and begins to function as bound cyt *c*, hypotonia can stimulate the process. The results also shows that cyt *c*-oxidase activity of intact liver mitochondria can be determined from rates of ascorbate (with TMPD) oxidation in medium with 290 – 110 mM sucrose and gradually increaseing TMPD concentrations within interval 20 – 80 μM after preliminary addition of 2-3 μM cyt *c* and 10 μM dinitrophenol. The study had financial support by RFBR (grant N 05-04-49700).

P2.1.6. THE EFFECTS OF ACUTE AND ENDURANCE EXERCISE ON UCP 3 PROTEIN EXPRESSION IN MOUSE GASTROCNEMIUS, PLANTARIS, SOLEUS AND EDL MUSCLES

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Previous studies have indicated that short-term bouts of exercise induces large increases in uncoupling protein 3 in skeletal muscle, whereas long-term bouts of training result in marked decreases in skeletal muscle uncoupling protein 3 at both mRNA and protein levels in rodents. Using peptide antibodies specific to UCP 3, we show that absolute uncoupling protein 3 expression is elevated in *gastrocnemius* and *plantaris*, but not *soleus* or *extensor digitorum longus* muscle homogenates following short-term swimming exercise (intervals over 2 weeks). Furthermore, we show that uncoupling protein 3 expression per unit mass of mitochondria is increased in *gastroncnemius* muscle of mice. We show that the increase in uncoupling protein 3 expression in short-term exercise is transient, and returns to pre-exercise levels, 22 hours after the final exercise bout. By contrast, endurance swimming training (intervals over 17 weeks) resulted in no significant changes in uncoupling protein 3 expression in whole muscle homogenates of *gastrocnemius, plantaris, soleus* or *extensor digitorum longus.*

P2.1.7. SUPPRESSION BY DIBUCAIN AND BY CYCLOSPORIN A OF INNER MITOCHONDRIAL MEMBRANE PORE OPENING IN LIVER MITOCHONDRIA BY ACIDIFICATION OF HIGH PHOSPHATE INCUBATION MEDIUM

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In 1973, it was found by Leikin and Vinogradov that a decrease in pH of a high P_i incubation medium results in a Ca^{2+} -induced stimulation of succinate oxidation with the typical for pore opening kinetics (ref. in [1]). These data were reproduced in two other laboratories [1, 2]. Recently we have found that the decrease in pH of sucrose incubation medium with 5 mM P*i* from 7.4 to 6.5 induced an EGTA- and cyclosporin A (CsA)-sensitive swelling of mitochondria, two-fold increase in respiration rate and release of a large part of the cytochrome *c* pool from mitochondria [2]. Increase in concentration of calcium-phosphate complex in mitochondria was shown to induce pore opening [3]. We found that under the same condition [1] addition of 10 – 20 μM $CaCl_2$ did not affect the stimulation of respiration rates at low pH. CsA was more effective in prevention of respiration stimulation at pH 6.5 than at the neutral one (pH 7.4). As a result, the rate of respiration in the presence of CsA was lower in incubation medium with pH 6.5, than with pH 7.4. 50 μM dibucaine (the phospholipase A_2 inhibitor) completely prevented the mitochondrial swelling increase at pH 6.5. These data suggest that cytoplasm acidification and increase in P*i* after ischemia/reperfusion may induce calcium-phosphate complex formation, pore opening, cytochrome *c* release and cell death.

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P2.1.8. ION CHANNELS FORMED BY MINI-GRAMICIDIN IN PLANAR BILAYER PHOSPHOLIPID MEMBRANES: SENSITIZED PHOTOINACTIVATION VERSUS SINGLE-CHANNEL ANALYSIS

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Changing the chemical structure of channel-forming peptides is a convenient approach for studying structure-function relations of ionic channels. Here we compared the kinetic properties of channels formed by gramicidin A (gA) and its truncated analogue, mini-gramicidin, in which D-Leu-L-Trp domain of gA is conserved while four amino acid residues from the N-terminus are omitted. Two approaches were used: sensitized photoinactivation method developed by Rokitskaya et al. (1996) and single-channel ahalysis. The first approach is based on irradiation of a bilayer lipid membrane (BLM) with visible light in the presence of a photosensitizer, which leads to reduction of electric current across BLM mediated by gA. It has been concluded that singlet oxygen generated upon excitation of the photosensitizer damages tryptophan residues of gA. Exposure of BLM to a single flash of visible light in the presence of aluminum phthalocyanine provoked a transient decrease in the current across BLM mediated by mini-gramicidin. The time course of this decrease was well fitted by a single exponential with a characteristic time (2.5 s) close to that of gA. According to our previous studies, this parameter corresponds to the single-channel lifetime. In contrast to the covalent dimer of gA showing no current relaxation upon flash excitation, the covalent dimer of mini-gramicidin displayed the flash-induced current decrease, the characteristic time of which shortened as the membrane thickness increased. In the thinnest BLM formed from the DPhPC/squalene solution, the time course of photoinactivation was about 10 times longer for the mini-gramicidin covalent dimer than for mini-gramicidin. The photosensitized current relaxation is assumed to reflect equilibration between dimers and monomers and between conducting and non-conducting dimers in the case of mini-gramicidin and mini-gramicidin covalent dimer, respectively. The results of single-channel measurements were in qualitative agreement with those of sensitized photoinactivation: the average single-channel lifetime of single channels formed by covalent dimers of mini-gramicidin amounted to 6.5 s, 0.66 s and 0.04 s in BLMs formed from DPhPC/squalene, DPhPC/hexadecane and DPhPC/decane solutions, respectively. Thus, the increase in the membrane thickness led to destabilization of mini-gramicidin channels.

P2.1.9. A SINGLE BASE EXCHANGE LEADS TO TISSUE SPECIFIC ABLATION OF UCP3 EXPRESSION

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Uncoupling protein 3 (Ucp3) is a nuclear encoded protein located within the inner mitochondrial membrane and predominantly expressed in skeletal muscle and brown adipose tissue (BAT). It is considered to play a role in lipid metabolism by preventing deleterious effects of fatty acids during states of high lipid oxidation[1]. In our colony of Djungarian hamsters (*Phodopus sungorus*) we identified animals with a BAT specific Ucp3 deficiency, a phenotype which proved to be heritable by intercrossing littermates. In an initial founder line the BAT specific absence of Ucp3 did neither impair nonshivering thermogenesis nor uncoupled respiration of isolated mitochondria[2]. Mutant hamsters did however display an increased body weight, which must be verified to exclude a founder effect.

Sequencing of the hamster Ucp3 gene revealed a single base exchange, that was strictly associated with BAT specific lack of Ucp3 expression in the homozygous state. In reporter gene assays we confirmed, that transcription of the mutant allele is strongly impaired in brown adipocytes. This allele appears to exist in natural populations, as it was identified in distinst laboratory colonies established independently from wild-trappes founders. Knowing the underlying mutation allows us to genotype animals and repeat phenotyping on a broad genetic background.

It is of great importance that a naturally occurring single base exchange is able to dramatically affect Ucp3 expression and to possibly influence body weight control. Numerous studies have shown an association of single nucleotide polymorphisms within the human Ucp3 gene and body mass index. However, until now there is no report on functional consequences of these alleles and it has been a matter of discussion, whether they may be just markers for functional polymorphisms within linkage disequilibrium3. This new mutant animal model proves that a single nucleotide exchange is able to markedly alter Ucp3 gene expression levels and will provide further insight whether such changes can lead to an altered energy balance.

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P2.1.10. THE ROLE OF FATTY ACIDS IN OXIDATIVE PHOSPHORYLATION UNCOUPLING OF WINTER WHEAT MITOCHONDRIA AND THE PARTICIPATION OF ADP/ATP-ANTIPORTER AND THE PLANT UNCOUPLING MITOCHONDRIAL PROTEIN (PUMP) IN THIS PROCESS

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The oxidative phosphorylation uncoupling by free fatty acids (FFA) in plant mitochondria depends on the function of FFA as protonophores and their interaction with such specific mitochondrial proteins from the family of mitochondrial anion carriers as ADP/ATP-antiporter, plant uncoupling mitochondrial protein (PUMP) and others. The aim of the present work was study of uncoupling action of saturated and unsaturated fatty acids C_{12}-C_{24} and determination of ADP/ATP-antiporter and PUMP role in uncoupling by these acids. The isolated mitochondria of etiolated shoots of winter wheat (*Triticum aestivum* L, cv. Zalarinka) germinated on moist paper at 26 ^{0}C were used in this work. We used FFA concentrations from 0.056 μM to 10 mM. The contribution of ADP/ATP antiporter and PUMP was measured, using inhibitors of these proteins – 0.5-2 mM GTP, GDP, ATP, ADP for PUMP and 1 μM carboxyatractyloside (CAT) for ADP/ATP-antiporter.

It was shown that among saturated fatty acids the most increase of oxygen consumption in state 4 by mitochondria was caused 50 μM palmitic acid (C 16:0) (two times in oxygen consumption), which also caused 30% induction of mitochondria swelling. The increase of oxygen consumption in state 4 in the presence of saturated fatty acids was associated with ADP/ATP-antiporter function; its contribution was about 80-100%. It was found that among unsaturated fatty acids the maximum of oxygen consumption by mitochondria were caused C 18 fatty acids, especially polyunsaturated linoleic (C 18:2) and α-linolenic (C 18:3). The increase in oxygen consumption at state 4 was a 3-fold for 250 μM linoleic acid and a 6-fold for 500 μM α-linolenic acid. However, we observed a 5-fold increase of mitochondria swelling in the presence of linoleic acid (in 90 seconds of incubation), whereas in the presence of α-linolenic acid it was a 3-fold. The data obtained showed that uncoupling of oxidative phosphorylation by unsaturated acids was associated with PUMP function; its contribution was about 65%.

So, uncoupling activity of FFA depends on its aliphatic chain length, presence and number double bounds. It is concluded that mechanisms of oxidative phosphorylation uncoupling in winter wheat mitochondria by saturated and unsaturated fatty acids are different.

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P2.1.11. GDP AND FATTY ACID INTERACTIONS WITH UCP1

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The activation of the thermogenic uncoupling protein 1 (UCP1) in brown adipose tissue (BAT) by fatty acids is well established, but the mechanism of activation and interaction with nucleotide inhibition is still debated. While fatty acids failed to affect nucleotide affinity in several radioactive binding studies, functional studies of palmitate stimulation of respiration in BAT mitochondria have suggested a simple competitive interaction with GDP (Shabalina et al, 2004).

Given the demonstration of simple competitive kinetics in functional studies, in this study we have re-examined the effect of palmitate on GDP binding to UCP1 in BAT mitochondria from warm-adapted rats using the fluorescent probe N-methylanthraniloyl-guanosine-5-diphosphate (mant-GDP).

Mant-GDP bound to BAT mitochondria with an affinity of 8.2±0.8 μM. This was comparable to an affinity for [^{3}H]-GDP of 6.7±3.3 μM. Palmitate, added at a free concentration calculated as 515 nM, did not alter the affinity for mant-GDP or [^{3}H]-GDP. However, palmitate did cause a 1.7-fold increase in the maximum number of binding sites (B_{MAX}) of mant-GDP. The EC_{50} for palmitate for this effect was 34.5±1.2 nM. Dimethyl anthranilate (DMA), the fluorescent group that has a methyl group in place of GDP, did not show high affinity binding to BAT mitochondria and was unaffected by palmitate.

We suggest that under conditions where palmitate is not present (0.3% BSA), a sub-population of UCP1 nucleotide binding sites is inaccessible to mant-GDP. Increasing palmitate modifies UCP1, making more mant-GDP binding sites available. We are investigating the relationship between the mant-GDP B_{MAX} and [^{3}H]-GDP B_{MAX} and whether this phenomenon is related to the intriguing aspect of nucleotide binding referred to as unmasking (Desautels et al, 1978).

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P2.1.12. INTRODUCING A MAMMALIAN CELL SYSTEM TO STUDY THE FUNCTION OF EVOLUTIONARY DISTANT UNCOUPLING PROTEINS

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In mammals, uncoupling protein 1 (UCP1) enables brown fat mitochondria to increase proton conductance and respire at maximal rates in the absence of ATP-synthesis thus resulting in nonshivering thermogenesis (NST). In contrast, the physiological roles of UCP2 and UCP3 have not been resolved yet but may be related to mitochondrial ROS production[1]. Our search for the evolutionary origin of thermogenic UCP1 by comparative genomics surprisingly revealed the presence of all three UCPs in fish[2], and recently in amphibians and marsupials (unpublished data). Isolated liver mitochondria of the Common Carp (*Cyprinus carpio*) expressing UCP1 displayed increased proton conductance by addition of palmitate or 4-hydroxynonenal (HNE). This inducible proton conductance was prevented by addition of GDP[3]. These properties strongly suggest that carp UCP1 is a functional uncoupling protein with broadly the same activatory and inhibitory characteristics as mammalian UCP1. Evolution and cross-species comparative studies of UCPs are of interest to elucidate their general physiological role(s) in vertebrates and to determine conserved structure-function relationships. However, comparative studies of UCP paralogues (UCP1, UCP2 and UCP3) and UCP orthologues (e.g. UCP1 of vertebrates) in native mitochondria are hampered by differential tissue specificity and different genetical backgrounds. We therefore established stable expression of UCPs at various levels in HEK293 (human embryonic kidney) cells allowing comparative measurements in the same cell system. Isolated HEK293 mitochondria expressing different levels of mouse UCP1 displayed GDP-sensitive uncoupling activity in response to palmitate. Fatty acid induced proton conductance strongly correlated with UCP1 content. In contrast, no change of proton conductance was observed in control mitochondria (-UCP1) in response to identical amounts of palmitate and GDP. For cell lines expressing mouse UCP3, the HNE-inducible uncoupling property correlated with protein content. These findings demonstrate the suitability of the HEK293 system to investigate native behaviour of ectopically expressed UCPs to directly evaluate uncoupling activity of mammalian UCP1 and UCP3 as well as their impact on ROS production and cellular damage. Comparing the uncoupling property of mammalian UCP1 and the novel vertebrate UCP1 orthologues casts light on the evolution of UCP1-mediated NST.

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P2.1.13. RAPIDLY UP-REGULATED EXPRESSION OF UCP3 AND ITS EFFECT ON ROS GENERATION IN MUSCLE MITOCHONDRIA DURING PROLONGED EXERCISE IN RATS

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Mitochondrial uncoupling is expected to result in a more oxidized state of the electron transport chain, thereby preventing superoxide generation (Korshunov, 1997). Superoxide-mediated activation of UCPs has been proposed to serve as a negative feedback mechanism to limit further superoxide production (Echtay, 2002). The present research is design to investigate whether does UCP3 involve in‘the negative feedback modulation’on generated ROS during a prolonged intensity exercise. We measured the efficiency of oxidative phosphorylation, ROS generation rate, transmembrane potential, the express of UCP3 mRNA and UCP3 protein in rat skeletal muscle mitochondria during exercise. The SD rats were divided into 5 groups (run for 0, 45, 90, 120 and 150 min respectively) on the treadmill according to the incremental protocol and sacrificed at zero time or immediately after every exercise time course. With the increase of the ROS generation in skeletal muscle mitochondria initially, the expression of UCP3 mRNA and UCP3 protein increased subsequently. Meanwhile, the state 4 respiration rate increased significantly and transmembrane potential was reaching to maximal. With mitochondrial UCP3 protein climbed to the peak at the time point of 120 min the ROS generation rate and ATP synthesis decreased slightly. The transmembrane potential and state 4 respiration rate remained the high lever during the process. The results confirmed that muscle mitochondrial UCP3 expression was rapidly up-regulated during acute prolonged exercise. Based on the hypothetical model proposed of Nedergaard, it is suggested that ROS may have a role in activation and/or induction of UCPs expression, and the coordination of both ROS and UCP3 may cause proton leak leading to protect against excessive superoxide production at the cost of slightly lowered efficiency of oxidative phosphorylation.

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P2.1.14. THE ATP/ADP–ANTIPORTER IS RESPONSIBLE FOR A GDP-SENSITIVE OLEIC ACID- AND OLEIC ACID HYDROPEROXIDE–INDUCED UNCOUPLING IN KIDNEY MITOCHONDRIA

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It is shown that in kidney mitochondria incubated in the presence of oligomycin and pyruvate or succinate with rotenone (1 mM malonate was added before oleic acid to increase ΔΨ changes in response to following addition), 5-10 μM oleic acid-induced ΔΨ decrease was partially prevented or reversed not only by carboxyatractylate (CAtr inhibitor of the ATP/ADP-antiporter) but also by 200 μM GDP. In some experiments GDP or CAtr added before oleic acid completely prevented this ΔΨ decrease. When added after oleic acid, GDP and CAtr reversed ΔΨ decrease by about 20 % ± 6 % and 50 % ± 10 % respectively. Addition of CAtr after GDP increased its coupling effect by 26 % ± 4 %, GDP added after CAtr being ineffective. The ratio $[(V_{Ole\text{-}OOH}-V_{Oligo})/ (V_{Ole}-V_{Oligo})]$ x 100% in liver mitochondria was equal to 21 % ± 5 % (sucrose incubation medium) and in kidney mitochondria was equal to 25 % ± 10 % (sucrose incubation medium) and to 25 % ± 7 % (salt incubation medium) when $V_{Ole\text{-}OOH}$, V_{Ole}, V_{Oligo} are the respiration rate in the presence of 10 μM oleic acid hydroperoxides, 10 μM oleic acid and oligomycin correspondingly. The decrease in ΔΨ induced by 10 μM oleic acid or oleic acid hydroperoxides was equal to 57 % ± 5 % or 8 % ± 3 % respectively.

The above data suggest that it is the ATP/ADP–antiporter rather than UCP that is responsible for the GDP inhibition of uncoupling effect of fatty acids in kidney mitochondria. As to oleic acid hydroperoxides, it is much less efficient as an uncoupler than oleic acid.

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P2.1.15. UNCOUPLING PROTEIN 1 IS EXPRESSED IN THE BRAIN OF ECTOTHERMIC VERTEBRATES

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Uncoupling proteins (UCPs) regulate proton conductance of the mitochondrial inner membrane. Until recently the thermogenic uncoupling protein 1 (UCP1) was considered to be unique to brown adipose tissue mitochondria of placental mammals where it dissipates proton motive force as heat (non-shivering thermogenesis, NST). We identified the ortholog of mammalian UCP1, as well as the two paralogs UCP2 and UCP3 in ectothermic bony fishes suggesting that the members of the core UCP family already existed 420 million years ago and are present in all living vertebrates (1). Accordingly, we found all three UCPs in the genomes of the Clawed frog (*Amphibia*) and the Opossum (*Marsupialia*), whereas in the Chicken genome only UCP3 can be found so far.

The biological function of thermogenic UCP1 in ectothermic vertebrates is not understood.
In the Common Carp (*Cyprinus carpio*) UCP1 is strongly expressed in the liver. In isolated Carp liver mitochondria fatty acids increase proton conductance in a GDP-sensitive manner. Thus, UCP1 orthologs of fish and mammals share key functional characteristics. UCP1 expression is also detected in the brain, albeit at lower expression levels than in liver. We studied the effect of cold acclimation on UCP1 gene expression in the Carp. Whereas UCP1 mRNA levels sharply declined in the liver of cold exposed Carp, Northern blot analysis of whole brain RNA revealed a two-fold increase of UCP1 expression. We therefore performed In Situ Hybridisation (ISH) of coronal and sagittal sections of the Carp brain with a ^{35}S-labelled Carp UCP1 cRNA probe. This analysis revealed distinct localised expression of UCP1 in the forebrain (periventricular grey zone of the optic tectum) and the brain stem (solitary and trigeminal tract). We hypothesise that cold-induced UCP1 expression may increase the capacity for local non-shivering thermogenesis in selected brain regions in order to maintain critical neuronal functions in cold water.

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P2.1.16. Tl^+ EFFECTS ON RAT LIVER MITOCHONDRIA WERE STIMULATED IN THE PRESENCE OF INORGANIC PHOSPHATE

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We were early found that effects of bivalent heavy metals like Cd^{2+}, Hg^{2+}, Zn^{2+}, and Pb^{2+} on isolated mitochondria were notably increased in the presence of inorganic phosphate (P_i). We studied effects of K^+ analogue Tl^+ (monovalent heavy metal) on respiration and swelling of isolated rat liver mitochondria in the presence of P_i in this connection. It was found that the basal respiration of succinate-energized mitochondria was constantly increased in enhancing Tl^+ concentration in medium containing 0-125 mM $TlNO_3$ or 0-50 mM Tl-acetate. The respiration in the presence of Pi was extra activated correspondingly in 1.7 and 1.2 times at common Tl^+ concentrations in a comparison with experiments lacking P_i. Respiration of mitochondria, uncoupled by 2,4-dinitrophenol (DNP), was not additionally stimulated in the media containing $TlNO_3$ or Tl-acetate in the presence of P_i. Swelling of succinate-energized mitochondria was gently enhanced in growing concentration of $TlNO_3$ from 0 till 125 mM in a medium. However, the swelling in the presence of P_i was strongly increased at common of $TlNO_3$ concentrations in a comparison with experiments lacking P_i. It was supposed that the increasing in respiration and swelling of energized mitochondria in medium containing $TlNO_3$ and P_i are coming out from facilitation of Tl^+ transport via the inner mitochondrial membrane under these experimental conditions.

P2.1.17. UCP3 ACTIVATION: MUTUAL RELATIONSHIP BETWEEN SUPEROXIDES AND POLYUNSATURATED FATTY ACIDS

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Several evidences suggest that many factors are involved in UCP3 activation. CoQ, superoxides, lipids, lipid peroxides and/or their derivatives seem to play a role in the mechanisms of activation.

We investigated on the interrelated role between superoxides production and fatty acids on UCP3 catalyzed - proton conductance. To this aim we evaluated proton leak kinetic by modulating i) the mitochondrial endogenous superoxides production ii) the levels of free fatty acid (FFA) iii) the formation of lipid peroxides, and iv) the UCP3 uncoupling activity.

In the absence of mitochondrial endogenous FFAs GDP inhibited proton leak kinetic of skeletal muscle mitochondria, confirming a role for UCP3 in the establishment of basal proton conductance. In the same condition, no differences in the kinetic of proton leak were observed between mitochondria respiring in the absence and in the presence of rotenone, despite a 6 fold difference in mitochondrial H2O2 release was detected. This suggests that the formation of O^{-2} at complex I per se is not able to induce mitochondrial proton conductance.

The exogenous addition of arachidic acid (i.e a saturated fatty acid) or arachidonic acid (i.e a polyunsaturated fatty acid) to mitochondria induces mitochondrial uncoupling as they stimulate proton leak kinetic, with the arachidonic acid having the higher effect.

The induction of proton conductance by arachidic acid does not involve UCP3, the formation of O2- at complex I and lipids peroxidation. On the other hand arachidonic acid can catalyze UCP3 mediated uncoupling only when it can undergo to a peroxidative process. When this process is not easily allowed, because of a low production of O2- or when lipid free radicals are removed by Vitamin E scavenging effect, arachidonic acid behave as arachidic acid and induces a mitochondrial uncoupling not mediated by UCP3.

These data are consistent with a role for mitochondrial lipid hydroperoxides and/or derivatives formation inside the matrix in UCP3 activation.

P2.1.18. BUTYLATED HYDROXYTOLUENE - A USEFUL MODEL FOR SAFER UNCOUPLERS IN OBESITY TREATMENTS?

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The concept of using mitochondrial uncouplers in weight loss treatments is feasible. However, this concept became unpopular due to various safety concerns. Dinitrophenol (DNP) reportedly caused undesirable side-effects and caused several incidences of overdosing [1]. Therefore, a wide therapeutic window (difference between effective and toxic doses) is one of the important aspects that need to be considered in the development of anti-obesity drugs.

Butylated hydroxytoluene (BHT), a common synthetic antioxidant used in foods, has been shown to uncouple mitochondria [2]. Here, we found that BHT significantly uncouples mitochondria at extremely low concentrations, even at 10^{-12} M. Furthermore, increases in the uncoupling rates were not proportional to the increase in concentration – uncoupling was sensitive to low concentrations of BHT, but this uncoupling became less sensitive when more BHT was added. This resulted in a very shallow slope (0.11 ± 0.02) in the plots of change in respiration and BHT concentration. In contrast, CCCP (carbonyl cyanide *m*-chlorophenylhydrazone), a classical mitochondrial uncoupler, gave a much steeper slope (0.92 ± 0.06). Therefore, based on the wide range of effective concentrations and shallow slope observed, BHT might have a wide therapeutic window *in vivo*.

Potential-dependence may explain the observed behaviour of BHT. Based on the relationship between mitochondrial membrane potential and increase in BHT-stimulated uncoupling, we concluded that BHT-stimulated uncoupling is indeed potential-dependent. CCCP, on the other hand, did not show any potential-dependent uncoupling.

BHT may provide useful clues in designing safer uncouplers for obesity treatment.

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P2.1.19. TOCOPHERYLQUINONE ACTIVATES UNCOUPLING PROTEIN IN MYOCARDIAL MITOCHONDRIA: A POTENTIAL NEW ROLE OF VITAMIN E AS AN ANTIOXIDANT

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Oxidative stress and mitochondrial oxidative damage are usually associated with oxidant/antioxidant imbalance. The mitochondrial respiratory chain is believed to be the major source of reactive oxygen species (ROS) production in the cells. Several events are implicated in oxidative stress condition; these include alterations in mitochondrial DNA, lipids (e.g., cardiolipin) and proteins [e.g., uncoupling proteins (UCP)]. On the other hand, the mitochondrion uses a range of antioxidants such as manganese superoxide dismutase and glutathione reductase to protect itself from oxidative damage. Recently, numerous experimental data have shown that mild uncoupling though UCP activation may also be involved in the mitochondrial antioxidant defense mechanisms.

Furthermore, recent findings indicate that mitochondrial membranes rich in vitamin E protect cells against the toxic effects of oxidative stress [1]. There is a general agreement that alpha-tocopherol serves as a potent radical chain-breaking antioxidant to protect membrane phospholipids against peroxidation, presumably by functioning as an electron donor to free radicals. Tocopherylquinone (TQ) is an oxidation product of vitamin E. The level of TQ presumably rises during oxidative stress. However, vitamin E and CoQ cooperate as antioxidants through UQH_2 to recycle vitamin E to its active form [2]. Recent data show that lipid peroxidation products require a C-C double bond and either an acyl or a carbonyl group to activate UCPs [3]. Interestingly, the oxidized (TQ) but not the reduced form of vitamin E (alpha-tocopherol) contains a similar structural motif. Thus, we investigated the possibility that TQ might activate UCPs.

In isolated and FFA-depleted myocardial mitochondria from C57/BL6J mice, a highly purified alpha-tocopherol (99%) and TQ (95%) were used separately to activate UCP-mediated proton leak. Preliminary results from our laboratory show that the oxidized form of vitamin E is about 70 times more potent than the reduced form in activating UCP in myocardial mitochondria.

Theoretically, vitamin E exists as an oxidized form only when there is a continued high amount of ROS generation under oxidative stress conditions. Therefore, our results suggest that vitamin E

might act as antioxidants outside of its free radical scavenging properties; that is, the oxidized form of vitamin E (TQ) may act as an indirect antioxidant by activating other proposed oxidative defence mechanisms such as UCPs.

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P2.1.20. STARVING TETRAHYMENA PYRIFORMIS RESPONDS TO SOUND FREQUENCY IRRADIATION OF CELLS AND MITOCHONDRIA

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Unicellular ciliated Tetrahymena pyriformis (Tetr) are very sensitive to photodynamic damage, the photosensitizer can be selectively localized in mitochondria. Starved Tetr cultures in suspension are highly sensitive to pressure; thereby it may be sensitive to sound waves. There are publications describing various effects of music on plants and animals (reviewed by Wicke R.W. - <http://www.rmhiherbal.org/review/2002-1.html>, authors are grateful to "Denny S." for this reference). Especially puzzling are similar music's effects in humans and plants. Due to their size, Tetr can not be an "antenna" for these sound waves and can not experience a resonance with them. However Tetr may have suitable receptors or some kind of oscillatory reactions that would enable them to respond to sound waves or acoustic frequencies signals. Larionov V. has designed an electronic device transforming sound signals into light signals. This sound-to-light modulator (SLM) operates a light-emitting diode (LED) capable of producing directed light in two modes, either as a non-interrupted light source (1) or as an interrupted light emitter modulated at 20 Hz – 15 kHz (2). Light modulation is achieved with an electronic switch circuit controlled by a sound or Swip-capable sound frequency generator. The SLM has also a line-in port for external modulating signal. This signal (e.g., from a CD player) is directed to an acoustic converter made of a small speaker placed in the vicinity of a piezoelectric sound sensor (PS). The output from PS is directed to modulate LED by means of an operational amplifier LM-358 powered by a +5 V unipolar power supply. The light intensity was estimated with a photodiode. The LED was used to either directly irradiate a suspension of cells or as a light source in the microscope used to observe Tetr. In preliminary experiments, we found changes in Tetr when a suspension of starving Tetr cells was either supplemented with localized in mitochondria photosensitizer, positively charged molecule diS-C_3 -(5), and irradiated with 10 kHz red light by a LED via the electronic device or placed on the membrane of a headphone connected to a CD player playing music at high volume.

P2.1.21. THE AMINE-INDUCED PROTON OUTFLOW THROUGH THE COMPLEX OF PHOTOSYSTEM II OF CHLOROPLASTS

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The processes of the transfer of electrons and protons are the main reactions in photosynthesis bioenergetics. In a thylakoid membrane these processes are coupled to ATP synthesis and must have the common centers of regulation. Now two centers are well known - the ATP synthase and the cytochrome b6/f complex carried out the plastohydroquinone oxidation at the lumenal side of a thylakoid membrane. Recently we have obtained the data that allow to suppose the existence of the third regulatory center localized in PS II, near the site of the secondary quinine Q(b) (V. Opanasenko, A. Agafonov, R. Demidova, *Photosynth. Res*., 72, 243-253, 2002).

Here we have shown that the regulatory center at the level of Q (b) is developed at the presence of various penetrating amines. Amines induce an original pathway of outflow of protons from a lumen, and this outflow depends on a functional state of acceptor part of PS II. As is known, amines cause the uncoupling of the electron flow and ATP synthesis in chloroplasts. We have shown that efficiency of uncoupling by amines is maximal in the reactions of PS II and minimal in the reactions catalyzing the plastoquinone reduction. DCCD - the modifier of carboxylic groups of the amino acids, acts on the amine-induced outflow in dependence on the ratio of concentrations of the modifier and chlorophyll: at DCCD/Chl=2 it increases the efficiency of uncoupling by amines (effect A) and at the ratio 4-5 it reduces this efficiency (effect B). Both of DCCD effects develop on light for 20-30 s, and they are not accompanied by the covalent binding of an isotope of the modifier. The effect A is irreversible - it is kept after washing the chloroplasts after DCCD processing. The effect B would be eliminated by washing, but it can be completely restored by the addition of a new portion of DCCD in the reaction medium. The effect B is absent in reactions of PS II and PS I (at presence of DCMU).

The data show that the amines induce a transmembrane channel conducting protons from a lumen through the PS II complex. The channel consists of two DCCD-sensitive parts, hydrophobic (A) and more hydrophilic one (B). The part A connects the lumen to Q(b)-site, and the part B connects Q(b)-site to the stroma. At the presence of DCMU or PS II acceptors part B becomes hydrophilic that prevents binding of DCCD to the carboxylic groups.

P2.1.22. SPECIFICITY OF GDP INHIBITION OF PROTON CONDUCTANCE IN ENERGISED MITOCHONDRIA

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Purine (and not pyrimidine) nucleotides are established as inhibitors of uncoupling protein (UCP) activity. This has been shown for UCPs 1, 2 and 3. Inhibition of proton conductance by purine nucleotides is commonly used as a diagnosis of UCP activity.

Here we demonstrate that isolated skeletal muscle mitochondria show a gradual but persistent increase in proton conductance after energisation by substrates such as succinate. This increase in conductance occurs even in the absence of exogenously added activator and is sensitive to GDP. The uncoupling of basal leak over time is seen in skeletal muscle mitochondria isolated from both wildtype and UCP3 knockout mice and is sensitive to GDP in both models. This suggests that the steady gain in proton conductance of energised, non-treated mitochondria *in vivo* is not catalysed by UCP and that GDP is inhibiting uncoupling via another route. We show that the increase in proton conductance observed is inhibited by carboxyatractylate and methylenediphosphonate, these compounds specifically block the activity of the adenine nucleotide translocase (ANT) and phosphate carrier respectively. Inhibition of proton conductance by CAT or MDPA and GDP is not additive suggesting that GDP is inhibiting proton conductance through the ANT and phosphate carriers. Additionally, the increase in proton conductance is inhibited by pyrimidine nucleotides. Finally we find that inhibition of this uncoupling by GDP is itself blocked by addition of exogenous free fatty acids. We conclude that, under certain conditions, GDP is able to inhibit mitochondrial proton conductance through mitochondrial carriers other than UCP. Interaction of GDP with these other carriers appears to be blocked by fatty acids. This shows that caution should be taken when using GDP-inhibition of uncoupling as a diagnostic of UCP activity.

P2.1.23. FATTY ACID-INDUCED UNCOUPLING IN STARVING CELLS OF TETRAHYMENA PYRIFORMIS

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Mitochondria (mito) isolated from Tetrahymena pyriformis (Tetr) resemble by some properties mammalian mito: P/O and respiratory control values, cyt *c* content/mg mito protein and practically complete inhibition (more than 90%) of respiration by cyanide (L. Kilpatrick, M. Erecinska, 1977). Linoleic acid stimulates respiration of mito isolated from amoeba (another protozoa), the stimulation is suppressed a little by carboxyatractylate or GTP (N. Antos et al., 2004). In the present work mito energetics of living Tetr cells was under study. The respiration was recorded in inorganic incubation media (IM), as a rule, the cell concentration was 0.3-0.5 x 10^6 cells/ml. Cyanide suppressed the respiration of fed and starving Tetr cells by 30-50% and 10-15% correspondingly. Tetr starving for 1-2 days were used in the most of experiments. Pyruvate (20 mM) increased endogenous cellular respiration in about 1.7 times in IM with pH 7.1-7.4. Uncoupler DNP stimulated the rate of pyruvate oxidation in 2.0-2.4 times, while oleic acid (8-15 μM) increased pyruvate oxidation rate in 2 times. As a rule, especially for cells starved for 2 days, this oleic acid-stimulated respiration was suppressed a little (by 5-14%) by glutamate (3 mM) and by carboxyatractylate (3 μM) added after glutamate. Starvation of Tetr for 2-4 days resulted in increase of IM pH from 7.1 to 7.5. In this situation the cells became more sensitive to pressure. For estimation of cyt *c* content from differential spectra, the starving Tetr cells previously was destroyed by transient increase in IM pH up to 11-12, then pH was adjusted to the initial value. Cyt *c* was reduced by cyanide + ascorbate + TMPD (in sample cuvette) and oxidized by ferricyanide (in reference cuvette). The results show that about the same methods can be used for the study of mito metabolic state in the isolated mito and the mito within Tetr cells and that long-chain fatty acids can induce uncoupling in living cells.

P2.1.24. MITOCHONDRIAL ADAPTATIONS TO CHOLINE-METHIONINE DEFICIENT DIET, A MODEL OF NON ALCOHOLIC STEATOHEPATITIS

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Obesity is often associated with diabetes mellitus and an emerging pathology called Non Alcoholic SteatoHepatitis (NASH). Further, NASH is now the most common liver disease in western countries. The ethiology of NASH and its mitochondrial consequences are still unknown. This study aimed to evaluate the hepatic mitochondrial alterations of rats fed a choline-methionine deficient diet (CMDD), a known model of NASH.

Male Wistar rats were fed a standard diet or a CMDD for 6 weeks. Liver mitochondria were isolated and their oxygen consumptions (JO_2) in state 4 (with oligomycin) and 3 (phosphorylating) were measured. Oxidative phosphorylation efficiency was determined from the relation between ATP synthesis and respiratory rate (P/O) using an ADP-regenerating system (hexokinase plus glucose) in the presence of succinate plus rotenone. Reactive oxygen species (ROS) production was monitored fluorimetrically.

In the CMDD group, histological observations confirmed steatohepatitis. With succinate mitochondria from CMDD rats showed increased oxygen consumption as compared to controls in state 4 (32 ± 2 *vs* 22 ± 1 nanoatomO/min/mg prot; $p<0.001$) and state 3 (203 ± 12 *vs* 137 ± 5 nanoatomO/min/mg prot; $p<0.001$). Respirations were also higher with octanoyl-carnitine as substrates both in state 4 (48 ± 3 *vs* 35 ± 2 nanoatomO/min/mg prot; $p<0.01$) and in state 3 (14 ± 1 *vs* 10 ± 1 nanoatomO/min. /mg prot; $p<0.01$). Mitochondrial ROS production with succinate was lower in CMDD (97 ± 9 *vs* 150 ± 21 pmoles H_2O_2/min/mg prot/min; $p<0.05$). Moreover, mitochondrial cytochrome aa_3 content (80 ± 8 vs 53 ± 8 pmol/mg prot; $p<0.05$) and cytochrome oxidase (COX) activity (+54%) were increased. The relation between ATP synthesis and respiratory rate (P/O) was shifted to the right in the CMDD group demonstrating a drop in mitochondrial efficiency (lower ATP synthesis at similar oxygen consumption). In the presence of sodium cyanide (NaCN), a COX specific inhibitor, state 4 respiratory rate of CMDD rats was brought back to that of controls and the P/O relations overlapped, indicating similar mitochondria efficiency.

To conclude, feeding rats with a choline-methionine deficient diet lowers the efficiency of liver mitochondria. Such drop in efficiency may involve COX on the basis that i) COX is a well recognized site of slipping between redox reactions and proton pumping and ii) NaCN reversed the mitochondrial consequences of CMDD. The rise in hepatic COX activity induced by CMDD may contribute to increase lipid oxidation and limit oxidative stress.

P2.1.25. OXIDATIVE STRESS INDUCE FORMATION IN LIVER MITOCHONDRIA OF THE COMPLEX OF ADP/ATP AND ASPARTATE/GLUTAMATE ANTIPORTERS AT FATTY ACID UNCOUPLING ACTIVITY

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In liver mitochondria the protonophore uncoupling activity of palmitate is suppressed by inhibitor of the ADP/ATP antiporter carboxyatractylate (Catr) and by substrates of the aspartate/glutamate antiporter glutamate or aspartate (Asp) [1]. Oxidizing agent *t*-butylhydroperoxide (TBH) at concentration of 0.2 mM does not influence on the rate of respiration of mitochondria both in the presence and in the absence of palmitate. At the same time in the presence of TBH recoupling effects of Catr and Asp are very small if these substances are added after palmitate; however, if Catr is added after Asp or Asp after Catr the recoupling effects of Catr and Asp are significantly increased. The effects of Catr and Asp are considered as evidence that at oxidative stress ADP/ATP and aspartate/glutamate antiporters involved in uncoupling function as uncoupling complex with common fatty acid pool. Following the Catr addition, the fatty acid molecules are able to move from ADP/ATP antiporter to aspartate/glutamate antiporter, and in the opposite direction, upon the Asp addition. It is known, that in mitochondria production and effect of reactive oxygen species are increased with aging of animals [2]. In liver mitochondria of old rats ADP/ATP and aspartate/glutamate antiporters are involved in uncoupling function as uncoupling complex with the common fatty acid pool in the absence of TBH. In the presence of pyruvate or β-hydroxybutyrate, which are able to reduce mitochondrial pyridine nucleotides, the complex does not form. Antioxidants ionol and thiourea also prevent complex formation.

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P2.1.26. ON THE MECHANISM OF NON-SHIVERING THERMOGENESIS: UCP3 OR ATP/ADP-ANTIPORTER CANNOT SUBSTITUTE FOR UCP1 IN THERMOGENESIS IN MICE LACKING UCP1

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Mice lacking uncoupling protein 1 (UCP1) are extremely cold sensitive but can develop cold tolerance through long-term preacclimation to mild “cold” (18°C) exposure. The mechanism of heat production in such UCP1(-/-) mice is skeletal muscle-derived shivering thermogenesis [1]. However, other mechanisms based on non-shivering thermogenesis (inefficient use of energy) have been suggested for UCP1(-/-) mice [2]. Therefore, we decided to investigate in these mice uncoupling activities of the main candidates for possible alternative non-shivering thermogenesis: the ATP/ADP-antiporter (ANT) and UCP3.

Brown-fat mitochondria lacking UCP1 are still able to be uncoupled by fatty acids, although the sensitivity to fatty acids is more the 10 times lower than in UCP1(+/+) mitochondria [3]. The ANT may participate in fatty acid-induced uncoupling [4] and it could be envisaged that in mice lacking UCP1, ANT could take over thermogenic function of UCP1. Indeed, ANT concentration analysed by Western blot was increased in UCP1(-/-) brown-fat mitochondria as compared to UCP1(+/+). However, paradoxically, the effect of an inhibitor of ANT (carboxyatractyloside) on fatty acid-induced uncoupling was smaller in UCP1(-/-) mitochondria than in UCP1(+/+), indicating less ANT-mediated uncoupling in UCP1(-/-) mice despite a higher content of this protein.

Skeletal muscle has been proposed as being responsible for a non-brown adipose tissue derived non-shivering thermogenesis mediated by UCP3 [5]. Expression of UCP3 at protein level was 4-fold elevated in skeletal muscle mitochondria from cold-acclimated UCP1(-/-) mice compared to wild-type. However, there was no effect of GDP (a suggested UCP3 inhibitor) on basal respiration or on fatty acid-induced uncoupling. A relative small GDP effect on respiration under conditions with the highest production of superoxide (reverse electron transport) was independent of the level of UCP3 expression. Noticeably, the sensitivity to fatty acids (potential UCP3 activators) was significantly *lower* in skeletal muscle from cold-acclimated UCP1(-/-) mice (with a *higher* UCP3 content).

Thus, there was no evidence for a higher ATP/ADP-antiporter- or UCP3-mediated uncoupling activity in mitochondria from UCP1-ablated mice than in wild-type.

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P2.1.27. REGULATION OF UCP1 ACTIVITY BY FATTY ACIDS, FATTY ACID ANALOGUES AND NUCLEOTIDES

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Elucidation of the regulation of UCP1 activity in its native environment, i.e. in the inner membrane of brown-fat mitochondria, has been hampered by the presence of UCP1-independent, quantitatively unresolved effects of investigated regulators on the brown-fat mitochondria themselves. We have utilized the availability of UCP1-ablated mice to dissect UCP1-dependent and UCP1-independent effects of regulators. UCP1 can mediate a 4-fold increase in thermogenesis when GDP-inhibited mitochondria are stimulated with the classical positive regulator fatty acids (oleate). By identifying the UCP1-mediated fraction of the response, we could conclude that the interaction between purine nucleotides (GDP) and fatty acids (oleate) unexpectedly displayed simple competitive kinetics (1). – We have further examined the properties required of the fatty acids within this system. We found that the (re)activation ability of fatty acids is not qualitatively linked to such properties of the fatty acids as being metabolizable, being able to flip-flop over the membrane etc. The ability of fatty acids with different chain lengths, with different degrees of unsaturation, or with different structural modifications to function as (re)activators of UCP1 has been mapped; in general, the structural demands are broad.

In isolated brown-fat mitochondria, the presence of UCP1 is also associated with a high Cl- permeability. By utilizing UCP1-ablated mice to dissect also here UCP1-dependent and UCP1-independent effects, we have examined the kinetics of the Cl- permeability ascribable to UCP1 and its sensitivity to GDP and other nucleotides. We have also examined the interaction between fatty acids and nucleotides on this parameter.

We conclude that when examined in its native environment, UCP1 functions as a proton (-equivalent) carrier in the absence of exogenous or endogenous fatty acids.

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Native UCP1 displays simple competitive kinetics between the regulators purine nucleotides and fatty acids.

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P2.1.28. COLICIN E1 IONIC CHANNELS AND LIPID FLIP-FLOP

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An intimate involvement of lipid in the structure of peptide ionic channels has been suggested for a series of antibacterial peptides leading to formulation of a toroidal model. The validity of this model for the channel formed by colicin E1 was recently proposed from the sensitivity of the colicin-induced macroscopic current to spontaneous curvature of membrane lipids and the strong dependence of the single-channel conductance of colicin E1 channels on the anionic lipid content. One implication of the toroidal pore concept is that the channel formation would allow the movement of lipid molecules from one monolayer of the bilayer to the other. Addition of colicin E1 to liposomes containing the fluorescent phosphatidylcholine analog 1-lauroyl-2-(1'pyrenebutyroyl)-sn-glycero-3-phosphocholine (pyPC) exclusively in the external monolayer led to the transfer of pyPC to the internal monolayer in a dose-dependent manner, resulting in a nearly equal distribution of the fluorescent analog between the two membrane leaflets. Thus we demonstrated the ability of colicin E1 to induce lipid flip-flop. Large channels (600 pS) of colicin E1 recently observed, can also be discussed in terms of the toroidal model. From the effect on single-channel conductance of differently sized non-electrolytes, we estimated the size of small and large channels of colicin E1 to be 12 Å and 16 Å, respectively. The difference in the channel sizes evidences against an oligomeric structure of the large channel and thereby provides additional support for a toroidal nature of the colicin E1 channel. [RFBR 03-04-48905 (YNA); Fogarty TW01235, NIH GM-18457 (WAC)].

P2.1.29. DECREASED ANT CONTENT IN ZUCKER FATTY RATS: RELEVANCE FOR ALTERED HEPATIC MITOCHONDRIAL BIOENERGETICS IN STEATOSIS

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Mitochondria from Zucker fatty (ZF) rats (a model for fatty liver disease) showed a delay on the repolarization after a phosphorylative cycle and a decrease on state 3 respiration, suggesting alterations at the phosphorylative system level. The ATPase activity showed no differences between control and ZF rats, implying alterations on other components of the phosphorylative system. A pronounced depletion in the content of the adenine nucleotide translocator (ANT) was observed by western blotting, while no alterations were found on the mitochondrial voltage-dependent anion channel (VDAC) content. This data suggests that hepatic accumulation of fat impairs mitochondrial function, reflecting the loss of oxidative phosphorylation capacity caused by a decrease in the ANT content.

P2.1.30. EFFECTS OF STARVATION ON HEART MITOCHONDRIAL PROTON LEAK AND UNCOUPLING PROTEIN EXPRESSION IN CANARY BIRDS AND MICE

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In the wild, it is reasonable to assume that animals would be subject to occasional bouts of starvation (due to drought, inclement weather etc). It is expected that heightened ability to adapt to starvation can enhance an organism's chance of survival. In this experiment, we wanted to test the possibility of uncoupling proteins (UCPs) and proton leak playing a role in starvation adaptation. To this end, canary birds (*Serinus canarius*) and C57BL/6J mice were starved for 24 and 48h; heart mitochondria was isolated and analyzed bioenergetically. Western blotting was also performed to gauge UCPs content.

The results showed that in both species, endogenous proton leak increased significantly after starvation periods of 24 and 48 h. The endogenous proton leak of heart mitochondria increased 2.4 and 12.7 folds in canary birds after 24 and 48 h of starvation. In mice, the increase in endogenous proton leak after 24 and 48 h of starvation was 3.7 and 6.5 folds over control respectively. Among the various conditions (Control, 24 and 48 h of starvation), palmitic acid (PA) induced proton leak (mainly via UCP activation) increased across the board, but there was a statically significant increase only in canaries that were starved for 48 h. There was a 3.8 fold increase over the control. After 48 h of starvation, both endogenous and PA-induced proton leak in canary birds were about 1.6 times that of mice. GTP inhibition of PA-induced oxygen consumption (which is a measure of UCP activity) increased significantly after 48 h of starvation in both species; and were about 2 times higher in canary birds compared to mice under all of the three conditions (control, 24 and 48 h starvation). Also, western blotting showed that UCPs were up-regulated after 24 and 48 h of starvation in both species. Moreover, in all starvation conditions, canary birds were found to contain far higher amounts of UCPs vs mice.

In conclusion, this comparative study demonstrated a significant difference between mice and canary heart mitochondria. While upregulation of UCPs and increased proton leak were observed in both canary and mice upon starvation, the response of canary mitochondria was far more apparent. The consequence of increased mitochondrial coupling on metabolism and adaptive thermogenesis in canary birds, remain to be determined.

P2.1.31. SPECIES-SPECIFIC STIMULATION OF THE MITOCHONDRIAL PERMEABILITY TRANSITION BY NORBORMIDE AND ITS DERIVATIVES

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Norbormide [5-(α-hydroxy-α-2-pyridylbenzyl)-7-(α-2-pyridylbenzylidene)-5-norbornene-2,3-dicarboximide] (NRB) is a toxic compound endowed with a unique species-specific vasoconstrictor activity that is restricted to the peripheral arteries of the rat. It is a mixture of eight racemate diastereoisomers, which strongly differ in their vasoconstrictor activity[1]. Early studies demonstrated that NRB also causes rat-specific mitochondrial dysfunction, which was recently attributed to the opening of the inner membrane permeability transition pore (PTP)[2]. A question posed by these findings was whether the species-specific PTP modulation and the species-specific toxicity are causally linked, as suggested by the fact that both processes are specific to the rat. To address this question, in the present study we investigated the PTP-regulatory properties of both vasoconstrictor and non vasoconstrictor NRB isomers by following the Ca^{2+} retention capacity and matrix swelling of rat, mouse and guinea pig liver mitochondria. All NRB isomers tested exihibited similar, rat-specific effects on mitochondrial permeabilization, which indicates that: i) species-specifity of NRB action on the PTP is not connected to the molecular isomerism; ii) regulation of pore activity and vasoconstrictor activity are unrelated phenomena.

Our previous work[2] showed that NRB induced rat-specific changes in the fluidity of mitochondrial membranes, which in turn were likely to increase the PTP open probability through conformational changes of the internal Ca^{2+} regulatory site. Based on these observations, the mechanism of NRB action on the PTP was supposed to involve a mitochondrial transport system unique to the rat that allows internalization of the drug across the membranes. This putative carrier should be absent in other species, or shielded from contact with the drug due to different membrane structural arrangements[2]. In order to better understand the mechanism of NRB action, we carried out studies with cationic derivatives of the drug, which are accumulated inside the mitochondria *via* the transmembrane potential of the inner membrane. Cationic NRB stimulated the opening of the PTP

at much lower concentrations than the neutral molecule (2 μM as compared to 35 μM in rat mitochondria) and was effective also on mouse and guinea pig mitochondria. The loss of species-specificity on the PTP opening by the NRB cationic derivative is consistent with our previous hypothesis that the neutral drug is taken up by rat mitochondria via a selective carrier.

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Poster session 2.2. ROS and redox regulations

P2.2.1. ON THE MECHANISM BY WHICH OPENING THE MITOCHONDRIAL ATP-DEPENDENT K^+ CHANNEL INCREASES MITOCHONDRIAL PRODUCTION OF REACTIVE OXYGEN SPECIES

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Opening the mitochondrial ATP-sensitive K^+ channel (mitoK_{ATP}) increases reactive oxygen species (ROS) levels in cardiomyocytes; however the mechanism by which mitoK_{ATP} opening leads to increased ROS levels has not been established. We examined ROS production in suspensions of isolated rat heart and liver mitochondria, using fluorescent probes that are sensitive to hydrogen peroxide. When mitochondria were treated with agents that cause opening of mitoK_{ATP}, including diazoxide and cromakalim, protein kinase G plus cyclic GMP, or 4-phorbol-12∃-myristate 13-acetate (PMA) we observed increase production of H_2O_2. This increase in ROS was inhibited by the mitoK_{ATP} blocker 5-hydroxydecanoate. Valinomycin (1 pmol/mg mitochondrial protein), a K^+ ionophore, caused an equivalent increase in K^+ inflow and an equivalent increase in ROS production, thus mimicking the effect of mitoK_{ATP} opening. Using a matrix-loaded fluorescent pH indicator we confirmed the predicted increase in matrix pH upon addition of cromakalim, PMA, or valinomycin. Equivalent matrix swelling (by suspending mitochondria in hypo-osmotic media) did not affect ROS production, whereas alkalinization of the medium increased ROS production. We therefore conclude that mitoK_{ATP}-mediated signaling in respiring mitochondria is due to an increase in ROS production, caused by raised matrix pH as K^+ ions replace expelled protons.

P2.2.2. STUDY OF THE ASSEMBLY OF THE BOVINE NADPH OXIDASE

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Neutrophils play an essential role in the body's innate defence against pathogens and are one of the primary mediators of the inflammatory response. To defend the host, neutrophils use a wide range of microbicidal products, such as oxidants, microbicidal peptides and lytic enzymes. The generation of microbicidal oxidants results from the activation of a multiprotein enzyme complex known as the NADPH oxidase, which catalyses the formation of superoxide anion $O_2^{\bullet -}$. The importance of the phagocyte NADPH oxidase in the immunity system is clearly demonstrated by a rare genetic disorder known as chronic granulomatous disease (CGD). The aim of our research is to uncover the key structural and functional features of the protein components that control the enzyme activation and inhibition in the cell. We are studying the physico-chemical properties of the various complexes formed during the activation process.

In the resting neutrophiles, four of the enzyme subunits are dispersed in the cytosol, the catalytic core of the NADPH oxidase, namely the flavocytochrome b558 is localised in the granules membrane. In response to stimulus, the inactive NADPH oxidase is transformed in an active enzyme by translocation of the cytosolic subunits onto the membrane and by the assembly of several of them (p67phox, p47phox et rac2) with the membrane core (gp91phox and p22phox) that contains all the redox components (NADPH, FAD, hemes) involved in the electron transfer pathway that bring to $O_2^{\bullet -}$ formation. This assembly induce electron and proton transports through the flavocytochrome, resulting in superoxide anion production. We will present our study on the assembly of the NADPH oxidase complex performed in a cell free system. We will show our results obtained with the different recombinant cytosolic sub-units and the native flavocytochrome and the effect of free radicals on these proteins.

P2.2.3. PHOSPHOLIPID-MEDIATED SCAVENGING OF PRO-APOPTOTIC CYT C BY ALPHA SYNUCLEIN: ROLE IN NEUROPROTECTION

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Cytochrome c (cyt c) is essential for uninterrupted energy metabolism in cells. Its release from mitochondria into the cytosol leading to the apoptosome formation and caspase activation is a "point-of-no-return" in the cell death program. We sought to determine if there are candidate cytoplasmic proteins that would bind cyt c released from mitochondria and prevent its interaction with Apaf-1 leading to caspase activation. Here, we provide experimental evidence that alpha-synuclein (Syn), one of the abundant proteins in presynaptic terminals, acts as a sacrificial scavenger of cyt c, released from damaged mitochondria. This anti-apoptotic function of Syn is mediated by anionic phospholipids facilitating binding of cyt c and resulting in triple complex Syn/Lipid/cyt c. In mitochondria, cyt c avidly binds to available cardiolipin (CL), a mitochondria-specific phospholipid to yield a complex with a peroxidase activity. During apoptosis, CL migrates from the inner to the outer membrane resulting in its significant presence on the surface of mitochondria. This favors availability of CL for the cytosolic Syn and the formation of a triple complex Syn/CL/cyt c. Using CL as a prototypical anionic phospholipid, we showed that the triple complex displays a potent peroxidase activity that induces covalent oligomerization of its components. By applying immuno-spin trapping, we demonstrated generation of tyrosyl radicals by the peroxidase activity of the complex. Typical phenolic peroxidase substrates – Amplex Red, DOPA – inhibited the protein aggregation. Syn acted as a competitive inhibitor of the cyt c/CL peroxidase activity. The peroxidase activity triggered by tert-butyl hydroperoxide caused accumulation of intensely stained round-shaped alpha-synuclein/cyt c accumulations in Syn-rich HeLa cells. In brain samples from Parkinson's disease (PD) patients, the co-localization of Syn and cyt c was detectable in Lewy bodies, a neuropathological hallmark of PD. We conclude that cyt c scavenging anti-apoptotic function of Syn is essential to protect death of neurons triggered by fortuitous damage to mitochondria in pre-synaptic terminals. However, damage of mitochondria causes production of H2O2 that feeds the peroxidase activity of the triple complex. Thus, anti-

apoptotic lipid-dependent binding of cyt c by Syn creates a “pro-oxidant monster” that triggers and propagates protein aggregation.

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P2.2.4. THE ROLE AND MECHANISMS OF MITOCHONDRIAL DYSFUNCTION IN NECROSIS AND APOPTOSIS INDUCED BY CADMIUM (II) IN AS-30D RAT ASCITES HEPATOMA CELLS

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Mitochondria are target organelles for heavy metals; however, mechanism(s) underlying heavy metal-induced mitochondrial dysfunction and cytotoxicity are not fully elucidated. Using AS-30D rat ascites hepatoma cells, we studied modulating action of various antioxidants, inhibitors of mitochondrial permeability transition (MPT) pore and respiratory chain on Cd^{2+}-induced mitochondrial dysfunction and tested their possible preventive effect on cell death produced by this heavy metal.

In AS-30D hepatoma cells Cd^{2+} induced both necrosis and apoptosis in a time- and dose-dependent fashion as revealed by trypan blue and propidium iodide assays, respectively. We found that MPT pore inhibitors: cyclosporin A and bongkrekate, and complex III inhibitors: stigmatellin and antimycin A substantially prevented necrosis observed after 3h exposure of the cells to Cd^{2+}. However, another complex III inhibitor myxothiazol and complex I inhibitor rotenone had no such effects. After 24h exposure to Cd^{2+} and the effectors, only stigmatellin and antimycin A partially inhibited Cd^{2+} necrosis, whereas cyclosporin A and bongkrekate were not effective; moreover, rotenone and thenoyltrifluoroacetone enhanced the necrosis. Notably, the MPT pore and respiratory chain inhibitors prevented neither Cd^{2+}-induced apoptosis revealed after 24h exposure to the metal nor its harmful effect on the cell cycle. In addition, mannitol and the antioxidant TEMPO partially inhibited the apoptotic but not the necrotic action of Cd^{2+}, while 2h pre-treatment N-acetylcysteine was effective both against Cd^{2+}-induced apoptosis and necrosis.

To elucidate molecular mechanism(s) of these phenomena, fluorescent probes for $\Delta\Psi_{mito}$ and ROS (JC-1 and DCFH-DA, respectively) and flow cytometry were applied. A dose-dependent increase of intracellular ROS generation observed in the presence of Cd^{2+} both after incubation of the cells in phosphate-buffered saline for 50 min and in RPMI medium for 1 and 3h was inhibited by protonophore CCCP as well as by stigmatellin, antimycin A and cyclosporin A; in the former case – completely, in the latter case – partially. Besides, $\Delta\Psi_{mito}$ dissipation followed the increase of intracellular ROS production. This indicates that the ROS production increase was directly connected with mitochondrial respiratory complex III and MPT pore opening was likely involved in

this process. This correlates well with our observations on isolated rat liver mitochondria in which low concentrations of Cd^{2+} evoked a high-amplitude swelling in isotonic sucrose medium, the swelling that was sensitive to the combined action of cyclosporin A, ADP, Mg and dithiothreitol. We also found that the swelling of the mitochondria respiring on ascorbate + TMPD (complex IV substrates) was weakly inhibited by bongkrekate, more strongly by rotenone and was completely eliminated by stigmatellin or antimycin A.

Thus, mitochondrial dysfunction, thiol status and ROS generation are the key events in Cd^{2+}-induced injury of AS-30D hepatoma cells.

P2.2.5. DOES THE FATTY ACID COMPOSITION AFFECT METABOLIC FUNCTIONS AND OXIDATIVE STRESS STATUS IN RAT HEART MITOCHONDRIA?

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In vertebrates' heart, the degree of unsaturation of cellular membranes has been negatively correlated with maximum life span[1]. A low degree of unsaturation of biomembranes may confer an advantage by decreasing their sensitivity to lipid peroxidation and by preventing lipoxidation-derived damage to other macromolecules such as proteins[1]. Our study aimed to investigate the effect of fatty acid composition of mitochondrial membranes on the mitochondrial functions and oxidative stress status in the heart. Four groups of rats were fed a controlled ration of diets differing in the nature of fat and antioxidant added; (1) coconut oil (CO), (2) olive oil (OO), (3) fish oil (FO), and (4) fish oil and probucol (FOP). After four months of feeding, the major differences observed in the fatty acid composition of heart mitochondria were: 1) a higher content in C18:2 in CO and OO groups compared to FO and FOP groups, and 2) a lower LC-PUFAs (C20:5n3, C22:5n3 and C22:6n3) content in the CO and OO groups compared to both fish oil groups. These important differences in fatty acid composition did not cause any differences in the mitochondrial respiration rate of complex I (malate and pyruvate), complex II (succinate) and complex IV (ascorbate and TMPD) as well as in the isocitrate dehydrogenase and citrate synthase activities. The activity of Lon protease, which is down regulated by oxidative stress, was higher in CO (2.84 ± 0.12) and FO (2.76 ± 0.11) groups than in the OO (2.20 ± 0.10) and FOP (1.85 ± 0.05) groups. Furthermore, the protein carbonyl groups, which were formed by the action of reactive oxygen species on proteins, showed lower content in the group fed with CO diet compared to all other groups. Our results suggest an advantage of cononut oil (rich in saturated fat) in the diet for protection of mitochondrial components against oxidative stress.

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P2.2.6. S-NITROSOTHIOLS INDUCE INHIBITION OF COMPLEX I AND ROS PRODUCTION BY MITOCHONDRIA

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Nitric oxide (NO) is a physiological messenger in the body but it can also be toxic to cells. Among other mechanisms, NO toxicity has been attributed to direct effects on mitochondria causing inhibition of mitochondrial respiration due to inhibition of cytochrome oxidase and production of reactive oxygen (ROS) or reactive nitrogen species (RNS). Although elevated mitochondrial ROS production is thought to be involved in pathology of oxidative damage as well as in cell signaling, the mechanisms by which mitochondrial ROS production is regulated are unclear. We were interested whether RNS can induce S-nitrosation and inhibition of complex I of the mitochondrial respiratory chain and whether such inhibition can cause production of ROS in isolated rat heart and liver mitochondria. We found that reversible inactivation of mitochondrial complex I by S-nitroso-N-acetyl-D,L-penicillamine (SNAP) in isolated rat heart mitochondria resulted in a three-fold increase in H_2O_2 production, when mitochondria were respiring on pyruvate and malate, (but not when respiring on succinate or in the absence of added respiratory substrate). The inactivation of complex I and the increased H_2O_2 production were present in mitochondria washed free of SNAP or NO, but were partially reversed by light or dithiothreitol, treatments known to reverse S-nitrosation. Specific inhibition of complex I with rotenone increased H_2O_2 production to a similar extent as that caused by SNAP. The results suggest that S-nitrosation of complex I can reversibly increase oxidant production by mitochondria, which is potentially important in cell signalling and/or pathology.

P2.2.7. OXYGEN DELIVERY TO THE TISSUES AND MITOCHONDRIAL RESPIRATION

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In mammals, the O_2 transport from the inspired air to the tissues is made by convective and diffusive mechanisms. The convective mechanisms are provided by the cardio-respiratory system and comprised by the basic variables of cardiac output and of blood O_2 content. Microcirculation in arterioles and capillaries is adjusted to match the O_2 demand of local tissues. Endothelium-generated NO diffuses to the smooth muscle of microvessels and produces vasodilation that increases circulatory time in the capillaries and allows a more effective O_2 extraction in the tissues. Once within the tissue, O_2 diffuses to mitochondria where it is reduced in an exergonic process coupled to ATP synthesis. Both, O_2 and ATP are the two most homeostatic intracellular species. In heart and muscle, both species show unchanged levels with 25-100 times increases in work load and ATP turnover rate. The linear rates of O_2 uptake shown by tissue slices and perfused organs are interpreted as a fast switching of mitochondria between metabolic state 3 (with a fast rate of O_2 uptake and ATP synthesis) and state 4 (with a slow rate of O_2 uptake and no ADP phosphorylation). Endogenous mitochondrial NO, produced by mtNOS, sustains the concept of a physiological functional activity of this enzyme in regulating mitochondrial and cellular O_2 uptake.

P2.2.8. INVOLVEMENT OF REACTIVE OXYGEN SPECIES AND RAS/ CAMP PATWAY IN THE REGULATION OF MITOCHONDRIAL BIOGENESIS

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During growth of the eukaryotic cell, mitochondrial activity must meet energy demand. More specifically, it has been shown in yeast that the respiratory capacity and the mitochondrial enzyme content simultaneously decrease when the growth rate slows down in order to maintain a constant growth yield (1). Previous work from our laboratory has shown that, upon aerobic growth, the activity of the Ras/ cAMP pathway is involved in such a process (2-3). More specifically, the yeast Tpk3p (one of the three cAMP protein kinase) plays a major role in the maintenance of the optimal conditions of this adjustment. TPK3- cells present important modifications in the mitochondrial enzymatic equipment (decrease of respiratory rates and cytochrome content) when energy demand decrease. Lack of Tpk3p lead to an increase in reactive oxygen species production and an oxidant stress as shown by the increase in catalase and superoxide dismutases activities in TPK3- cells. This induces a growth delay for TPK3- cells. Recently, we have shown that addition of an antioxidant (N-acetyl cystein) in the growth medium restores the mutant growth rate which is then comparable to the wild type. This is associated with a full restoration of distinct mitochondrial features. Moreover the activity of transcription factors involved in mitochondrial biogenesis is decreased in TPK3- cells and restored by antioxidant addition, thus indicating that mitochondria to nucleus signalling might go through reactive oxygen species. This study points out a crucial role of reactive oxygen species generated in a tpk3p deficient strain for the modulation of mitochondria content in response to cellular energy demand.

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P2.2.9. CONFOCAL MICROSCOPY APPLICATION IN MONITORING OF MITOCHONDRIAL REACTIVE OXYGEN SPECIES PRODUCTION WITHIN SELECTED CELL CULTURES

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Using fluorescent probe MitoSOX, as a selective mitochondrial superoxide indicator, we quantified mitochondrial contribution to superoxide production in HEPG2 and HeLa cells. We have confirmed mitochondrial localization of MitoSOX by an overlays with a Mitotracker Red CMXRos and TMRE, respectively. The rate of superoxide production was increased by rotenone due to retardation of electron transport and reduced by a chemical uncoupler FCCP. Specifically, image analyses have shown that addition of 20 μM rotenone lead up to 30 fold increase in superoxide production compared to the basal rate and was suppressed when cells were incubated with 1 μM FCCP prior to addition of rotenone.

Correlation of changes in mitochondrial ROS production with their redox status was performed in cells constitutively expressing mitochondrially addressed green fluorescent protein sensitive to local redox potential changes (Rossignol *et al*. 2004)*. With this system we detected 9.6% decrease in fluorescence after treatment with 1 μM FCCP. Susbsequently, addition of 20 μM rotenone resulted in an immediate 9% increase in fluorescence, which gradually restored the basal level of fluorescence.

Finally, we compared the measurements with HEPG2 *in situ* with assays of isolated liver mitochondria, in which we observed 35% increase in hydrogen peroxide generation after rotenone treatment and its suppression by FCCP.

In conclusion, our data indicate that retardation of mitochondrial electron transport leads to an increase in superoxide generation in HEPG2 and HeLa cells and that uncoupling can suppress this effect.

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P2.2.10. TIME COURSE OF INFLAMMATION MARKERS AND MITOCHONDRIAL FUNCTION IN LIVER FROM RATS SUBJECTED TO ENDOTOXIC SHOCK

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Endotoxic shock is a condition in which the cardiovascular system fails to perfuse tissues adequately and cells fail to extract oxygen from blood. In each cell the mitochondria are responsible for oxygen extraction from the blood. Therefore, it is an important problem to understand the mechanisms modulating mitochondrial function under endotoxic shock. To this aim we have investigated the kinetics of parameters of respiratory function of mitochondria. In parallel the expression of genes involved in mitochondria derived apoptotic pathways and genes known to exhibit a protective effect against endotoxin mediated tissue damage, as well as expression markers for endotoxaemic conditions were included. Adult male Sprague-Dawley rats weighing 280±21 g were injected with lipopolysaccharide (LPS) at a dose of 8 mg/kg (i.v.). At different time points (0h, 2h, 4h, 8h and 12h (n=6)) the animals were sacrificed and liver tissue and blood were taken for analytical examination. Gene expression was investigated by real-time PCR using SYBR®-green in tissue samples. The mitochondrial function was determined directly in liver homogenates by monitoring the oxygen consumption in presence of different substrates and ADP. Additionally activity of haem oxygenase was determined in liver homogenates.

Expression levels of iNOS and TNF-α were already significantly increased at 2h post LPS challenge, with peak levels at 4h. Increased expression of HO-1 was detected only after 4h and declined thereafter. The increase of HO-1 expression was correlated inversely with the respiration of mitochondria in state 3. It was increased at 2 hours, dropped down to normal levels at 4 and 8 hours and again increased at 12 hours. In order to elucidate the possible involvement of mitochondrial damage in the up regulation of HO-1 we have further analysed the expression of relevant markers for apoptotic pathways targeting mitochondria. In conclusion our data suggest that HO-1 under endotoxic shock conditions may unfold a protective effect by modulating mitochondrial function and apoptotic pathways targeting mitochondria.

P2.2.11. OXIDATIVE STRESS ENZYMES AND MITOCHONDRIAL BIOENERGETICS IN WILD LIZA SALIENS EXPOSED TO HEAVY METALS

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The Esmoriz-Paramos coastal lagoon represents an ecosystem of great physical and ecological significance. However, as a result of industrial, agricultural and anthropogenic activities this habitat has been progressively degrading over the last decades. The heavy metal contamination is an important factor to the decline of sediments quality and may adversely affect fish health. In the present work the leaping grey mullet Liza saliens was studied because it is the dominant endemic species in the lagoon. It is a filter feeder and also being a detritus-mud feeder, it is therefore exposed to contaminated sediments.

Previous work has shown that the mean sediment metal concentrations were 234 mg Zn/Kg d.w. and 84 mg Cu/Kg d.w. Concentrations of zinc and copper were also determined in the gill, muscle and liver of Liza saliens, older than 6 years. The highest metal concentrations were observed in the liver (254 mg Cu kg-1) and gill (114 mg Zn kg-1). A positive correlation between copper content in liver and fish age was found, suggesting the loss of copper homeostatic capacity and its bioaccumulation.

In this study, the variations of mitochondrial bioenergetics (O2 consumption), physiological parameters (condition factor and liver somatic index), antioxidant defences (superoxide dismutase and catalase), total protein and lipid peroxidation, were evaluated in mullet liver. The purpose of this work was to assess fish responses to chronical metal exposure using accepted biomarkers and others such as mitochondrial bioenergetics.

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P2.2.12. SKELETAL MUSCLE MITOCHONDRIAL FUNCTION AND ROS PRODUCTION IN RESPONSE TO EXTREME ENDURANCE EXERCISE IN ATHLETES

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Although it is well known that endurance exercise induces oxidative stress (1) there is no evidence of deteriorated mitochondrial function after 1-2 hours intensive exercise (2). However, the effects of extreme endurance exercise on mitochondrial function and mitochondrial ROS production have not been investigated previously.

Nine healthy well-trained men (age 27.1 ± 0.87 (mean ± SE), BMI 24.2 ± 0.64 and VO_2 peak 62.5 ± 1.78 ml/kg. min) performed 24 hours exercise, consisting of equal parts running, cycling and paddling. Muscle biopsies were taken from vastus lateralis pre-exercise (PreEx), immediately post-exercise (PostEx) and after 28 hours of recovery (PostEx-28). Mitochondria were isolated and mitochondrial respiration was analyzed with palmitoyl-carnitine (PC) and pyruvate (Pyr). Mitochondrial H_2O_2 release was measured with the Amplex Red-horseradish peroxide method. The reaction was initiated by addition of succinate with following addition of antimycin A (reversed electron flow).

UCP3 protein expression, evaluated with western blot technique, was not changed by exercise. Both state 3 (Pyr and PC) and state 4 (PC) rates of oxygen consumption (estimated per maximal ETC-activity) were increased PostEx (+29%, +11% and +18%). State 3 remained elevated PostEx-28, whereas state 4 (Pyr) decreased below that at PreEx (-18%). Mitochondrial efficiency (P/O) decreased PostEx (Pyr -8.9%, PC -6.1%) and remained reduced PostEx-28. The relative substrate oxidation (state 3 PC/Pyr) increased after exercise PreEx: (0.71 ± 0.06) vs. PostEx (0.90 ±0.04) and (0.77 ±0.06) PostEx-28. Mitochondrial H_2O_2 release (succinate) increased dramatically after exercise (+189 ± 64%). Treatment with Antimycin A resulted in a twofold-increased rate of mitochondrial H_2O_2 release PreEx but a decreased rate in PostEx samples. The exercise-induced changes in mitochondrial ROS production was totally abolished PostEx-28.

In conclusion extreme endurance exercise decreases mitochondrial efficiency and increases mitochondrial ROS production. Both of these changes would increase the oxygen demand during

exercise. Relative fatty acid oxidation as measured in isolated mitochondria increased after exercise indicating that the capacity to oxidize fat is improved during prolonged exercise.

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P2.2.13. SEX-DEPENDENT CONTROL OF OXIDATIVE HOMEOSTASIS OF RAT BRAIN MITOCHONDRIA

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Current studies have shown the gender influence on the generated oxidative stress level, highlighting that females could have less reactive oxygen species (ROS) production and more antioxidant capacity than males. This could be related with longer female life span in several species. Oxidative stress has been postulated to be one of the major factors of cellular aging and to be responsible for many age-related neurodegenerative pathologies. The aim of this work was to analyse whether there was any difference between males and females on antioxidant enzymatic mitochondrial systems and on the ROS production from brain mitochondria of 18 months old rats. To this end, 12 Wistar rats, 6 males and 6 females were analysed. Antioxidant enzyme activities, oxygen consumption and ROS production were studied in several conditions: in the presence of succinate and pyruvate + malate-like substrates and GDP-like inhibitor of mitochondrial chain uncoupling. There was similar oxygen consumption in state 3 and in state 4 of mitochondrial respiration in both genders. As well, lower activities of antioxidant enzymes such as superoxide dismutase (SOD), glutation peroxidase (GPx) and glutation reductase (GRd) were observed in females in comparison with males. In spite of all this, ROS production with succinate and pyruvate + malate-like substrates was higher in male mitochondria than in female ones. However, the ROS production increased in the presence of GDP up to the same level in both genders. In conclusion, under these experimental conditions, females produced less ROS than males although they had the same oxygen consumption. This observation could not be explained by antioxidant enzymatic action since females showed lower activity. Therefore, this could be due to a greater uncoupling in female rat brain mitochondria, since the ROS production rose up to the same level in both genders when uncoupling was inhibited. Thus, the greater respiratory mitochondrial chain uncoupling in females may avoid more efficiently the ROS production and perhaps contribute to the lesser impact of several neurodegenerative pathologies in females.

P2.2.14. ROLE OF HYPOXIA INDUCIBLE FACTOR AND REACTIVE OXYGEN SPECIES IN OXIDATIVE PHOSPHORYLATION DOWN-REGULATION OF RENAL CANCER CELLS

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Many types of cancer and, in particular, clear cell renal carcinoma (CCRC) exhibit a low rate of mitochondrial ATP synthesis partly compensated by an increase in glycolytic flux. The highest decrease in oxidative phosphorylation (OXPHOS) is correlated with the most aggressive types of renal cancers [1]. The increase in glycolytic flux is explained by stabilization of Hypoxia Inducible Factor (HIF) that induces the transcription of genes coding for enzymes involved in the glycolytic pathway. HIF alpha subunits are expressed in CCRC because they are devoid of pVHL (von Hippel Lindau protein). Indeed, in the presence of oxygen, prolyl hydroxylases bind OH to HIF prolines 402 and 564. HIF is then ubiquitinated by the pVHL complex and HIF is degraded by the proteasome. While transfection of CCRC with *vhl* restored HIF degradation and increased OXPHOS, no change in the level of transcripts encoding OXPHOS subunits has been observed in these cancer cells after transfection with ectopic *vhl* [2]. Inhibition of HIF alpha subunit synthesis by RNA interference in VHL-deficient CCRC also increased OXPHOS. On the contrary, stabilization of HIF-α subunit by $CoCl_2$, an inhibitor of prolyl hydroxylases, reduced OXPHOS in spite of the presence of pVHL in CCRC cells transfected with *vhl*. However, $CoCl_2$ also prevented the maturation of cytochrome c oxidase subunit 4 precursor (pre-COX4), very likely by inhibiting the mitochondrial intermediate peptidase cleaving an octapeptide in the pre-sequence [3]. Indeed, a pre-COX4 with a size increased by about 1 kDa as compared to the mature COX4 could be detected in the presence of $CoCl_2$. This effect could interfere with the presumed role of HIF in OXPHOS biogenesis. Surprisingly, deferoxamine, that also stabilizes HIF by preventing its hydroxylation, modified neither OXPHOS subunit contents nor COX4 precursor maturation. However, if $CoCl_2$ and deferoxamine both increase HIF content, $CoCl_2$ stimulates ROS production while deferoxamine decreases it by preventing Fenton reactions that are iron-dependent. OXPHOS subunit synthesis was also decreased by H_2O_2 treatment. It is therefore concluded that HIF-related changes combined

with ROS production should be responsible for the decrease in OXPHOS subunit contents observed in cancer cells such as CCRC.

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P2.2.15. INCREASED OXIDATIVE STATUS IN RAS2VAL19 AFFECTS MITOCHONDRIAL ADENINE NUCLEOTIDE TRANSLOCATOR FUNCTION VIA ROS SIGNALING IN S. CEREVISIAE

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Oncogenic mutation in Ras2p, $RAS2^{Val19}$, experiences elevated chronic intracellular oxidative stress caused by downregulation of majority of antioxidant defence enzymes (through cAMP/protein kinase A dependent signaling) and extensive mitochondrial non-phosphorylating respiration (cAMP/protein kinase A independent phenotype)[A]. Reactive oxygen species (ROS), as a signaling mediator, were found to be involved in cellular processes connected to cancer (invasiveness and proliferation) on one hand and to antineoplastic regulation (apoptosis and cellular senescence) on the other hand in variety of organisms. Here we show that activity of mitochondrial adenine nucleotide translocator (AAC) is greatly cut down in cells carrying $RAS2^{Val19}$ mutation and such regulation might be induced by a ROS signaling molecule (such as hydrogen peroxide), which will prompt proteases to specific cleavage of AAC protein. Reduced AAC activity is not linked to ATPsynthase failure in any other way than through its ROS contribution to the considerable oxidative stress within the cell. Thus, low activity of AAC is rather caused by increased intracellular oxidative stress in $RAS2^{Val19}$ as seen from suppression by overexpression of superoxide dismutase, SOD1, in $RAS2^{Val19}$. This type of protein modification is unique.We discuss its possible physiological role.

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P2.2.16. THE ROLE OF PLASTOQUINONE POOL IN PRODUCTION AND SCAVENGING OF REACTIVE OXYGEN SPECIES IN CHLOROPLAST THYLAKOIDS

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To estimate the contribution of oxygen reduction in PQ-pool to overall oxygen reduction in photosynthetic electron transport chain (PETC) the rates of oxygen uptake in isolated pea thylakoids in the absence of any additions and in the presence of DCMU together with donor pair (ascorbate + TMPD) were compared. It was found that a contribution of segments of PETC besides Photosystem I to oxygen reduction increased with increasing in light intensity, and at high intensities achieved 50 % at pH 5.0, and was higher than 60 % at pH 6.5 and pH 7.8. The data are explained as result of O_2 reduction in PQ-pool as well as in Photosystem I followed by reduction of superoxide radicals generated in both processes, by plastohydroquinone in thermodynamically advantageous reaction. Such collaborative participation of PQ-pool and PSI in oxygen reduction we named 'co-operative oxygen reduction'. According to proposed scheme, superoxide and even more dangerous its protonated form, HO_2, can be suppressed. Thus, PQ-pool may be considered as one of elements of an intramembrane scavenging system, where plastohydroquinone, operating as a trap of superoxides, produces H_2O_2. To evaluate the place of H_2O_2 production in thylakoids this production was measured in the presence of Cyt *C*, superoxide trap not penetrating into a membrane, to prevent superoxide dismutation outside the thylakoids. The flow of electrons participating in H_2O_2 production in the presence of Cyt *C* was calculated as the share in a total electron flow along PETC. At saturating light intensities, this share reached 60 %. The share of electrons participating in possible production of H_2O_2 in thylakoid lumen, in a total electron flow was found to be in average 9 %. There are many evidences of the dependence of adaptation reaction start-up in plant cells on the redox-state of PQ- pool. Possibly, just H_2O_2 produced with participation of plastoquinone pool components can play the role of a signal. The opportunity of H_2O_2 generation inside the membrane may have the fundamental meaning since the molecules produced there can leave the thylakoids for stroma in any point of a membrane, while the membrane H_2O_2 scavenging system is concentrated in the vicinity of PSI. Moreover, such H_2O_2 molecules can leave for the lumen and then for cytoplasm, bypassing also the stromal scavenging system.

P2.2.17. PHOTODYNAMIC TREATMENT WITH MITOCHONDRIAL PHOTOSENSITIZER INDUCES OXIDATIVE STRESS AND CELL DEATH IN HELA CELLS

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It is well known that mitochondria play a key role in development of oxidative stress and apoptosis. The effects of mitochondrial ROS were studied using Mitotracker Red (MR) as a photosensitizer, which was selectively accumulated in mitochondria due to membrane potential and produce reactive oxygen species (ROS) upon photoactivation. We have shown that photoactivation of MR caused production ofROS, detected with fluorescent dye 5-(and-6)-chloromethyl-2′, 7′-dichlorodihydrofluorescein diacetate (CM-DCFH-DA). Immediately after illumination fluorescence of CM-DCF was revealed in mitochondria. After 5-10 min incubation in the dark, fluorescence became higher and spread homogenously in the cells. The same effect was observed after local illumination of a part of the cell: immediately after illumination fluorescence of CM-DCF was detected only in mitochondria of illuuminated part and during incubation in the dark spread all over the cell. The rate of ROS production was stimulated with inhibitors of complex I (rotenone and piericidin) and complex III (myxothiazol) of respiratory chain. This enhanced ROS production was fully prevented with diphenyleneiodonium (inhibitor of flavin-containing enzyme) and mitochondria-targeted antioxidant mitoQ. Mild illumination of MR-loaded cells caused cytochrome *c* release from mitochodria into cytosol and apoptosis after 24h incubation in the dark. Apoptosis but not the release of cytochrome *c* was fully prevented with inhibitor of caspases zVADfmk. Overexpression of antiapoptotic protein Bcl-2 prevented the both cytochrome *c* release and cell death. Increase in the fluence of illumination dramatically changed the features of cells death and led to necrosis during 3-5h after illumination. Preincubation with zVADfmk and overexpression of Bcl-2 had no protective effect in this case. MitoQ protected against necrosis caused by strong illumination and revealed some features of apoptosis. These data suggest that MitoQ suppress the massive intramitochondrial ROS production after strong illumination. MitoQ (or other mitochondria-targeted antioxidants) could be suggested as a promising co-treatment in photodynamic therapy. They do not interfere with apoptotic killing of the target cells but prevent necrosis, which is followed by inflammation of the surrounding regions of the tissue.

P2.2.18. ESCHERICHIA COLI YTFE, A NOVEL DI-IRON PROTEIN INVOLVED IN IRON-SULPHUR BIOGENESIS

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Phagocytes (*e.g.* macrophages) employ diverse antimicrobial mechanisms to destroy invading pathogens, including the production of nitric oxide (NO), as judged by the release of high fluxes of NO and reactive nitrogen species by eukaryotic macrophages upon infection and by the decreased resistance observed in eukaryotes in which the generation of NO was blocked. To understand how pathogens respond to nitrosative stress we have analysed the global gene transcription profile of *Escherichia coli* exposed to nitric oxide while growing anaerobically, a favourable condition for pathogen colonisation. It is shown that NO modifies the transcription of genes encoding for proteins involved in a wide range of cell functions, being *E. coli ytfE* one of the highest induced genes. We present results showing that *E. coli ytfE* mutant strain has increased sensitivity to nitric oxide and iron starvation, and that it grows poorly under several anaerobic respiratory conditions. We also found that, the enzymatic activities of all examined iron-sulphur proteins are decreased in the *ytfE* mutant. These results led us to propose that YtfE is involved in the iron-sulphur cluster biogenesis. By producing the recombinant YtfE we could conclude that the protein contains a di-iron centre of the histidine-carboxylate family.

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P2.2.19. THE DEPENDENCE OF H_2O_2 FORMATION IN MITOCHONDRIA ON SUCCINATE DEHYDROGENASE ACTIVITY

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Heart mitochondria (MCH) in the presence of succinate and antimycin A serve as the convenient model to study generation of reactive oxygen species (ROS) [1].

We have shown in this model that changes in SDH activity influence considerably on the intensity of H_2O_2 formation in MCH. Experiments were performed in heart and brain MCH of Wistar rat males. Respiration was measured polarographycally and H_2O_2 formation by chemiluminescence of luminol with peroxidase. Brain MCH were incubated at higher temperature (37 ° C) than heart (26° C) in order to increase H_2O_2 formation, which is diminished in brain because of more effective antioxidant system in contrast to heart MCH. Under such conditions the rate of H_2O_2 formation in heart MCH was still higher than in brain, while respiration was higher in brain.

The addition of malonate, inhibitor of SDH together with antimycin led to a considerable rise in the rate of H_2O_2 formation in both heart and brain MCH. The effect of glutamate on SDH is opposite to malonate. Glutamate abolishes oxalacetate inhibition of SDH. Addition of glutamate stimulated respiration in MCH of both tissues and led to a decrease in H_2O_2 formation.

SDH inhibition by oxalacetate occurs during storage of MCH in ice, aging. We have observed increase in H_2O_2 formation in aged heart MCH.

H_2O_2 formation in MCH competes with coupled respiration for oxygen. Active respiration decreases pO_2 in MCH and therefore diminishes superoxide-H_2O_2 formation, while inhibition of oxidation, particularly of the most intensive, succinate oxidation, results in a rise of pO_2 and ROS formation. The essential role of SUC oxidation is also due to that only this maintains high level of Q reduction that prevents appearance of semiquinon directly supporting superoxide formation [2].

Decrease in SDH activity is the general property of pathological tissues, modeled by experimental stress [3]. It is also inherent to aging of the organism. Our data shows that SDH inhibition may initiate oxidative stress. In contrast, support of SDH activity may provide increase in resistance of MCH to pathogenous influences. It is probably the mechanism of antioxidant effect of SUC *in vivo* and its therapeutic [4] and geroprotective action [5].

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P2.2.20. QUANTIFICATION OF MITOCHONDRIAL GENERATION OF REACTIVE OXYGEN SPECIES IN BRAIN TISSUE

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Reactive oxygen species (ROS) are implicated in pathogenesis of several neurological diseases. But the exact sites of ROS generation in brain mitochondria remains unclear [1,2] and even the involvement of mitochondria in cellular oxidative stress is still questioned [3]. In our work we tested if mitochondria are the main source of reactive oxygen species in brain tissue and localised the exact site of ROS generation in mitochondrial respiratory chain. We used for the quantitative determination of ROS generation Amplex Red/ and *p*-hydroxyphenylacetic acid/peroxidase-based methods to detect H2O2 and the SOD-sensitive reduction of acetylated cytochrome C to detect superoxide. Using different brain samples, like rat hippocampal homogenates, isolated rat and mouse mitochondria and rat submitochondrial particles (SMP) we found a linear correlation between complex I-dependent oxygen consumption rate and complex I-dependent ROS generation rate. This result indicates that in brain tissue the mitochondrial respiratory chain complex I is the major source of ROS.

Applying different respiratory chain inhibitors we compared ROS production by complexes I and III and observed significantly higher production rates by complex I in both isolated rat brain mitochondria and rat brain SMP. Similar to isolated mitochondria also rat brain SMP show no differences in the NADH-dependent production of superoxide with the respiratory chain inhibitors rotenone, myxothiazol and stigmatellin. These data are in accordance with our previous work [1] (but in contrast to [2]) and indicate that the flavin moiety of respiratory chain complex I is very likely the major site for superoxide generation in the respiratory chain of brain mitochondria.

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P2.2.21. ROS AND MITOCHONDRIAL Ca^{2+} ARE CRITICAL TARGETS FOR SURVIVAL SIGNALING BY C-RAF

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Gene ablation and overexpression studies provided evidence for the antiapoptotic activity of C-Raf. It has been shown in the past that survival signaling by C-Raf includes inactivation of pro-apoptotic proteins (MST2, ASK1, BAD), transcriptional upregulation of pro-survival proteins and the crosstalk with additional survival pathways, which maintain mitochondrial integrity and thereby assure continued energy production and cell survival. However, the molecular mechanisms through which C-Raf suppresses apoptosis remain largely enigmatic. Prevention of the release of apoptogenic factors from mitochondria may be central to C-Raf's survival function. Both, reactive oxygen species (ROS) and Ca^{2+} are critical regulators of cell survival under physiological conditions, whereas excessive ROS production and mitochondrial Ca^{2+} overload are linked to induction of cell death. To get a detailed understanding of the interplay between C-Raf, Ca^{2+} and ROS during apoptosis, we used the growth factor removal-induced apoptosis of IL-3 dependent 32D cells as model system. Expression of oncogenic C-Raf (v-Raf) efficiently delays the onset of apoptosis in these cells. Our results show that apoptosis induction through growth factor removal results in enhanced production of ROS, which is not seen in cells protected either by IL-3 or v-Raf. Antioxidants (*N*-acetylcysteine and trolox) prevented enhanced ROS production and significantly delayed apoptotic cell death, also following treatment with staurosporine or oxidative stress-inducing agent *tert*-butylhydroperoxide, against which v-Raf provided efficient protection. Furthermore, mitochondrial uncouplers (FCCP and 2,4-dinitrophenol), but not NADPH oxidase inhibitor diphenyleneiodonium (DPI), delayed cell death and decreased ROS formation, suggesting mitochondrial origin of ROS. Moreover, we demonstrate that apoptosis under conditions of enhanced ROS production consistently associated with increased mitochondrial Ca^{2+}, which is not observed in cells protected by IL-3, v-Raf, ruthenium red or antioxidants. Taken together, our data suggest that survival control by C-Raf aims at prevention of oxidative stress and maintaining mitochondrial Ca^{2+} homeostasis.

P2.2.22. INCREASED SOD2 EXPRESSION IS RESPONSIBLE FOR ANTI-OXIDATION IN MUSCLE MITOCHONDRIA DURING PROLONGED EXERCISE IN RATS AFTER ENDURANCE TRAINING

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The recent research in the transgenic mice overexpressing the human mitochondrial superoxide dismutase 2 (SOD2) strongly suggested that endogenously generated superoxide does not regulate UCP activity and energy expenditure in vivo (Silva, and Nedergaard, 2005). The present research was to compare the effect of SOD2 and UCP3 expression on anti-oxidation in muscle mitochondria during prolonged exercise protocol *pre* and *post* endurance training. SD rats were divided into un-training (UT) and six–week endurance training (T) groups. Every group was applied with above given incremental exercise protocol (See Part I). The following parameters were determined: ROS generation rate, MDA content, SOD2 activity, expression of SOD2 and UCP-3 mRNA in muscle and SOD2 and UCP-3 protein in muscle mitochondria. We found that ROS generation was significantly lower in T group than that in UT group at every parallel time points. There were no significant difference in MDA content, SOD2 activity and SOD2 mRNA or protein expression level between different time points within UT group or T group, but these parameters were higher in T group than in UT group at every parallel time points. There were remarkably higher levels of UCP3 mRNA at the point of 90, 120 and 150 min, and protein content at the point of 120 and 150 min than that at 0 time in both groups, but UCP3 mRNA and protein were lower in T than in UT group at every points. Based on the results, we suggest that up-regulated SOD2 expression may play a more significant role in anti-oxidation during a prolonged intensity exercise after endurance training. That up-regulated activity and SOD2 expression enhance mitochondrial tolerance to excessive superoxide production and lower effect on UCP3 activity during intensity exercise.

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P2.2.23. HORSERADISH PEROXIDASE AND MYOGLOBIN ACTIVE SITE STUDIES BY ATR-FTIR SPECTROSCOPY

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Vibration changes in the catalytic site of horseradish peroxidase (HRP) and myoglobin (Mb) were investigated by Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR) spectroscopy in the 2000-900 cm^{-1} range. Reduced *minus* Oxidised (ferrous *minus* ferric) difference spectra were generated electrochemically and Compound II *minus* Oxidised difference spectra by substrate dialysis. These spectra show vibrational changes around the active site arising from protein backbone and amino acid residues, together with vibrational changes of the haem B cofactor that might provide a means of identifying these redox states in other systems. Assignments of bands can be made from effects of pH and H/D exchange, from comparisons with spectra of model materials and from known crystallographic structures.

The Reduced *minus* Oxidised spectra of HRP [1] and Mb [2] showed differences consistent with their known structural and functional differences. The spectra of HRP were pH-dependent whereas those of Mb were relatively pH insensitive, consistent with the known effects of pH on their midpoint potentials. The amide I region of Mb was relatively simple whereas that of HRP was strongly overlapped by bands arising from the arginine-38 in its distal haem pocket. Both proteins had a band attributable to the proximal histidine haem ligand. At low pH, additional histidine bands were present in HRP spectra, probably arising from protonation change of the distal pocket histidine. Other bands in both proteins could be assigned to the haem cofactor itself.

Compound II *minus* Oxidised spectra were generated with a new dialysis method that allowed reaction with H_2O_2 or peracetic acid to form compound II [3], providing the first FTIR spectra in this region for the Fe^{IV}=O state. The spectra of the two proteins again differ in a manner consistent with their known structural and functional differences and a number of band changes that are associated with the ferryl *minus* ferric haem B transition can be identified.

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P2.2.24. FERRICYTOCHROME C PROTECTS CYTOCHROME C OXIDASE AGAINST STRUCTURAL AND FUNCTIONAL DAMAGE INDUCED BY HYDROGEN PEROXIDE

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Cytochrome *c* oxidase (CcO) (EC 1.9.3.1) catalyzes the transfer of electrons from ferrocytochrome *c* to oxygen, a reaction coupled to proton translocation across the inner mitochondrial membrane. The conversion of oxygen to water proceeds through several oxy-intermediates. A powerful approach to studying the mechanism of CcO is based on the observation that two of these intermediates, "peroxy" and "ferryl", can be generated *in vitro* by H_2O_2. However, in addition to generating the "peroxy" and "ferryl" forms, the reaction of H_2O_2 with oxidized CcO causes a loss of electron-transport activity, oxidation of Trp19 and Trp48 within nuclear-encoded subunits VIIc and IV, respectively, and also partial dissociation of subunits VIa and VIIa [1]. H_2O_2 - induced oxidation of nuclear-encoded subunits involves long-range migration of mobile free radicals through the tryptophan-rich hydrophobic network localized within the highly hydrophobic mitochondrial-encoded subunits and results in oxidation of Trp334 within subunit I [2].

In this work the reaction between CcO and H_2O_2 was studied in presence of ferricytochrome *c*. The generation of the "peroxy" and "ferryl" CcO oxy-intermediates is not affected if H_2O_2 is added to the CcO – ferricytochrome *c* mixture. However, less than 10% of Trp19 and almost none of Trp48 are modified by H_2O_2 in presence of ferricytochrome *c*. This result is in sharp contrast to the ~50% and ~20% modification of these tryptophan by H_2O_2 in the absence of ferricytochrome *c.* The percent dissociation of subunit VIIa and VIa is also considerably reduced. CcO maintains all of its electron-transport activity if ferricytochrome *c* is present. Lastly, ferricytochrome *c* co-elutes with CcO from an anion-exchange column suggesting that ferricytochrome *c* is tightly bound or covalently attached to CcO after the mixture has been exposed to H_2O_2.

These results are consistent with a protein-to-protein radical transfer mechanism and suggest that ferricytochrome *c* has an important role in the mitochondrial defense mechanism.

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P2.2.25. MITOCHONDRIA-TARGETED ANTIOXIDANT MITOQ PROTECTS GRAMICIDIN CHANNELS FROM PHOTODYNAMIC INACTIVATION IN PLANAR PHOSPHOLIPID BILAYERS

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To characterize the protective action of a mitochondria-specific antioxidant, 10-(6′-ubiquinolyl)decyltriphenylphosphonium (MitoQ), we used sensitized photoinactivation of gramicidin A (gA) channels in a planar bilayer lipid membrane (BLM) as a model of oxidative damage to membrane proteins. Inactivation of the channels has been shown to result from the damage to tryptophan residues in gA caused by reactive oxygen species that are generated upon excitation of a photosensitizer. In our experiments, the addition of MitoQ to the bathing solution of BLM at a concentration of 0.5 μM protected gA channels from photoinactivation sensitized by Rose Bengal (RB). Both quinone and triphenylphosphonium moieties were ineffective in the protection of gA channels up to a concentration of 5 μM. The protective effect of MitoQ was independent of its redox state and of the sign of voltage applied to BLM. We surmise that MitoQ diminished RB-sensitized gA channel photoinactivation by quenching singlet oxygen, because sodium azide, the well-known singlet oxygen quencher, but not superoxide dismutase, showed the protective effect in this system. MitoQ also protected gA channels from photoinactivation sensitized by the cationic fluorescent dye Mitotracker Red. In this case the protection was obviously due to the scavenging of superoxide anions in addition to singlet oxygen. The effect of MitoQ exhibited low sensitivity to the surface charge of BLM, thereby showing that the binding of MitoQ to the membrane is determined by hydrophobicity rather than by electrostatics.

P2.2.26. APPLICATION OF HYDROXYLAMINE-BASED SPIN PROBES TO DETECT MITOCHONDRIAL REACTIVE OXYGEN SPECIES IN TISSUES AND MITOCHONDRIAL SUSPENSION

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Three similar hydroxylamine-based spin probes (methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine = CMH, 1-hydroxy-3-carboxy-2,2,5-tetramethyl-pyrrolidine hydrochloride = CPH, 4-phosphono-oxy-2,2,6,6-tetramethyl-piperidine-N-hydroxyl = PPH) reacting with reactive oxygen species (ROS), first of all with superoxide ($O_2^{\cdot-}$), have different hydrophobicity increasing in the following order: PPH<CPH<CMH. Incubation with rat heart mitochondria (RHM) resulted in the formation of spin adducts with CPH, and CMH (but not PPH), which was not sensitive to superoxide dismutase (SOD) and correlated with hydrophobicity of tested spin traps. Levels of spin adducts were drastically increased by antimycin A and became sensitive to SOD, which decreased levels of PPH, CPH, and CMH adducts by 96%, 72% and 45%, respectively. None of the spin probes influenced respiratory control, P/O ratio, respiration rate in state 3 and 4 of RHM. Further experiments have shown that CPH can be applied to tissue homogenates, and that the resulting spin adduct, 3-carboxy-proxyl, is stable in such models. CPH can be infused into blood allowing detection of ROS in tissues frozen in liquid nitrogen after the infusion. We have found that in hearts obtained from old rats ROS generation was significantly increased compared to their young counterparts. Experiments with isolated RHM have shown increased $O_2^{\cdot-}$ generation in mitochondria from old rats compared to mitochondria obtained from young rats. Parallel experiments aimed at detecting H_2O_2, the dismutation product of $O_2^{\cdot-}$, using peroxidase and scopoletin failed to detect any difference between mitochondria from old and young rats. However, in the presence of antimycin A, both methods displayed similar results, an increase in ROS production. Our data suggest that (i) hydroxylamine-based spin traps are not toxic and sensitive probes for detection of ROS in mitochondrial suspension, tissue homogenate, and tissues; (ii) $O_2^{\cdot-}$ generated by intact mitochondria does not escape from mitochondria while in the presence of antimycin A $O_2^{\cdot-}$ leaks out to the medium and becomes available for SOD and hydrophilic spin traps; (iii) the increased levels of ROS in hearts from old rats originate from mitochondria.

P2.2.27. MITOQ INDUCED MIOFIBROBLAST DIFFERENTIATION OF HUMAN FIBROBLASTS

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There are no doubts that reactive oxygen species (ROS) produced by mitochondria and other cellular sources are not only injurious by-products of metabolism but also essential mediators of cell signaling during proliferation, differentiation and apoptosis. It was shown in various models that known, that some cytokines and spreading of the cell on the substrate stimulated generation of ROS although the enzymatic source(s) are largely unknown.

We have investigated the role of mitochondrial ROS in intracellular signaling using mitochondria-targeted antioxidant, 10-(6'-ubiquinolyl)decyltriphenilphosphonium (MitoQ). The several cell cultures (primary human fibroblasts, rat fibroblasts (Rat-1), mouse fibroblasts (L line), rat epithelial cells (IAR-2) and HeLa carcinoma) were treated with 20 nM MitoQ for 5-7 days and changes in cytoskeleton and morphology were examined. A specific targeting of MitoQ to mitochondria was confirmed using fluorescent rhodamine-labeled MitoQ. Furthermore, uncouplers FCCP and CCCP inhibited all the morphological changes induced by MitoQ preventing accumulation of the antioxidant in the matrix of mitochondria. It was found, that treatment with MitoQ increased the area of spread cells in all cell lines tested. Elongation of fibroblasts strongly decreased. Analysis of cell population revealed a fraction of very large (3-5 times larger then control) well-attached and symmetrical cells. The changes of cell shape were accompanied with fundamental cytoskeleton reorganization and increasing of focal contacts. Significant growth in number and thickness of actin microfilament bundles (stress-fibers) was observed. Moreover, the population of enlarged fibroblasts was enriched in α-smooth muscle actin (α-SMA) that is a selective marker of myofibroblasts. In epithelial cells significant destruction of circular actin bundle and appearance of stress-fibers-like bundles was observed. These changes indicated to possible beginning of epithelial-mesenchimal transition (EMT) induced by MitoQ. The observed EMT was far from being complete, since E-cadherin (a marker for epithelial cell-cell contacts) was still presented in these cells.

It was found that sort-term (2-3 h) treatment with MitoQ followed by prolonged (7-60 days) culturing of the cells without added MitoQ caused the same effects on morphology, cytoskeleton and expression of α-SMA. It was suggested that scavenging of mitochondrial ROS by MitoQ

initiated production of cytokine(s) which could be supported for a long time due to autocrine mechanism. In fibroblasts the candidate cytokine could be Transforming Growth Factor (TGF-β), which is a well-known inducer of myofbrablast and EMT differentiation.

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P2.2.28. MECHANISM OF TOXICITY OF THE BRANCHED-CHAIN FATTY ACID PHYTANIC ACID, A MARKER OF REFSUM DISEASE, IN BRAIN CELLS INVOLVES MITOCHONDRIAL DEPOLARISATION AND GENERATION OF REACTIVE OXYGEN SPECIES (ROS), WITH ROS PRODUCTION IN BRAIN MITOCHONDRIA BY ROTENONE-LIKE ACTION

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The saturated branched-chain fatty acid phytanic acid (3,7,11,15-tetramethylhexadecanoic acid; phyt) is taken up by dairy diet. The β-methyl group prevents β-oxidation. Therefore, phyt degradation is initiated by α-oxidation in peroxisomes. Refsum disease, an inherited peroxisomal disorder with neurodegeneration and muscle dystrophy is characterised by high level phyt accumulation. The mechanisms of toxicity of phyt are not yet understood. We investigated the influence of phyt in dissociated rat brain cells (hippocampal astrocytes, neurons, oligodendrocytes). In response to phyt, cytosolic Ca^{2+} was quickly transiently increased, involving intracellular Ca^{2+} stores. Phyt caused cell death of astrocytes within a few hours of exposure (1). In isolated rat brain mitochondria, phyt dissipated mitochondrial membrane potential. Moreover, phyt released cytochrome c. Phyt induced substantial reactive oxygen species (ROS) generation in isolated mitochondria like in intact cells. Phyt at low concentrations deenergized mitochondria, as seen by depolarization, stimulation of non-phosphorylating oxygen uptake, and inhibition of the reduction of the tetrazolium dye MTT. Palmitic acid exerted only small effects. In addition, phyt reduced state 3 respiration, partly due to inhibition of the ADP/ATP carrier. Important for functional consequences is our finding that preloading with small amounts of Ca^{2+} highly sensitized mitochondria to rapid permeability transition even with low phyt concentrations (2). Furthermore, in mitochondria from rat brain free Phyt enhances superoxide ($O_2^{\bullet-}$) generation, in state 3, and in state 4. Phyt stimulates $O_2^{\bullet-}$ generation with substrates glutamate/malate, pyruvate/ malate or succinate in the presence of rotenone. The enhanced $O_2^{\bullet-}$ generation by Phyt in state 4 is in contrast to the mild uncoupling concept. There uncoupling by nonesterified fatty acids should abolish $O_2^{\bullet-}$ generation. Interference of Phyt with the electron transport was shown (3). In conclusion, phytanic acid initiates glial and neuronal cell death by activating the mitochondrial route of apoptosis.

Complex I is the main site of Phyt-stimulated $O_2^{\bullet -}$ generation. Furthermore, inactivation of aconitase and oxidation of the mitochondrial glutathione pool show oxidative damage with chronic exposure to Phyt. Thus, in neural tissue, enriched with phytanic acid, the reduction in mitochondrial ATP supply and the facilitation of the opening of the permeability transition pore seem to be major mechanisms by which phytanic acid induces the onset of degenerative processes.

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P2.2.29. ROLE OF AVIAN UNCOUPLING PROTEIN IN PREVENTING REACTIVE OXYGEN SPECIES PRODUCTION OF SKELETAL MUSCLE MITOCHONDRIA

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An avian homolog of mammalian uncoupling proteins (avUCP) has been identified in several bird species but its biological role still remains hypothetical. We used various physiological or pharmacological situations to induce large changes in expression of avUCP and examined the functional properties of isolated mitochondria *in vitro*.

Control 5-wk-old muscovy ducklings (*Cairina moschata* L, R31 INRA) were kept at thermoneutrality (25°C from the age of 1-wk, TN). Experimental ducklings were rendered i) hypothyroid by giving 6-n-propyl-2-thiouracil (PTU; Sigma, 0.15 % w/v) in drinking water, ii) hyperthyroid by daily subcutaneous injections of 3,5,3'-triiodo-L-thyronine (T_3, Sigma, 10 µg/100 g) in addition to PTU, or iii) acclimated to cold (4°C from the age of 1-wk, CA). Ducklings were killed by decapitation and tissues (liver, skeletal muscle) were sampled for mitochondrial isolation or subsequent determination of avUCP expression and antioxidant enzyme activity. Mitochondrial oxygen consumption was measured with a Clark-type electrode and reactive oxygen species (ROS) production was monitored by the linear increase in fluorescence due to the oxidation of homovanilic acid by H_2O_2 in the presence of horseradish peroxidase using a SFM-25 fluorometer (Kontron).

avUCP mRNA was detected by RT-PCR in gastrocnemius muscle but not in liver. By comparison with controls, transcript relative abundance was markedly increased in CA (+188%) or T_3-treated (+430%) ducklings but down-regulated in hypothyroid birds (-69%). Mitochondrial ROS production (pmoles H_2O_2 / nmoles oxygen) with succinate as substrate was lower in muscle mitochondria from CA (27 ± 2) or T_3-treated (12 ± 1) than in those from control (38 ± 2) and hypothyroid birds (47 ± 4). Addition of GDP (UCP inhibitor) markedly increased the mitochondrial ROS production of CA or T_3-treated birds back to the level of control or hypothyroid ducklings. No such effect was observed in liver. Differences in ROS production between groups could not be related to changes in antioxidant enzyme activities (superoxide dismutase or glutathione peroxydase).

Present results show that the expression of avUCP is associated with a GDP-sensitive reduction in mitochondrial ROS production. These data suggest that AvUCP may play a role in preventing muscle cells from oxidative damage in situations of enhanced metabolic activity.

P2.2.30. CELLS, MITOCHONDRIA AND NITROSATIVE STRESS IN HEALTH AND DISEASE

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Control of mitochondrial respiration is one of the relevant physiological functions of NO [1]. This occurs via the rapid (milliseconds) and reversible inhibition of cytochrome c oxidase (CcOX) [2-4]. Two reaction mechanisms have been described leading to formation of either a nitrite-(CcOX-NO_2^-) or a nitrosyl-derivative (CcOX-NO), both inhibited adducts though with different K_I and cell destiny. The two mechanisms are both operative, although one may prevail on the other depending on the turnover conditions and substrates concentration (cytochrome c and O_2). The patho-physiological validity of the mechanisms has been tested in the whole cell. We have shown that under normal cell-culture O_2 tension (~ 270 μM) neuroblastoma cell mitochondria degrade NO to nitrite, switching to nitrosylation upon rising the electron flux level of the respiratory chain at the level of CcOX. Moreover, a persistent activation of the NMDA receptor of cultured glioma cells treated with morphine, via a persistent rise of cytosolic Ca^{++}, induces a NO-mediated depression of the mitochondrial membrane potential that can be prevented by specifically inhibiting the NO-synthases [5]. We have also observed that the CcOX response to nitric oxide (NO) may differ in different cell lines, such that either mechanism of NO reaction with CcOX may prevail under comparable conditions. In this frame, preliminary results suggest that mitochondria of EBV-transformed lymphocytes, and particularly those from a patient affected by Ataxia Teleangiectasia, are able to degrade NO to nitrite more efficiently than normal.

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P2.2.31. cAMP REGULATION OF OXYGEN FREE RADICAL BALANCE IN MAMMALIAN CELLS

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In mammalian cells aerobic energy metabolism adapt itself to the continuous changing energy demand (1). cAMP, produced by the plasma membrane adenylyl cyclase in response to hormones and neurotransmitters (2) and by the bicarbonate activated soluble adenylyl cyclase localized to nucleus, mitochondria, microtubules and other intracellular structures (3) plays a central regulatory role in energy supplying and energy requiring processes (2). We have found that cAMP regulates cellular oxygen metabolism. Different human and murine cell lines were analysed, either in their exponential growth phase (serum-saturated), or in a quiescent state by 48 hour cultivation under serum-limitation. In the serum-limited cells a large accumulation of H_2O_2 which was localised in spots superimposed on mitochondria was observed. Serum limited cells showed, also, a large accumulation of $O_2^{\cdot -}$ in the mitochondrial matrix. Exposure (60 min) of serum limited cell cultures to dibutyryl cAMP, a permeant derivative of cAMP, resulted in disappearance of H_2O_2 from cells and $O_2^{\cdot -}$ from mitochondria. The increase in the level of H_2O_2 and $O_2^{\cdot -}$ observed in serum limited cells and their disappearance upon cAMP treatment is a general phenomenon, observed in six different lines of murine and human cell tested. H89, a specific inhibitor of cAMP dependent protein kinase (PKA) depressed the ROS removing effect of cAMP, thus the effect involves PKA. Analysis of the ROS scavenger system revealed that the accumulation of ROS was associated with an extensive oxidation of glutathione. Serum limitation and subsequent treatment with cAMP had no significant effect on the level of glucose-6-phosphate and glucose-6-phosphate dehydrogenase activity. Also no change was produced by serum limitation and cAMP treatment in the activity of glutathione reductase, glutathione peroxidase and catalase. Thus the ROS accumulation induced by serum limitation and the ROS removing effect of cAMP were not due to depression and reactivation of these scavenger systems respectively. Analysis of the impact of serum limitation and cAMP on the activity of complex I revealed an inverse relationship between the observed changes in cellular ROS and the forward NADH-ubiquinone oxidoreductase activity of the complex. In two lines of fibroblasts and Hela cells serum limitation was associated with a depression of the Vmax of the NADH-ubiquinone oxidoreductase activity, which was largely rescued by 60 min cAMP. Two

unrelated patients, carrying a G44A nonsense mutation in the NDUFS4 gene (4) and a C1564A mutation (Q522K substitution) in the NDUFS1 gene (5) of complex I, revealed remarkably different profile of biochemical dysfunctions. The G44A mutation caused the disappearance of the 18 kDa subunit and the loss of normally assembled complex I in the NDUFS4 patient fibroblasts, while the C1564A mutation determined the appearance of a subcomplex in adding to the normally assembled complex I. Rotenone sensitive NADH-UQ oxidoreductase activity in NDUFS4 mutant was undetectable, compared to control fibroblasts, while in NDUFS1 mutant it was 20% respect to control fibroblasts. Direct monitoring of ROS in the fibroblast cultures revealed that the NDUFS1 mutation was associated with a large increase in the level of $O_2^{\cdot-}$ and H_2O_2 whilst in the NDUFS4-mutant fibroblasts no ROS were detectable. Exposure of the *NDUFS1* mutant fibroblasts to dibutyryl-cAMP stimulated the residual NADH-ubiquinone oxidoreductase activity, induced disappearance of ROS and restored the mitochondrial potential. These are relevant observations for a possible therapeutical strategy in the *NDUFS1* mutant patients.

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P2.2.32. UNCOUPLING PROTEINS: DO ROS OR ROS PRODUCTS ACTIVATE - AND DO THE UNCOUPLING PROTEINS PROTECT AGAINST OXIDATIVE DAMAGE?

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A physiological function of the original uncoupling protein, UCP1, is well established: UCP1 is the molecular background for nonshivering thermogenesis. The functions of the "novel" UCPs, UCP2 and UCP3, are still not established. Recent discussions imply that all UCPs may play a role in protection against reactive oxygen species (ROS) (reviewed in (1)). Here we examine critically whether UCP1, UCP2 and UCP3 are stimulated by ROS (superoxide) or ROS products (4-hydroxy-2-nonenal (HNE)), and whether the UCPs actually can diminish oxidative damage. By comparing the response of brown-fat mitochondria from wild-type mice (i.e. UCP1-possessing mitochondria) with mitochondria from UCP1-ablated mice, we have examined the ability of HNE to (re)activate UCP1 and the effect of HNE on the conductance ascribable to UCP1. Whereas we confirm that HNE affects brown-fat mitochondria, we see no evidence that this effect is mediated via UCP1. By examining mitochondria from mice that moderately overexpress mitochondrial superoxide dismutase, we have investigated whether physiological levels of superoxide activate UCP1, UCP2 or UCP3 (2). In brown-fat mitochondria (UCP1) we observe unchanged UCP1 activity characteristics. In muscle mitochondria (UCP3) we see no alterations in basal proton leak, nor any difference in the response to fatty acids and GDP. We also examined the characteristics of brain mitochondria and saw no alterations due to superoxide dismutase overexpression but the level of UCP2 in these mitochondria is not established. We examined oxygen damage (nonenal/protein adducts) in brown-fat mitochondria from cold-acclimated wild-type and UCP1-ablated mice but did not observe any difference in level of adduct formation. This would indicate that a function of UCP1 as a protection against oxidative stress is not evident. We conclude that, concerning UCP1, it is unlikely that it has a role in protection against reactive oxygen species; concerning UCP2/UCP3, most evidence for physiologically significant roles in this respect is still circumstantial.

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P2.2.33. ACTIVATION OF UNCOUPLING PROTEIN 3 BY ENDOGENOUS REACTIVE OXYGEN SPECIES

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Uncoupling proteins 2 and 3 have been postulated to play a protective role in reducing damage caused by reactive oxygen species (ROS) in mitochondria, and can be activated *in vitro* by exogenously added superoxide or lipid peroxidation products such as 4-hydroxynonenal. Purine nucleotides are the canonical inhibitors of UCP activity; by adding guanosine diphosphate to isolated rat skeletal muscle mitochondria respiring on succinate, it was proposed that UCP3 could be activated by endogenous ROS generated predominantly at Complex I through reverse electron transfer. However, purine nucleotides may affect mitochondria through pathways not mediated by UCPs so other interpretations remain possible.

We now demonstrate that respiring isolated skeletal muscle mitochondria from transgenic mice lacking UCP3 have a significantly higher rate of ROS production than wild type control mitochondria. Addition of guanosine diphosphate increased the ROS production of both wild type and UCP3-knockout mitochondria to the same rate, suggesting that UCP3 was activated in this system.

Superoxide production by the electron transport chain is acutely sensitive to changes in the proton motive force. Thus the membrane potential and pH gradient were measured to determine whether UCP3 reduces the production of ROS by reducing the proton motive force, or through a different mechanism.

This study shows that ROS production in isolated skeletal muscle mitochondria respiring on succinate is dependent on the presence of UCP3, supporting a role for UCP3 in protecting against ROS-induced damage.

P2.2.34. CALORIC RESTRICTION DECREASES ROS PRODUCTION IN BROWN ADIPOSE TISSUE OF OLD RATS BY UNCOUPLING ACTIVITY

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Brown adipose tissue (BAT) is a model tissue for the study of mitochondrial uncoupling activity due to its specific uncoupling protein 1 (UCP1). Uncoupling has been postulated as a mechanisms able to reduce the production of reactive oxygen species (ROS) by favouring the electron flow in the electron transport chain. The free radical theory of aging proposes that the accumulation of oxidative damage is a key event in the aging process. Caloric restriction (CR) has been shown to extend longevity and delay the onset of age-related impairment of functionality in several tissues. The aim of this work was to test wether CR effects in BAT could be mediated by changes in uncoupling protein. To this end, we used 18 month old rats subjected to 40% CR or ad libitum feeding for three months, and determined oxygen consumption, ROS production, mitochondrial respirometry, antioxidant activities, total protein and UCP1 levels. Oxygen consumption was decreased in restricted rats. Mitochondrial ROS production was decreased by CR using glycerol-3-phosphate (G3P) as substrate. CR-induced decrease in ROS production was reverted in the presence of GDP, a specific dinucleotid-phosphate which inhibits UCP1. GDP also was able to produce a marked drop in mitochondrial oxygen consumption in restricted rats. Restricted rat mitochondria showed higher protein and UCP1 content. These findings are indicative of a higher functional state and activity in mitochondria of CR rats, which could prevent ROS production. On the whole, our results suggest that, at least, part of the decreasing effect of CR on ROS production could be mediated by a greater uncoupling activity.

P2.2.35. THE ROLE OF MITOCHONDRIA IN THE CONTROLLING OF CALCIUM CHANNELS IN THE PLASMA MEMBRANE: PAM AND MAM AS A POINT OF THE CONTROL MACHINERY

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The majority of the endoplasmic reticulum (ER) is located in the proximity of the plasma membrane (plasma membrane-associated membranes, PAM), but certain parts protrude into the cytosolic compartment where association with mitochondria occurs (mitochondria-associated membranes, MAM). During cell stimulation, when the ER is depleted of Ca^{2+}, store-operated calcium channels (SOCs) in the plasma membrane are activated. This is one of the paradigms in the field of cellular signalling. Mitochondria, due to their highly negative membrane potential, can readily accumulate Ca^{2+}. This uptake is achieved by a low affinity (K_d >10 μM) Ca^{2+} activated calcium channel (the mitochondrial Ca^{2+} uniporter), located in the inner mitochondrial membrane [1]. Mitochondria can also take up Ca^{2+} entering the cells via plasma membrane channels due to their close vicinity to the plasma membrane.

Using electron microscopy, we observed that some regions of the endoplasmic reticulum form close contacts with mitochondria. We analysed composition and molecular structure of the MAM and PAM fractions using blue-native electrophoresis, Superose 6 column filtration and SDS-polyacrylamide gel electrophoresis. In the MAM fraction, we found proteins characteristic for the plasma membrane (e.g. plasma membrane calcium ATPase). On the other hand, the PAM fraction contained the voltage-dependent anion carrier (VDAC) and the sarco-endoplasmic reticulum calcium ATPase (mitochondrial and endoplasmic reticulum markers, respectively). We have previously described that VDAC is a key determinant of Ca^{2+} permeability at ER-mitochondria contacts [1], what is compatible with the present results.

Mitochondria can control SOCs in a spectrum of cell types [2]. We investigated mechanism of this regulation. Using fluorescent probe for Ca^{+2} (Fura-2), we found that the antioxidant TEMPOL partially inhibited the rate of capacitative calcium influx into human lymphoblastoid (Jurkat) cells. This points to a possible role of free radicals in the regulation of capacitative calcium influx.

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P2.2.36. INVOLVEMENT OF HEART MITOCHONDRIAL NITRIC OXIDE SYNTHASE AND TIME COURSE OF RAT ADAPTATION TO HIGH ALTITUDE

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The present work analyzes the time course of the adaptive response to high altitude (Cerro de Pasco, Perú, 4,340 m) considering classical physiological parameters and mtNOS activity and expression. The hematocrit of rats exposed to high altitude showed a hyperbolic increase and were up to 40 % higher than those of sea level rats with a $t_{½}$ of 11 days. Right ventricle weight was increased by 128 % after 84 days of exposure with a $t_{½}$ of 26 days. Heart mtNOS activity showed an hyperbolic increase almost parallel to hematocrit with a $t_{½}$ of 19 days. A linear relationship was found between hematocrit and heart mtNOS activity (R^2=0.88, $P \leq 0.05$). After 84 days of exposure, heart mtNOS activity was 70 % higher in high altitude than in sea level animals. The observed enhancement of mtNOS biochemical activity was accompanied by a 60 % increase in mtNOS expression recognized by anti-iNOS antibodies. This fact suggests a common signaling pathway leading to increased transcription of the genes encoding for erythropoietin and mtNOS. According to our results, we conclude that mtNOS and mitochondria are the main source of cardiomyocytes NO and constitute an important factor in the adaptive response to sustained heart hypoxia: mtNOS accounted for about 86 % of total cellular NO production in sea level rats and for about 96 % in rats exposed to high altitude for 84 days.

Poster session 2.3. Aging

P2.3.1. MITOCHONDRIAL FUNCTIONS IN ACANTHAMOEBA CASTELLANII AGING

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Acanthamoeba castellanii was studied as an unicellular eukaryotic model organism for aging. The aim of the present study was to identify specific alternations in mitochondrial respiratory functions during the aging process. The rates of state 3 (ADP-stimulated) respiration, uncoupled (FCCP-stimulated) respiration and respiratory control ratios decreased as function of age, using either external NADH or succinate as substrates. There was also an age-related decline in the activity of cytochrome c oxidase and total amount of membranous coenzyme Q. These changes were accompanied by a decrease in basal mitochondrial ROS production, and almost no change in superoxide dismutase activity and protein level, indicating a negligible importance of this enzyme in oxidative stress defence in *A. castellanii* aging. However, a significant decrease in alternative oxidase activity and protein level as well as a less pronounced decrease in uncoupling protein activity and protein level were observed when cells shifted from exponential growth to stationary phase. Therefore, a function of both energy-dissipating systems in unicellular organisms may be to cause mild uncoupling in response to ROS produced by mitochondria, leading to lowered proton motive force and decreased superoxide production. This function seems to be important in exponentially growing cells, decreasing with *A. castellanii* aging leading to cysts.

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P2.3.2. CENTRAL ROLE FOR MITOCHONDRIAL MEDIATED APOPTOSOS WITH AGE

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Apoptosis is a highly regulated form of cell death characterized by specific morphological, biochemical, and molecular events. However, its role during aging, particularly in post mitotic tissues such as the brain, heart and skeletal muscle has not been completely understood. The mechanisms by which apoptosis occurs with advancing age and adaptations that may protect against apoptosis remain to be identified and are under investigation. However, mitochondrial mediated apoptosis is believed to play a central role in the observed losses in cells and biological function. Using several models of normal aging (calorie restriction and life long exercise) we have discovered several key proteins involved in the regulation of apoptosis and found that the age associated increase in apoptosis can be attenuated with specific interventions. For example, caspase independent pathways mediated by apoptosis inducing factor and endonuclease G release from the mitochondria appears to be critical for apoptosis with age and are halted by interventions. Moreover, mice which express a proof-reading deficient version of the mitochondrial DNA polymerase G (POLG) accumulate mtDNA mutations and display features of accelerated aging. This provides us with a model to investigate mitochondrial mediated apoptosis in vivo. We determined that mitochondrial dysfunction is a critical event and are currently examining the role of specific caspase-dependent and independent mitochondrial pathways. Elucidating the precise signaling pathways leading to apoptosis will lead to targeted interventions to prevent cell loss (1-5).

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P2.3.3. HOW EVOLUTIONARY THINKING AFFECTS OUR IDEAS ABOUT AGEING INTERVENTIONS

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Evolutionary theory has guided the development of anti-ageing interventions in some conscious and some unconscious ways. We assume that the body is optimized in some sense, and seek medical strategies that help and support the body's efforts to maintain itself. If we think that ageing is caused by many parts of our bodies wearing out simultaneously, then we must address these problems one-by-one.

Meanwhile, a range of experimental evidence points to the startling hypothesis that aging has evolved as an adaptation in its own right: Two ancient mechanisms of programmed death in protists have survived half a billion years of evolution, and still figure in the ageing of vertebrates today (apoptosis and telomere shortening). Single gene deletions seem to have the power to postpone ageing, sometimes at no cost in fertility. (This indicates that there are mechanisms, programmed by genes, that have no other purpose than to disable and ultimately to kill the bearer of those genes.) Semelparous organisms die promptly after reproduction (though their death is not directly caused by reproduction). Caloric restriction and other hormetic effects attest to the body's capacity to extend life span at will when under stress. (Why, then, does it revert to a short life span when the stressors are removed?)

Aging seems to be a full-blown adaptation, a developmental program under full and explicit genetic control.

P2.3.4. DIMEBON AS MITOPROTECTIVE AND ANTIAGING DRUG

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The disturbances of calcium homeostasis, enhanced Ca^{2+}-dependent activation of mitochondrial permeability transition (MPT) and increased generation of free radicals are the earliest and the most common features of aging and of age-dependent neurodegenerative disorders [1,3]. It was shown earlier that the neuroprotective action of some widely used cognition enhanced drugs, endogenous neuroprotectors might be the result of its action on mitochondria. These agents can increase the resistance of neurons (and other cells) to apoptotic (necrotic) process by increasing of mitochondria threshold onset to MPT inductors [2,3]. The good example is Dimebon, which was successfully tested on Alzheimer's patients in clinical trials and was patented as a new agent for the therapy of neurodegenerative disorders. In experiments with amyloid-induced neuronal cells death this compound reveal significant neuroprotective effect [4]. At least in part, it may be connected with its effect on mitochondria. Dimebon effectively increase of mitochondria threshold onset to different inductors of MPT– first of all to physiological effectors – calcium and inorganic phosphate - and to neurotoxins (MPP+ and β-amyloid peptide). This compound also prevents the mitochondrial lipid peroxidation induced by butylhydroxyperoxide and ferrous ions. These findings and low toxicity allow us to assume the possibility of antiaging exhibition of Dimebon. It was shown in long-continued experiments that the average duration of life of female mice C57Bl/6 treated with Dimebon (1.5 mg /kg per os) was increased by 2.3% ($P \leq 0.007$). This effect is accompanied with the increasing of quality of life of Dimebon-treated animals: decreasing of the senile alopecia ($P \leq 0.002$), body weight loss ($P \leq 0.05$), cataract development ($P \leq 0.002$).

So Dimebon may be helpful as the the antiaging neuroprotective agent, which can decrease the vulnerability of cells to death signals to target mitochondria and combine mitochondria protective potential with capacity to enhance the cognitive function.

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P2.3.5. AGE-RELATED DIFFERENCES ON SERUM PARAOXONASE ACTIVITY IN MALE AND FEMALE RATS

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Paraoxonase 1 (PON1), a protein specifically associated with high density lipoproteins (HDL) containing apolipoproteins A-I and J (apo A-I and apoJ), has been shown to protect low density lipoproteins (LDL) against lipid peroxidation. A progressive increase in oxidative stress is commonly associated with aging and its related diseases. Despite the PON1 antioxidant capacity, the effect of aging on PON1 activity is not clearly understood. Thus, the aim of this study was to investigate the effect of aging on PON1 activity in adult (6 months) and aged (24 months) male and female Wistar rats. With this purpose, serum lipid profile, lipid peroxide levels, paraoxonase and arylesterase activities and PON1, apoA-I and apoJ levels were measured. The LDL/HDL cholesterol ratio significantly increased with aging in both genders. Paraoxonase and arylesterase activities also increased with aging in both genders, although that increase was greater in male rats. Changes in apoJ and PON1 levels accounted for the higher PON1 activities observed in aged animals. ApoA-I levels were higher in female adult rats compared to males and decreased with age, thus reaching male values and dovetailing the HDL-cholesterol profile. The lack of difference on lipid peroxide levels between adult and aged rats would agree with the increased PON1 activity.

The reported increase in PON1 activity could be a physiological response to the higher oxidative stress associated with aging. In addition, our results reveal that this response is gender-dependent, since female rats showed a lower-level response compared to males, in which the effect of the female age-related hormonal changes could not be discarded.

P2.3.6. AGE-DEPENDENT CHARACTER OF MITOCHONDRIA TARGETED ANTIOXIDANTS (MTA) MEDIATED PROTECTIVE EFFECT ON CARDIOLIPIN PEROXIDATION AND CREATINE KINASE FUNCTIONING IN RAT HEART MITOCHONDRIA

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Stability and proper functioning of mitochondrial creatine kinase (mtCK) octamer are important for support of intracellular energy buffering system [1]. Octamerisation of mtCK and its capacity to be bound on mitochondrial membrane is crucial for inhibiting of permeability transition pore opening in heart mitochondria under Ca^{2+} overload or oxidative/nitrosative stress conditions [2]. Recently it was shown that membrane binding properties of mtCK octamer is strongly depends on state of mitochondrial lipids and essentially from cardiolipin peroxidation and its turnover rate [3]. Recently we have found that by using of mitochondria targeted antioxidant described in [4] can prevent cardiolipin peroxidation in isolated heart mitochondria under oxidative stress conditions. Determination of cardiolipin and its lysoforms content was done by high performance thin layer chromatography - HPTLC [5]. Careful analysis of mtCK octamer state and content in such mitochondria was performed and included size-exclusion chromatography, blue native and izoenzyme electrophoresis. It was shown that enzyme activity and mtCK octamer/dimer ratio both elevated for mitochondria treated with targeted antioxidants compare with untreated mitochondria under oxidative stress condtitions. Further investigation of linkage between level of cardiolipin peroxidation and mtCK octamer stability and functioning revealed that Shiff-base formation occurred between Lys in N-terminal end of polypeptide chain and aldehydes formed during lipid peroxidation. Amount of Shiff base was measured by its intrinsic fluorescence [6] and was found that strong correlation exists between amount of targeted antioxidant, respiratory chain activity and level of cardiolipin oxidation. Dietary supplementation of rats with MTA and subsequent analysis of described parameters revealed age-dependent character of phenomenon. Possibility for reseach and cure age- associated pathologies in tissues with high energy demand is discussed.

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Poster session 2.4. Cancer, ischemia and degenerative disorders

P2.4.1. THE EFFECT OF HYPOXIA UPON THE TRANSCRIPTION PATTERN OF ISOFORMS OF CYTOCHROME C OXIDASE AND OXIDATIVE ENERGY PRODUCTION IN ASTROCYTES AND NEURONS

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The brain is the organ with the highest energy demand in mammalian organisms. Two different brain cell types, neurons and astrocytes, are structurally, functionally, and metabolically tightly coupled with astrocytes playing a central role in the regulation of cerebral energy metabolism in dependence on neuronal activity. Oxygen is, besides glucose, the most important substrate to fulfil astrocytic and neuronal energetic requirements and is also the substrate of cytochrome c oxidase (COX). This enzyme is engaged in mitochondrial oxidative energy metabolism. It catalyses the electron transfer from ferrocytochrome c to oxygen, a process coupled to the translocation of protons across the inner mitochondrial membrane and subsequently to production of ATP, an indirect product of COX, through the ATP Synthase.

Mammalian COX is composed of three catalytic, mitochondrially encoded and ten regulatory, nuclear encoded subunits. The regulatory COX subunit IV plays an important role in adjusting energy production to cellular energetic requirements by binding of ATP to the N-terminus of subunit IV thereby causing an allosteric inhibition of COX activity at high energy level, i.e. high ATP/ADP ratio [Arnold and Kadenbach (1999) FEBS Lett. 443: 105-108]. COX subunit IV exists in different isoforms (IV-1 and IV-2) [Huttemann et al. (2001) Gene 267: 111-123]. While the isoform IV-1 is ubiquitously transcribed in all adult mammalian tissues including brain, the isoform IV-2 showed high transcription levels only in the lung. Therefore, we examined the transcription pattern of subunit IV isoforms in two different brain cell types under normoxic and hypoxic conditions. Besides the expression of COX isoform IV-1 in primary astrocytes and cerebellar granule cells from mouse brain, we detected also low levels of mRNA transcripts for the COX isoform IV-2 in cerebellar granule cells pointing at a cell type specific expression of COX subunit

IV isoforms in the brain. Under conditions of oxygen deprivation mRNA transcription of COX IV-2 is induced in astrocytes and further up-regulated in cerebellar granule cells. Increased expression of the COX IV-2 isoform caused an abolition of the allosteric inhibition of COX by ATP at high energy levels as determined by polarographic measurements. We conclude that the expression of COX isoform IV-2 under hypoxia suppresses the sensitivity of COX to its allosteric regulator ATP and overrules the regulation of COX by the cellular energy level.

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P2.4.2. LARGE CONDUCTANCE POTASSIUM ION CHANNEL FROM RAT BRAIN MITOCHONDRIA

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In the inner mitochondrial membrane potassium selective ion channels are present. They are probably involved in cytoprotective action in various cell types. Mitochondrial response to changes of cytosolic calcium concentration has a strong impact on neuronal cell viability. Ca^{2+} induced mitochondrial depolarization and an increase in mitochondrial respiration was studied with isolated rat brain mitochondria. These effects were specific for potassium ions. Both depolarization and respiration of mitochondria was blocked by iberiotoxin and charybdotoxin – a well known inhbitors of large conductance potassium channel (BK_{Ca} channel). Furthermore, NS1619 a BK_{Ca} channel opener induced the potassium specific effect similar to that one induced by Ca^{2+}. The activity of potassium channel was recorded with the use of planar lipid bilayer technique. The mean conductance of the channel was about 250 pS in gradient 50/450 mM KCl solutions. Single channel activity of this reconstituted protein showed properties of the big conductance potassium channel (BK channel). It was activated by Ca^{2+} and blocked by charybdotoxin or iberiotoxin. Additionally, stimulation of the channel activity was observed upon application of BK channel openers, benzimidazolone derivatives: NS1619 and NS004. We also observed immunoreactivity between anti-β4 subunit (of BK_{Ca} channel) antibody with ~26 kDa proteins in brain mitochondria. Immunochemical analysis's confirmed the predominant occurrence of β4 subunit in neuronal (of hippocampal origin) but not glial mitochondria. We hypothesize that mitochondrial BK_{Ca} channel represents a new mode that mitochondria, acting as calcium sensor, can play a part in neuronal signal transduction.

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P2.4.3. X-LINKED ISOLATED COMPLEX I DEFICIENCY

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Complex I deficiency, the most common respiratory chain defect, is genetically heterogeneous: mutations in 9 nuclear and 7 mitochondrial DNA genes encoding complex I subunits have been described. It has been previously suggested that the male/female ratio of patients with an isolated complex I deficiency would underlie a genetic defect in a complex I X-linked structural gene. However, no X-linked complex I-deficient patients have been reported so far.

We investigated the presence of mutations in the two complex I structural genes located in human chromosome X, *NDUFA1* and *hESSS*, in a cohort of 26 patients presenting with a mitochondrial complex I defect. Novel missense hemizygous mutations were identified in the *NDUFA1* gene of two patients by direct sequencing and confirmed by RFLP analysis. The mothers from both patients were heterozygous carriers for each mutation. Biochemical measurement of the respiratory chain enzymatic activities demonstrated a clear isolated complex I defect in the muscle and fibroblasts from these patients. Human complex I NDUFA1 subunit is a 70 amino acid, highly conserved membrane protein. Both mutations lead to aminoacid substitutions in evolutionarily conserved positions of NDUFA1. Previous studies have shown that the bovine/rodent homologous of human NDUFA1, MWFE, has an essential role for a complete functional complex I in mammals. Our findings agree with the relevant role of the NDUFA1 subunit in the biogenesis and function of mitochondrial complex I and provide new insights into complex I deficiency.

P2.4.4. ENERGETIC DEPRESSION DUE TO Ca^{2+} INDUCIBLE MITOCHONDRIAL DYSFUNCTION CONTRIBUTES TO HUNTINTON'S DISEASE IN STRIATUM, OTHER BRAIN REGIONS AND SKELETAL MUSCLE OF TRANSGENIC HD RATS AND MICE

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Huntington´s Disease (HD) is a progressive neurodegenerative disorder caused by a CAG repeat expansion in the coding region of the *IT15* gene resulting in an elongated polyglutamine stretch in the HD htt protein. The mechanism of cytotoxicity of HD htt is unknown. We hypothesized that mitochondrial dysfunction and disturbed Ca^{2+} signalling contribute to energetic depression [1] and atrophy of HD tissues [2].

Recently we investigated isolated mitochondria of atrophic skeletal muscle from 14 weeks old transgenic R6/2 mice in a medium A (A) containing 120 mM mannitol, 60 mM KCl, 100 µM EGTA, and 5 mM Pi and in medium B (B) containing 250 mM sucrose, 20 mM MOPS, 1 mM Pi. In A and in the absence of added Ca^{2+} the respirometric properties of HD mitochondria were not changed compared to the wild type but after addition of 10 or 20 µM Ca^{2+}, complex I dependent respiration was inhibited in HD mitochondria. Fluorimetric measurement of Ca^{2+} accumulation in B with Ca-green revealed a decreased ability of HD mitochondria to accumulate Ca^{2+}. Due to the large number of 150 glutamine repeats R6/2 mice is a model of juvenile HD, therefore it could be possible that the detected changes in the function of HD mitochondria were not detectable in animals with shorter repeat length.

We therefore started to investigate HD rats [3], which express 51 CAG-repeats and which closely resemble the adult onset phenotype of HD in humans. These animals are the first transgenic rat model for a neurodegenerative disease [3]. In preliminary experiments mitochondria from striatum, brain and skeletal muscle of 24 month old HD rats were compared. In contrast to R6/2 mice,

respiration of HD rat muscle mitochondria in A was not dependent on Ca^{2+} additions. This is an indication that changes of muscle mitochondrial function in 24 month old HD rat are less pronounced than in 14 weeks old R6/2 mice. However in medium B clear differences were detectable: Complex I-dependent respiration of HD mitochondria was instable even without Ca^{2+} – additions compared to WT.

In isolated mitochondria from HD rats we measured the rate of Ca^{2+} accumulation after stepwise Ca^{2+} additions. These accumulation rates were decreased in HD mitochondria from striatum, brain and in skeletal muscle mitochondria.

Data support our hypothesis that mitochondrial Ca^{2+}-metabolism is impaired in HD.

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P2.4.5. IDENTIFICATION OF THE LARGE CONDUCTANCE Ca^{2+}-ACTIVATED POTASSIUM CHANNEL IN RAT BRAIN MITOCHONDRIA

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A large amount of evidence has implicated mitochondria as the potential target for cytoprotective strategies. It has been shown that increased mitochondrial K^+ uptake may induce protection in different models of cell death. Electrogenic K^+ uptake into mitochondria could be catalyzed by K^+ selective channels such as mitochondrial ATP—regulated potassium channel (mitoK_{ATP} channel) and mitochondrial large conductance Ca^{2+}-activated potassium channel (mitoBK_{Ca} channel).

The mitoBK_{Ca} channel was initially described in human glioma cells LN229. Later it was also identified in the mitochondrial inner membrane of guinea pig ventricular cells. It has been shown that the pharmacological preconditioning with the use of BK_{Ca} channel opener – NS1619 protects heart against infarction. Hence, as in the heart, ischemic preconditioning can also increase the resistance of neurons to lethal ischemic insults, the aim of our studies was to test the presence of mitoBK_{Ca} channel in rat brain mitochondria. For this purpose, we employed immunohistochemical studies performed on paraffin-embedded rat brain sections with the use of specific antibodies against α and β_4 subunit of BK_{Ca} channel and anti-COX antibody as a mitochondrial marker. Findings derived from double-label immunohistofluorescence experiments revealed that the distribution of immunoreactivity generated by β_4 subunit antibody colocalizes with the mitochondria labeled with anti-COX antibody. We have shown that the punctate mitochondrial β_4 immunoreactivity is preferentially expressed in neurons with much less dense staining in glial cells. Taken together, these results suggest the presence of novel K^+ channel in rat brain mitochondria.

P2.4.6. ALTERATION OF ROS PRODUCTION AS WELL AS BCL-2 AND BCL-XL EXPRESSION UNDER DEVELOPMENT OF CANCER CELLS RESISTANCE

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In many cases the development of acquired resistance of cancer cells is connected with suppression of cellular susceptibility to induction of apoptosis by chemotherapeutic agents. Overexpression of Bcl-2 and Bcl-xl proteins in transfected cells has been reported to be associated with elevation of resistance to apoptosis effected by a variety of drugs (Reed, 1995; Ibrado et al., 1996). The aim of the study was a comparable investigation of possible link between alteration of cellular ROS level and expression of bcl-2 and bcl-xl genes under development of resistance of K562, MCF-7 and SCOV-3 cells to doxorubicin (DOX) possessed of pro-oxidant properties. It was found that the development of resistance of cancer cells to DOX was accompanied by the decrease of ROS production determined by different extent of the decrease of activities of some flavoenzymes including xanthine oxidase, NADPH-cytochrome P450 reductase which activate redox cycling of DOX followed by generation of $O_2^{\cdot}$, H_2O_2 and $^{\bullet}OH$. In contrast to resistant K562/DOX and SKVLB cells the cellular ROS level did not change in MCF-7/DOX cells in compare with sensitive cells. ROS level of resistant cancer cells was associated with changes of bcl-2 and bcl-xl genes expression. The growth of bcl-xl mRNA was detected by RT-PCR assay in all three types of cells. In contrast to K562/DOX and SKVLB cells bcl-2 mRNA was suppressed in MCF-7/DOX cells. Based on the data it can be suggested the existence of different types of relations between changes of cellular ROS level and expression of bcl-2 and bcl-xl genes under development of cancer cells resistance.

P2.4.7. DIVERGENT EFFECTS OF MUTATIONS THAT CAUSE TWO DIFFERENT MITOCHONDRIAL DISEASES, LHON AND MELAS, ON THE BACTERIAL NDH-1

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The mitochondrial NADH-ubiquinone oxidoreductase (complex I) converts the energy liberated in the oxidoreduction reactions into electrochemical potential across the membrane. Seven of the 46 subunits of complex I are encoded by the mitochondrial genome (mtDNA). The ND1 and ND6 genes in mtDNA are hot spots for Leber hereditary optic neuropathy (LHON) mutations and they accommodate also several mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes syndrome (MELAS) mutations.

We have built a system to model mitochondrial disease mutations in *Escherichia coli* NDH-1. Here we present the modeling of the human LHON mtDNA mutations 14459, 14484 and 14498 that cause the ND6 subunit A72V, M64V and Y59C alterations, respectively, as well as the MELAS missense mutations 3946 and 3949 that lead to E214K and Y215H changes in the ND1 subunit, respectively. Both modeled domains are well conserved and the mutated amino acids are the same in human and *E. coli* enzymes (except that Ala-72 is Met in *E. coli*). The corresponding NDH-1 mutants were generated and analyzed, using deamino-NADH, decylubiquinone and hexammineruthenium as substrates.

The modeled LHON mutations 14459 and 14484 decreased NDH-1 activity by 17-47 %, consistently with previous reports [1-3]. In addition, these mutations induced a slight decrease in the affinity for decylubiquinone. The 14498 mutation lowered activity by 27 % and, strikingly, induced a marked substrate inhibition at higher decylubiquinone concentrations. Interestingly, the double mutant, 14459 and 14484, also displayed substrate inhibition, pointing to an analogous effect of the different LHON mutations on the enzyme. The MELAS mutations decreased NDH-1 turnover but, unlike the LHON mutations in the ND6 subunit, did not alter ubiquinone binding. To our knowledge, this is the first time that biochemical differences between separate mitochondrial diseases or clinical entities affecting mitochondrial complex I can be shown in the same model system.

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P2.4.8. STUDY IN MECHANISM OF DIAZOXIDE EFFECTS ON Ca^{2+}-LOADED RAT HEART MITOCHONDRIA

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INTRODUCTION: In ischemia/reperfusion experiments on isolated rat heart it was shown that in the presence of diazoxide as for ischemic preconditioning contractile parameters of heart muscle restored and Ca^{2+} overload of mitochondria decreased. However, the mechanism of protective action of diazoxide inducing the pharmacological preconditioning is not clear as yet. MATERIAL AND METHODS: The effect of diazoxide on Ca^{2+}-loaded rat heart mitochondria was studied. Condition of the oxidative phosphorylation and the electron-transport chain enzymes was detected by mitochondrial respiration in state 3 and basal or state 4 or in the presence of uncoupler - 2,4-dinitrophenol (DNP). Permeability of the inner mitochondrial membrane to K^+ and H^+ ions was accordingly estimated by swelling of nonenergyzed mitochondria in KNO_3 and NH_4NO_3 media and by state 4 respiration. RESULTS: The presence of Ca^{2+} into a respiration medium has not affected on biochemical effects of diazoxide: diazoxide increased the permeability of the membrane to K^+ and H^+ ions and partly depressed state 3 and DNP-uncoupled respiration. CONCLUSION: It was supposed that the decrease of mitochondrial respiration at the same time increase of ion permeability of the inner mitochondrial membrane in the presence of diazoxide causes partial depolarization of the membrane and its thereby depresses energy-linked uptake of Ca^{2+} in the matrix protecting rat heart mitochondria from calcium overload in ischemia/reperfusion conditions.

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P2.4.9. ION CHANNELS FROM RAT SKELETAL MUSCLE MITOCHONDRIA

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Chloride and potassium selective ion channels are present in inner mitochondrial membrane. Potassium uptake upon mitochondrial energization may partly compensate the electric charge transfer produced by proton pumping and thus enable the formation of a pH gradient along with transmembrane electric potential. Chloride channels may be involved in several crucial processes such as regulation of mitochondrial volume or membrane potential. Single channel activity was measured after reconstitution of the inner mitochondrial membranes from rat skeletal muscle into a planar lipid bilayer. Two potassium channels types and one chloride channel were observed. After incorporation, in gradient 50/450 and 50/150 mM KCl (*cis/trans*) the potassium channels were recorded with a mean conductance of ~ 300 pS (mito BK_{Ca}) and 91 ± 10 pS, respectively. Chloride channel conductance was 56 ± 4 pS under gradient 50/450 mM KCl (*cis/trans*) conditions.

In gradient 50/450 mM KCl we observed increase propability of the big potassium channel opening in the presence of Ca^{2+} (*cis/trans*) (n = 6) and inhibition by iberiotoxin (IbTx) (*cis/trans*) (n = 2). The presence of mito BK_{Ca} channel in skeletal muscle mitochondria was further confirmed with the use of isolated skeletal muscle mitochondria. Furthermore, it was shown that chloride channels are inhibited by 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) and 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid (SITS) but only from *trans* side.

Our results suggest that there are potassium and chloride selective ion channels in inner mitochondrial membrane from rat skeletal muscle.

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P2.4.10. RECOMBINATION OF MITOCHONDRIAL DNA IN A DOUBLE HETEROPLASMIC FAMILY

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The absence of recombination of human mitochondrial DNA has been considered to be an established fact of mitochondrial genetics. However, direct experimental evidence for somatic recombination of human mtDNA in skeletal muscle has been provided recently [1,2]. To extend this study we investigated potential mtDNA recombination in a family harboring the heteroplasmic A8344G MERRF mutation in the mitochondrial $tRNA^{Lys}$ gene and additionally the heteroplasmic A16182C D-loop mutation in all tissues studied: blood cells, buccal cells, fibroblasts, urine sediment, skeletal muscle and brain. For both mutations we observed in four maternally inherited individuals roughly comparable allelic frequencies of 8344G and 16182A, respectively, with the lowest amounts in blood cells, followed subsequently by buccal cells, the urine sediment, fibroblasts, brain and skeletal muscle tissue. Detailed quantitative analysis of individual mutation loads revealed, however, in two individuals considerable deviations from a strict co-segregation with 8344G/16182A and 8344A/16182C as main genotypes. The putative original wild type allelic combination 8344A/16182A was additionally present in tissue samples of the sister of the patients mother while the double mutant 8344G/16182C was detectable in blood samples of the mother and the sister of the patient. This result, indicating tetraplasmy - a hallmark of recombination - was directly confirmed by single cell genotyping of fibroblasts from the sister of the patient. Re-mutation of 16182A could, however, also lead to the observed tetraplasmy. This possibility is improbable, since phylogenic studies indicate a 1.3-fold lower mutation rate of this particular site with respect to the entire D-loop [3]. Therefore, our data indicate recombination of human mitochondrial DNA in a family of maternally related individuals. We propose that the presence of recombination of human mitochondrial DNA has potential consequences for the interpretation of reticulations in the phylogenetic network of human mitochondrial DNA.

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P2.4.11. REDUCED LEVELS OF FRATAXIN IN FRIEDREICH'S ATAXIA CAN BE MEASURED QUICKLY AND ACCURATELY WITH A SIMPLE DIPSTICK IMMUNOASSAY

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Friedreich's Ataxia (FA) is a progressive neurodegenerative disorder caused by reduction in expression of the mitochondrial protein Frataxin. Reduced Frataxin levels result in disregulated iron metabolism and increased mitochondrial free iron levels. The increased free iron, in particular Fe^{3+}, in turn are believed to cause increased levels of free radicals and reactive oxygen species. Current hypotheses regarding the molecular pathology of FA suggest that it is the accumulated damage caused by these free radicals and reactive oxygen species that accounts for the progressive degenerative course characteristic of Friedreich's Ataxia. Although the genetic basis of FA is well characterized and gene-based tests are commonly used for diagnosis of FA, simple tests to measure levels of the Frataxin protein are needed: 1) to help better characterize the genotype-phenotype relationship, and 2) to monitor the efficacy of therapies directed towards a cure, which will require upregulating levels of the Frataxin protein. To this end, we have developed a simple immunodipstick test for Frataxin protein. The dipstick tests can be used to measure Frataxin levels accurately and rapidly in cells and tissues, and have a dynamic linear range of 10 to 1000 picograms Frataxin per test (using recombinant Frataxin as a reference standard). Using these tests, we have measured reduced Frataxin levels in lymphoid cells taken from FA patients, and have documented patient levels as low as 1% of control values. Finally, we have shown that the tests can be used to measure Frataxin levels in easily obtained tissues such as routine blood samples and in cheek epithelial cells collected by buccal swabs. The buccal swabs in particular are an attractive sample to consider for the repetitive testing needed to monitor efficacy of potential FA therapies, as the sampling is gentle and essentially non-invasive. Therefore, we are currently working to determine the clinical utility of these Frataxin dipstick/buccal swab tests for diagnosis and molecular characterization of FA and as theranostics to monitor efficacy of FA therapies.

P2.4.12. ERYTHROCYTE MEMBRANE ELECTRON TRANSFER, DIET AND LIFESTYLE IN HEALTHY AND IN TYPE 1 DIABETIC FAMILIES

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Human erythrocyte membrane contains electron transfer systems, which protect against extracellular pro-oxidant challenge and whose activity appears to be closely related to the metabolic state of the cell and could be modifiable by lifestyle. Particularly, erythrocytes export electrons across the cell membrane to external oxidants such as ferryicyanide (RBC vfcy). The rate of ferricyanide reduction varies as a function of cytoplasmic electron donor concentration. Having previously characterised RBC vfcy in patients with type 1 diabetes (T1D), present study aimed at evaluating modulating effects of diet and lifestyle on RBC vfcy in healthy and T1D relatives.

We measured RBC vfcy in 95 healthy controls and 76 non-diabetic relatives of type 1 diabetics whose dietary habits and lifestyle were assessed by using the European Prospective Investigation of Cancer and Nutrition questionnaires.

RBC vfcy was 12.1±3.6 μmol/ml h (mean±SD) in 95 healthy controls and 12.6±4.2 in 76 T1DM relatives. Daily intake of vitamin C was 121±47 mg in 95 healthy controls and 131±64 in 76 T1DM relatives; regular exercise was 2.4±1.8 hour/week and 2.1±1.9, respectively.

Both Spearman's rank correlation and stepwise multiple regression analyses including lifestyle information found the following independent variables to be positively associated with RBC vfcy: daily dietary intake of vitamin C among healthy controls (Rho 0.23, Z-value 2.22, p 0.03), whereas time spent in regular exercise among relatives (0.36, 3.05, 0.002).

Dietary intake of vitamin C and sporting activities modulate erythrocyte electron transfer efficiency. In the cytosol, ascorbic acid or vitamin C can donate electrons to trans-plasma membrane electron transfer activity in erythrocytes. Thus, intracellular electron donors available from dietary sources can be very important in maintaining the redox environment of a cell. Our data also support indirect evidence suggesting that regular exercise may improve electron transport efficiency. However, the reason why independent lifestyle variables associated with RBC vfcy markedly differed among population subgroups remains unknown.

P2.4.13. MITOCHONDRIAL DNA (MTDNA) MUTATIONS IN HELICOBACTER PYLORI CHRONIC GASTRITIS AND GASTRIC CARCINOMA

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Mitochondrial DNA (mtDNA) is a likely hot spot for mutations in cancer as it is preferentially modified by many carcinogens. mtDNA alterations have been observed in various types of tumors, including gastric carcinoma. Gastric carcinoma is considered a long-term consequence of H. pylori chronic gastritis. Therefore, we aimed at elucidating the significance of mtDNA mutations in the context of H. pylori chronic gastritis and gastric carcinoma.

A total of 150 H. pylori infected patients (99 with chronic gastritis and 61 with gastric carcinoma) were included in this study. DNA isolated from gastric samples and matched blood or adjacent normal tissue, was screened for mutations in mitochondrial genes encoding NADH dehydrogenase 1, 3, 4, and 5 (ND1, ND3, ND4, and ND5), cytochrome c oxidase subunit I (COI), ATP synthase (ATPase6), and two regions of the displacement loop (D-Loop; D310, corresponding to a mononucleotide C repeat, and D514, corresponding to a dinucleotide CA repeat). H. pylori vacA and cagA genotypes were determined by multiplex PCR and reverse hybridization (LiPA).

Overall, mtDNA mutations were more frequent in gastric carcinoma (28/61; 46%) than in chronic gastritis patients (20/99; 20%) ($p<0.0001$). D-Loop mutations were more frequently detected in gastric carcinoma (22/61; 36%) than in chronic gastritis patients (17/99; 17%) ($p=0.008$). Both gastric carcinoma and chronic gastritis patients harbored D310 mutations (19/61; 31% vs. 17/99; 17%, respectively), whereas D-Loop D514 mutations were restricted to gastric carcinoma cases (9/61; 15%).

In coding genes, mutations preferentially affected complex I genes, and were more frequent in gastric carcinoma (13/61; 21%) than in chronic gastritis (5/99; 5%) ($p=0.003$). ND1 mutations were present in both groups of patients, whereas ND3, ND5, COI, and ATPase6 mutations were gastric carcinoma specific. Mutations in coding genes were frequently transitions, preferred targets for oxidative DNA damage.

Unconditional logistic regression was performed, with both mtDNA mutations and H. pylori genotypes being tested in the model. In the final model, both the term for mutations in the D-Loop region, and the term for H. pylori vacA m-region were statistically significant. The threshold of

statistical significance was not attained for H. pylori vacA s-region, H. pylori cagA, as well as for mutations in coding genes, probably due to lack of statistical power.

Altogether, these results suggest that mtDNA mutations are frequent events in gastric carcinoma, and that these mutations are independent of H. pylori genotypes.

P2.4.14. MITOCHONDRIAL ROLE IN OXIDATIVE STRESS UNDER ISCHEMIA/REPERFUSION IN THE RAT KIDNEY

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Oxidative stress is probably the major result of ishemia followed by reoxygenation (I/R). Mitochondria are considered to be one of the major source for the cellular reactive oxygen species (ROS), and they play a key role in the balance between a *signalling ROS* and *pathological ROS*. Signalling ROS make a significant contribution to the process of the cell anti-oxidative stress defence (hypoxic/ischemic preconditioning (PC)).

The main goal of the present study was to investigate damage and defence mechanisms under the conditions of I/R in rat kidney.

Vital kidney slices we found to be a reliable model which could be tested in a model of ischemic/reoxygenation known as a representative example of the oxidative stress.

Confocal microscopy of the cells, loaded by TMRE, revealed a dramatic loss of the mitochondrial membrane potential through out the kidney tissue under oxidative stress caused by I/R. This effect was significantly blocked by cyclosporin A (CSA), i.e. apparently was due to the induction of mitochondrial permeability transition (MPT). Mitochondria in a kidney cells exposed to I/R demonstrated a significant volume increase and fragmentation of whole reticulum.

ROS production examined by DCF fluorescence in renal tissue exposed to I/R was about doubled as compared to control sample. DCF fluorescence profiles were colocalized with mitochondria, thus showing that mitochondria were responsible for the ROS burst caused by I/R.

Similar results were obtained for nitric oxide production evaluated by DAF-2 fluorescence. Mitochondrial nature of NO burst was demonstrated by DAF-2 colocalization to TMRE signal. Thus we have demonstrated that in the kidney I/R besides oxidative also causes nitrosative stress. Mitochondria become not only an origin but also a target of this stress.

One of the most potent forms of protection against I/R damage is a tissue adaptation (PC such as ischemic or pharmacological). In case of both ischemic, hypoxic and pharmacological PC (LiCl infusion), we were able to provide a protection for a kidney by a partial restoration of the mitochondrial membrane potential, lowering ROS and NO production These protective effects correlated with the appearance of the phosphorylated form of GSK-3b which corresponded to its inhibition.

Apparently, mitochondria play a crucial role in the mechanism of the I/R-induced kidney damage as well as in a signaling protective mechanism.

P2.4.15. MITOCHONDRIA IN PERIPHERAL NEUROPATHY: CORRELATION OF MOLECULAR GENETIC AND FINE STRUCTURAL CHANGES

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Mitochondria in neurons, Schwann cells, and blood vessels are essential for the normal structure and function of the peripheral nervous system (PNS). They may undergo functional and structural changes in primary disorders of the mitochondria due to mutations of mitochondrial and nuclear genes [1]. Or they may be secondarily affected which is apparent in a large variety of endogenous or exogenous conditions, i.e., metabolic, traumatic, ischemic, immunologic, and toxic states. Their structure may be altered by swelling or shrinkage, calcium or iron ingestion, enlarged matrix granules, increased or decreased numbers or abnormal shape of mitochondrial cristae, and characteristic inclusions such as granular, paracrystalline, or 'amorphous' structures. Abnormalities of their outer membranes are less obvious although of special interest because of recently detected diseases affecting genes involved in the formation, fusion and fission of mitochondria. These include mitofusin 2 (*MFN2*) mutations [2] and ganglioside differentiation associated protein 1 (*GDAP1*) [3]. While these disorders are caused by mutations of nuclear genes, mitochondria in the PNS are also involved in disorders caused by mutations of the mitochondrial DNA itself. Some of these may cause multisystem disorders (KSS), or more localized manifestions primarily affecting the PNS (NARP), skeletal muscle [4, 5], cardiac muscle, gastrointestinal system (MNGIE), the optic system (LHON), or the central nervous system (KSS, MELAS, MERRF, MILS, Leigh syndrome), and other organs. Thus far there are very few structural, light or electron microscopic studies available illustrating fine structural and other alterations of mitchondria in disease of *MFN2* and *GDAP1* mutations. These and other ultrastructural changes of mitochondria in the PNS and skeletal muscle will be illustrated for better understanding the molecular genetic, biochemical, and pathophysiological disturbances characterizing such diseases.

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P2.4.16. THE LHON-ASSOCIATED MUTATIONS 4136, 4160 AND 4171, AFFECTING THE ND1 SUBUNIT OF COMPLEX I, AND THE CONSERVED REGION IN THEIR VICINITY STUDIED IN ESCHERICHIA COLI

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Complex I is the largest of mitochondrial respiratory chain complexes, and knowledge of its functional mechanism is still incomplete. Seven of its subunits are mitochondrially encoded and have been shown to harbor pathogenic mutations. The first disease linked to mtDNA was Leber hereditary optic neuropathy (LHON), which was traced to a mutation in the ND4 gene. Since then, mutations in several other ND genes of mtDNA have been shown to cause LHON.

Several mutations in ND1 subunit are associated with LHON and, apart from the common 3460 mutation, their enzymological consequences are not known. Here we mutated amino acids in a highly conserved region in the fourth matrix side loop of the NuoH subunit of *E. coli* NDH-1 and modeled the neighbouring LHON-associated mutations 4136/Y277C, 4160/L285P, C4171A/L289M [1,2], with corresponding amino acid substitutions. The deamino-NADH (d-NADH) dehydrogenase activity, decylubiquinone binding and assembly of the enzyme were assayed with hexammineruthenium (HAR) or decylubiquinone (DB) as electron acceptors.

When the d-NADH:DB oxidoreductase activities were normalized on the basis of the d-NADH:HAR oxidoreductase activities, the D295A mutation caused the largest decrease (44%) in enzyme activity compared to the control while the effects of the other mutations were milder. The Q296T mutation impaired the enzyme activity by 19 %. Nevertheless, neither of these mutations caused decrease in enzyme affinity to decylubiquinone. In spite of the decrease in enzyme activity, all analyzed mutant strains were able to grow on malate, which necessitates sufficient intact NDH-1 activity. In light of the high degree of evolutional conservation of the amino acid residues studied, the effects of their mutations were milder than expected.

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P2.4.17. PHENOLIC COMPOUNDS FROM HYPERICUM PERFORATUM PROTECT RAT HIPPOCAMPAL NEURONS FROM EXCITOTOXIC AND AMYLOID-BETA TOXICITY: A ROLE FOR MITOCHONDRIA

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Recently, we showed *Hypericum perforatum* extracts to be neuroprotective against amyloid-beta 25-35 (Aβ (25-35))-induced toxicity **[1]**. In this study, we assessed the neuroprotective role of *Hypericum perforatum* phenolic compounds against excitotoxic and Aβ (25-35) using cultured rat hippocampal neurons. Additionally, the neuroprotective effect of the compounds was also evaluated following an insult with N-methyl-D-aspartate (NMDA) plus kainate (KA), due to previous reports indicating that exposure to Aβ (25-35) renders neurons more vulnerable to excitotoxicity. Viability was assessed by using the Syto-13 and propidium iodide live/death assay, and nuclear morphology was evaluated by using Hoechst 33342 staining. Quercetin, kaempferol and biapigenin (10 μM) significantly reduced Aβ (25-35) (55±4%, 66±4% and 75±2%, respectively) and NMDA plus KA-induced toxicity (49±4%, 48±6% and 72±6%, respectively).

To test the involvement of mitochondria in the mechanisms of neuroprotection, we evaluated delayed calcium deregulation (DCD) and loss of mitochondrial transmembrane potential ($\Delta\Psi_m$) induced by continuous exposure of hippocampal neurons to NMDA plus KA. Intracellular calcium was assessed by using Fura-2, and $\Delta\Psi_m$ was monitored by using tetramethylrodamine methyl ester ($TMRM^+$). Among the tested phenolics, biapigenin was the most effective compound in delaying the loss of $\Delta\Psi_m$ and DCD, which are events that can be causally associated to cell death. Further experiments were conducted by using isolated brain mitochondrial fractions and tetraphenylphosphonium ion (TPP+)-selective electrode and a Clark oxygen electrode to assess mitochondrial $\Delta\Psi$ and oxygen consumption, respectively. To evaluate the possible protective role of the compounds against oxidative stress and calcium-induced dysfunction, mitochondrial fractions were exposed to ADP plus iron and supra-physiological calcium pulses.

Taken together, the results of the present study strongly indicate that HP extracts may be endowed with neuroprotective phenolics and also that the mechanisms underlying neuroprotection may

involve antioxidant properties, the preservation of mitochondrial function and buffering of intracellular free calcium concentration.

[1] – Silva et al. Neuroprotective effect of *H. perforatum* extracts on beta-amyloid-induced neurotoxicity. Neurotox Res. 2004;6(2):119-30.

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P2.4.18. DEVELOPMENT OF NEW MODEL FOR SEARCH AND STUDY OF ACTION MECHANISM OF NEUROPROTECTING COMPOUNDS

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New model of brain cortex tissue slices survived in anoxia was developed: 1) The dynamics of mitochondrial functions changes (membrane potential, respiration rate, effectiveness of oxidative phosphorylation) was studied. The mitochondrial membrane potential, respiration and effectiveness of oxidative phosphorylation was found completely saved after 1 hour of incubation in anoxia at room temperature. Mitochondria isolated from these slices after 24 hours of incubation was found to keep succinate and malate oxidative activity, but loose membrane potential and oxidative phosphorylation coupling. Suppressed I complex of respiratory chain activity was detected in mitochondria isolated from these slices after 48 and 72 hours, but activity of the II complex was maintained. 2) The internucleosomal DNA fragmentation was detected as apoptosis marker. 3) The electron microscopy study of ultrastructure of slices incubated in anoxia for 72 hours was carried out. It was found that tissue ultrastucture was highly damaged and can be characterized by concentrical myelin membranes profiles. Obtained model was tested for sensitivity to addition of glycine – known neuroprotecting compound. Glycine was shown to delay the dynamics of internucleosomal DNA fragmentation, and prevents the tissue ultrastructural damages.

P2.4.19. ASSEMBLY OF THE OXIDATIVE PHOSPHORILATION SYSTEM IN X-LINKED ISOLATED COMPLEX I DEFICIENCY

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Isolated complex I deficiency is a frequently diagnosed defect of the mitochondrial OXPHOS system. Mammalian complex I is made up of at least 46 subunits, 39 encoded by the nuclear genome and 7 encoded by the mitochondrial DNA. Most mutations in the complex I nuclear structural genes involve a problem in the correct assembly/stabilization of the complex, leading to a low complex I activity, increased ROS production and cell damage in the most affected tissues. Gaining insight in complex I assembly will thus lead to a better understanding of the molecular mechanisms underlying complex I deficiency.

We have performed 2D-blue native gel electrophoresis using fibroblasts from two patients with a X-linked isolated complex I deficiency attributable to mutations in a structural gene of the complex, *NDUFA1*. An important decrease in the levels of intact complex I can be observed in both patients, suggesting that complex I assembly and/or stability is compromised. NDUFA1 would be incorporated into the complex at a late assembly stage. The patterns of incorporation of other complex I subunits into subcomplexes suggest that the stabilization rather than the assembly of the membrane arm might be affected by the *NDUFA1* mutations. Mutations in the *NDUFA1* gene seem also to affect the expression and activity of other mitochondrial complexes. Our results might give an explanation for the different clinical phenotypes of these patients and become useful for future diagnostic purposes.

P2.4.20. N-ARACHIDONOYLETHANOLAMINE (ANANDAMIDE) INFLUENCES MITOCHONDRIA IN LIVING CELLS

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N-Arachidonoylethanolamine (anandamide), along with other N-acylethanolamines (NAEs), is present in various mammalian tissues in small amounts. The content of NAEs in heart and brain increases dramatically during ischemia. Mitochondria play a major role in ischemic tissue damage due to the production of reactive oxygen species (ROS) and release of proapoptotic molecules. NAEs have been shown to affect isolated mitochondria by inhibition of the respiratory chain and increase in the permeability of the inner mitochondrial membrane [1,2]. The present study was aimed to investigate whether anandamide can also alter functions of mitochondria within living cells. HL-60 human leukemia cells were used for this purpose. It was found that anandamide inhibited respiration and increased the $NADH/NAD^+$ ratio immediately after addition. In cells treated with anandamide oligomycin dissipated mitochondrial electrochemical potential. Similar results were also obtained with the non-hydrolysable derivative, methanandamide. Other NAEs and arachidonic acid did not cause such effects. In a longer time scale these changes were followed by reduction of ROS production and eventually by cell death. These findings suggest that anandamide inhibits the respiratory chain and affects ROS production in living cells – critical processes for cell fate in ischemic tissue. More generally, anandamide could modulate ischemic damage by its influence on mitochondria.

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Poster session 2.5. Program death of cells and mitochondria

P2.5.1. MOLECULAR MECHANISMS UNDERLYING BAX/MITOCHONDRIA INTERACTIONS DURING APOPTOSIS: A STUDY IN YEAST

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Bax is a member of the Bcl-2 family which plays a key role during apoptosis. In healthy mammalian cells, Bax is essentially located in the cytosol. After the induction of apoptosis, it undergoes a conformational change and is translocated to the mitochondrial outer membrane (MOM), where it participates to the release of cytochrome c.

We have developed a tool, the budding yeast *Saccharomyces cerevisiae* which is naturally devoid of the presence of any Bcl-2 family member, to study the different steps involved in the translocation and activation of Bax. When expressed in yeast, the human full-length Bax remains cytosolic and no cytochrome c is released from mitochondria.

Through site-directed mutagenesis by substitution of selected residues, we investigated the role of the N and C-terminal ends of Bax in its addressing and insertion in the MOM.

The results obtained showed that the mobility of the two ends of the protein is crucial for its conformational change needed in order to be targeted to and inserted in the MOM. Indeed, the movement of these two extremities will enable the protein to uncover the domain (the α1 helix) necessary to interact with mitochondrial protein(s), thus facilitating its insertion in the correct conformation in the MOM. In parallel, our data demonstrates that the insertion of the hydrophobic C-terminal helix α9 is not required for Bax-insertion.

Post-translational modifications such as phosphorylation of certain residues may play an important role in the regulation of translocation and activation of Bax for initiating conformational changes. It has been observed that in polynuclear cells, the phosphorylation of the Ser184 by AKT is essential for Bax to remain cytosolic. We expressed full-length Bax and its active variants in a yeast strain

ΔSch9 (Sch9 being the yeast functional homologous kinase of human AKT). We observed that in ΔSch9 strain, full-lenth Bax and other inactive variants were activated whereas active variants partially lost their activity. These results strongly suggest that the phosphorylation process has an important function in the regulation of Bax and that one or more phosphorylable residue(s) distinct from Ser184 may also play a role in this regulation.

We also observed that mutations of substitution in the C-terminus of Bax impaired the interaction with the anti-apoptotic protein Bcl-x_L and thus its inhibitory effect. These observations suggests that the conformation and/or the primary sequence of the C-terminus of Bax has a role in the interaction with Bcl-x_L.

P2.5.2. EARLY CHANGES IN MITOCHONDRIAL PHYSIOLOGY DURING TNF-INDUCED APOPTOSIS OF LYMPHOID U937 CELLS

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We analysed the time course of the cytotoxic effect of tumor necrosis factor - α (TNF) applied together with the inhibitor of protein synthesis, emetine. These drugs induce mitochondria-mediated apoptosis of the lymphoid U937 cells. It has been shown that at the early stage of cytokine action about 30 min after addition, i.e. long before the cytochrome *c* release from mitochondria, considerable changes in mitochondrial physiology occurred. They consisted of rapid increase in rates of respiration (30% increase) and glycolysis (2-fold increase). As a result, mitochondrial transmembrane potential and ATP-generation level increased too. In 1-2 h, the coupled as well as uncoupled respiration and ATP level declined to 50-60% of the control values. At that time, the respiratory control ratio decreased 2-fold. At this stage, the low rate of uncoupled respiration could be restored by addition penetrating respiratory substrates, i.e. β-hydroxybutyrate or ascorbate with TMPD. It is suggested that substrate entry into mitochondria is somehow blocked at this early stage.

The last events (3-5 h after TNF addition) are characterized by further decrease in respiration rate (by 80%), insensitivity of respiration to substrate addition, cytochrome *c* release from mitochondria to cytosol, loss of mitochondrial potential and finally fragmentation of nucleus.

Thus, three stages of respiration changes could be distinguished during the TNF-induced apoptosis: 1, respiration increase; 2, reversible respiration decrease, 3, irreversible respiration decrease.

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P2.5.3. MECHANISM OF A GLUTAMATE-INDDUCED MITOCHONDRIAL DEPOLARIZATION IN CULTURED CORTICAL NEURONS

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The hypothesis on the dominant role of mitochondrial permeability transition pore (PTP) in the mechanism of glutamate-induced delayed Ca^{2+} deregulation (DCD) in cultured neurons is largely based on the finding that PTP inhibitor, cyclosprin A (CsA), is able to antagonize DCD development. However, this effect of CsA proved to be poorly reproducible. Therefore here we used another approach to this problem: we studied the effect of a prolonged glutamate challenge on the relationships between mitochondrial and cytosolic pH (pHm and pHc, respectively). To this aim the pH-sensitive yellow fluorescent protein (YFP) and the green fluorescent ratio-metric pericam were selectively expressed in the mitochondria and cytosol of cultured rat cortical neurons. The changes in pHc and pHm were compared with those in $[Ca^{2+}]_i$ and mitochondrial potential (measured by fura2 FF and rhodamin 123). We found that the secondary $[Ca^{2+}]_i$ and MD increase were accompanied with a strong mitochondrial acidification caused by proton influx into the matrix presumably through PTP, as this acidification was reverted to a transient alkalization after external Ca^{2+} replacement by Sr^{2+} or by addition of CsA to the Ca^{2+} -containing medium. Such a mitochondrial alkalinization ruled out a possibility that a nonspecific protons permeability increase underlies a delayed MD in the presence of Sr^{2+} or CsA. These data suggest the existence of two alternative mechanisms of a delayed MD triggered by mitochondrial Ca^{2+} overload: PTP opening and matrix alkalinization. Supported by grants of RFBR, Ministry of Education and Science and NATO.

P2.5.4. INVOLVEMENT OF REACTIVE OXYGEN SPECIES IN THE MITOCHONDRIAL PATHWAY OF ARACHIDONIC ACID-INDUCED APOPTOSIS

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Arachidonic acid at micromolar concentrations functions as a weak uncoupler of oxidative phosphorylation in hepatoma AS-30D cells, as it decreased the mitochondrial membrane potential within the cells and increased cell respiration. The effect on cell respiration was partially prevented by blocker of the mitochondrial permeability transition pore, cyclosporin A. Arachidonic acid also significantly increased the rate of reactive oxygen species production (ROS) and was a potent inducer of apoptosis as visualized by externalisation of phosphatidylserine and oligonucleosomal DNA fragmentation. This process was accompanied by a release of cytochrome c from mitochondria to the cytosol, activation of caspase-3 and association of the proapototic protein Bax with mitochondria, indicating for the mitochondrial pathway of apoptosis [1].

Here we show that the water-soluble vitamin E derivative, Trolox, a known potent antioxidant, completely protects against arachidonic acid-induced apoptosis in AS-30D cells along with prevention of the increase in ROS production by this fatty acid. Similar effect was also observed in human promyelocytic leukaemia HL-60 cells. These results point to ROS as a possible important mediator in arachidonic acid-induced apoptosis

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P2.5.5. EFFECT OF PROTEIN SYNTHESIS INHIBITORS ON THE COURSE OF PROGRAMMED CELL DEATH IN PEA GUARD CELLS

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Pea leaf epidermis incubated with cyanide displayed ultrastructural changes in guard cells that are typical of apoptosis [1]. Cycloheximide, an inhibitor of cytoplasm protein synthesis, and lincomycin, an inhibitor of protein synthesis in chloroplasts and mitochondria, produced different effects on the dynamics of CN^--induced programmed death of guard cells. According to light microscopy data, cycloheximide reinforced and lincomycin suppressed the CN^--induced apoptosis of guard cells based on the detection of disintegration and disappearance of cell nuclei. Lincomycin lowered the effect of cycloheximide in the light and prevented it in the dark. Based on electron microscopy data, the most appreciable effects of cycloheximide in the presence of cyanide were autophagy and a lack of apoptotic condensation of nuclear chromatin, the prevention of the rupturing of chloroplast envelops and its invagination inside the stroma, the appearance of particular compartments with granular inclusions in mitochondria. Lincomycin inhibited the CN^--induced ultrastructural changes in guard cell nuclei. Lincomicin, unlike cycloheximide, suppressed the oligonucleosomal DNA fragmentation in cells of leaf epidermis. The data obtained show that the programmed death of guard cells may have a combinated scenario involving both apoptosis and autophagy and may depend on the action of both cytoplasm-synthesized and chloroplast- and mitochondrion-synthesized proteins. In all likelihood, chloroplast- and mitochondrion-synthesized proteins act as proapoptotic factors and cytoplasm-synthesized proteins protect pea guard cells against programmed cell death. This work was supported by the Russian Foundation for Basic Research (grant № 04-04-48121).

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P2.5.6. THE ROLE OF CYCLOPHILIN D IN THE RESISTANCE OF BRAIN MITOCHONDRIA TO INDUCTION OF THE MITOCHONDRIAL PERMEABILITY TRANSITION

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The mitochondrial permeability transition (MPT) plays an important role in both necrosis and apoptosis. Cyclophilin D (CyD) protein is an important component of the MPT pore complex. Recent studies on CyD knockout mice indicated the importance of this protein for MPT opening. Brain mitochondria have been found to have a decreased ability to undergo the MPT when compared to heart or liver mitochondria. We hypothesized that this may be due to low expression of CyD in brain tissue. We have found that CyD expression progressively decreases in rat brain during development. Sensitivity of brain mitochondria to the Ca^{2+}-induced MPT decreases in parallel to the decrease in CyD expression. When compared to heart or liver, CyD expression is significantly reduced in adult rat brain. We also used an in vitro model of neuronal development comprised of PC12 cells, which differentiate into a neuronal-like phenotype following NGF treatment. Mitochondria in NGF-treated PC12 cells have a decreased ability to undergo the MPT and express significantly less CyD in comparison to mitochondria in non-differentiated cells, but sensitivity to the MPT is restored after vector-mediated overexpression of CyD. Specific anti-sense knockdown of CyD in non-differentiated PC12 cells results in reduced sensitivity to induction of the MPT. Together our data indicate that the decreased sensitivity of brain mitochondria to the MPT correlates well with low expression levels of CyD in brain; and that neuronal cells lose CyD during differentiation and become more resistant to MPT induction. This may be an adaptive protection mechanism that raises the threshold of brain tissue against injuries.

P2.5.7. SUPPRESSION OF LIVER ENERGY METABOLISM AS AN ATTRIBUTE OF LAMPREYS (LAMPETRA FLUVIATILIS) DURING PRESPAWNING MIGRATION

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The depression of cellular energy metabolism is characterized by alterations of mitochondrial properties. Decreased concentrations of ATP and ADP, decreased activities of respiratory chain enzymes, leaky mitochondrial membranes promote many pathological processes and diseases in mammals, including human beings [Gellerich et al., 2004]. However, these mitochondrial changes are quiet acceptable and sometimes essential for poikilothermic vertebrates. We have investigated the mechanisms of metabolic depression in liver mitochondria of Baltic lamprey during the pre-spawning migration. Lampreys are monocyclic animals, which die after the spawn in spring. Having come into the Neva River from the Baltic sea in autumn, the lampreys stop eating. During winter the alimentary canal atrophies, the liver drainage system regresses, and reversible metabolic suppression is observed in the hepatocytes [Sterling et al., 1967; Savina and Gamper, 1998]. The respiration rates of isolated lamprey liver mitochondria oxidizing pyruvate + malate and succinate in state 4 are 3.5-4.5 and 6.3-6.8 nmol O_2/min/mg protein, respectively. The oxidation rates of NAD - and FAD – dependent substrates in state 3 are correspondingly 7.1 and 12.5-15.3 nmol O_2/min/mg protein. The low efficiency of oxidative phosphorylation system accounts for the marginal respiratory controls lying within 1.8-2.0, ADP/O 1.2-1.5 and phosphorylation rates 15.6-22.5 nmol ADP/min/mg protein. The intramitochondrial exchangeable (ATP + ADP) pool is also low (2.0-2.8 nmol/mg protein). Besides, we have found that lamprey liver mitochondria swell in NH_4NO_3 medium over winter as a result of proton permeability of membranes. The swelling in the presence of pyruvate + malate is significantly inhibited by either CsA + ADP + Mg^{2+} or EGTA. The inhibitory effects of heparin, BSA, or CsA, if taken individually, are insignificant. In Aprile the oxidation rates of pyruvate and malate in state 4 increase by a factor of 3.5 and succinate by a factor of 2. Moreover, the respiratory controls amount to 2.2-3.5, ADP/O to 2.0-2.8, and phosphorylation rates reach 105.2-229.3 nmol ADP/min/mg protein. There is no significant swelling in NH_4NO_3 medium in the presence of pyruvate + malate, as well as there is no effect of either CsA or any other pore inhibitors. We suppose that the suppression of energy metabolism provokes opening of the pore in low conductance state in lamprey mitochondria over winter. There exists an analogy between some molecular mechanisms underling the metabolic depression in lamprey liver cells and

those in cells of patients suffering from mitochondrial encephalomyopathies, neurodegenerative diseases and sepsis [Luft, 1994; Halestrap et al., 2004].

P2.5.8. MULTIPLE MECHANISMS OF CYTOCHROME C RELEASE IN ARSENIC-INDUCED CELLS DEATH

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Arsenic trioxide, As(III), is a known environmental toxicant, co-carcinogen, and also a potent chemotherapeutic agent. As(III) was shown to induce apoptosis in a variety of transformed cell types, which also contributes to its anti-tumor activity [1]. Previous studies have demonstrated that As(III) and its derivatives cause direct damage to mitochondria [2], but it is not clear if these effects initiate apoptosis.

Here we show that in model experiments with isolated rat liver mitochondria, As(III) stimulates a dose-dependent, cyclosporin A-sensitive release of cytochrome *c* via induction of mitochondrial permeability transition, subsequent swelling of mitochondria and the rupture of the outer mitochondrial membrane. Mitochondrial GSH does not seem to be a target for As(III), which, however, appears to cause oxidative modification of thiol groups of pore forming proteins, notably adenine nucleotide translocase [3].

In mouse embryonic fibroblasts, 10 μM As(III) stimulated cytochrome *c* release and apoptosis via a Bax/Bak-dependent mechanism. The release was observed in wild-type but not in $Bax^{-/-}/Bak^{-/-}$ cells. At high concentrations (125 μM and higher), release of cytochrome c occurred in both cell lines; cells died by Bax/Bak-independent necrosis. At this concentration range As(III) targets mitochondria directly, particularly complex I of the mitochondrial respiratory chain. Since pyruvate, a substrate of complex I, is a predominant mitochondrial substrate in the cell, inhibition of complex I will cause mitochondrial instability and a decrease of the mitochondrial membrane potential that facilitates permeability transition and causes necrotic cell death.

Our data demonstrate for the first time that the cytochrome *c* release, which initiates apoptosis in cells exposed to low therapeutically relevant concentrations of this classic mitochondrial poison, occurs indirectly via the activation of Bax/Bak rather than via direct mitochondrial damage. Furthermore, the results implicate reactive oxygen species in a concentration-dependent mechanistic switch between apoptosis and necrosis.

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P2.5.9. MITOCHONDRIAL DISODERS: DIAGNOSIS AND CLINICAL SPECTRUM

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Mitochondrial disorders (defect of oxidative phosphorilation) are a group of diseases, mostly originating from mutations in the mitochondrial or sometimes nuclear DNA. In patients with mitochondrial disorders unexplained combination of symptoms from unrelated organs and tissues (previously neuromuscular) and progressive course was observed.

We revealed 33 patients from two months to 17 years with mitochondrial disorders with an mtDNA mutations in our center. Neurological symptoms (from muscular hypotonia, recurent apnoe, seizures, myoclonia to cerebral ataxia, leukodystrophy) have all our patients of this group. Myopathy has two children. Heart injures -progressive hypertrophy and dilatation cardiomyopathy, WPW-syndrome has have displaced in eight patients. Endocrine injures have revealed in six children: hypothyroidism, growth retardations, diabetes mellitus). One child has the gastrointestinal pseudoobstruction.

Mutations in 16S RNA gene was revealed in 11 patients, ATF-syntetase gene – in 6 children, NADH dehydrogenase gene – in 4 children, ND1 region - in 2 patients, ND6 region - in 2, 12S RNA gene – 3 patients, tRNA lysine – in 1 patient, tRNA arginine – 1 child. Two children was with MELAS syndrome, one – with multiplied depletions of mt DNA.

P2.5.10. CYANIDE-INDUCED APOPTOSIS IN PEA LEAFS

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Cyanide is an inducer of the programmed death (PD) of the stoma guard cells (GC) and the basic epidermal cells (EC) in the peels of the lower epidermis isolated from pea leaves [1]. The cyanide-induced PD of GC was determined as apoptosis by means of electron microscopy [2] and fluorescent microscopy. H_2O_2 addition strengthened the CN^--induced PD in the GC and to lesser degree in the EC. The maximum effect of H_2O_2 on the GC was revealed to occur with 100 μM H_2O_2. Menadione and methyl viologen as electron acceptors in chloroplasts strengthened the H_2O_2 generation in the light, but prevented CN^--induced apoptosis in GC. Menadione and methyl viologen did not suppress cyanide-induced PD in EC that, unlike GC, contain only mitochondria, but not chloroplasts. Nitroblue tetrasolium oxidizing by superoxide anion-radical suppressed CN^--induced apoptosis in GC.

NADPH oxidase of cell plasma membrane is a multienzymatic complex that generates superoxide. Quinacrine and diphenylene iodonium know as inhibitors of this NADPH oxidase did not affect on the respiration and photosynthetic O_2 evolution by leaf slices, but prevented the cyanide-induced death of GC and had no effect on cyanide-induced death of EC. The data obtained enable us to suggest that NADPH oxidase of plasma membrane in GC is apparently a source of reactive oxygen species (ROS) for cyanide-induced apoptosis. Chloroplasts and mitochondria were inefficient as ROS sources for the PD of guard cells. If ROS are generated to insufficient degree, exogenous H_2O_2 exerts a stimulatory influence on PD. Quinacrine and nitroblue tetrasolium suppressed the generation of ROS caused by the addition of H_2O_2 or menadione based on the measurements with the ROS-sensitive fluorescent probe 2′,7′-dichlorofluorescin diacetate. Most likely, endogenous and exogenous ROS trigger ROS-generation by NADPH oxidase of plasma membrane.

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P2.5.11. SUPEROXIDE AS A MEDIATOR OF ACETATE-INDUCED APOPTOSIS IN YEAST

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It has been convincingly shown that chronological aging in stationary cultures induces apoptosis in yeast. The death can be prevented by inhibition of the aging-linked acidification of the media. At the same time there is evidence that combination of acetate (an intermediate of glucose metabolism in yeast) and low pH can induce apoptosis in yeast. We wanted to test whether the main reason for apoptosis in both models could be due to the fact that combination of acetate and low pH induces protonation of superoxide anion transforming it to the $HO_2\cdot$ - one of the most aggressive forms of reactive oxygen species. To do that we tried to induce acidification of intracellular pH by means other than the addition of acetic acid. We found that propionate induces cell death in the way similar to the acetate-induced death. Moreover, K^+/H^+ ionophore nigericin in combination with low pH of the medium caused yeast cell death with apoptotic markers. Importantly, antioxidants prevented cell death induced by either propionate or nigericin in combination with low pH of incubation medium. Thus, our data support the idea of the superoxide protonation as a driving force for the apoptotic progression.

P2.5.12. PSEUDOMONAS AERUGINOSA-INDUCED HUMAN MAST CELL APOPTOSIS INVOLVES MITOCHONDRIAL-DEPENDENT AND CASPASE 8 PATHWAYS

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Mast cells play a critical role in the host defense against bacterial infection. Recently, apoptosis has been demonstrated to be essential in the regulation of host response to *Pseudomonas aeruginosa*. Here we show that human mast cell line HMC-1 and human cord blood-derived mast cells undergo apoptosis as determined by the single-stranded DNA formation after infection with *P. aeruginosa*. *P. aeruginosa* induced activation of caspase-3 in mast cells as evidenced by the cleavage of D4-GDI, an endogenous caspase-3 substrate and the generation of an active form of caspase-3. Interestingly, *P. aeruginosa* treatment induced up-regulation of Bcl-xS and down-regulation of Bcl-xL. Bcl-xS and Bcl-xL are alternative variants produced from the same Bcl-x pre-mRNA while the former is pro-apoptotic and the latter is anti-apoptotic likely through regulating mitochondrial membrane integrity. Treatment of mast cells with *P. aeruginosa* induced release of cytochrome c from mitochondria and loss of mitochondrial membrane potentials. Moreover, *P. aeruginosa* treatment reduced levels of Fas-associated death domain protein-like interleukin 1β-converting enzyme-inhibitory proteins (FLIPs) which are endogenous apoptosis inhibitors through counteraction with caspase 8. Thus, human mast cells undergo apoptosis after encountering *P. aeruginosa* through a mechanism that likely involves both Bcl family protein-mitochondrial-dependent and FLIP-associated caspase 8 pathways.

P2.5.13. BISPHOSPHONATE-INDUCED APOPTOSIS OF OSTEOCLASTS. ROLE OF MITOCHONDRIAL ADP/ATP TRANSLOCATOR

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Bisphosphonates are used for treatment of conditions of excessive bone resorption. Their main targets are the osteoclasts, in which they trigger apoptosis. The nitrogen-containing bisphosphonates (N-BPs) are known to inhibit the mevalonate pathway with subsequent effects on protein isoprenylation. Non-nitrogen bisphosphonates (non-N-BPs) are metabolized to cytotoxic ATP-analogs. A typical example of the latter is adenosine 5'-(β,γ-dichlormethylene)triphosphate ($AppCCl_2p$) a metabolite of clodronate [1].

It is shown here that although the nitrogen containing bisphosphonates themselves are not metabolized to ATP analogs, inhibition of the mevalonate pathway leads to formation of triphosphoric acid 1-adenosin-5'-yl ester 3-(3-methylbut-3-enyl)ester (ApppI) a novel isoprenylated ATP analog [3]. Formation of ApppI correlated well with the capacity of various N-BPs to inhibit the mevalonate pathway. Inhibition of farnesyl pyrophosphate synthase leads to increased intracellular concentration of isopentenyl pyrophosphate and the most probable mechanism of ApppI formation is aminoacyl-tRNA synthetase-catalyzed condensation of isopentenyl pyrophosphate with AMP.

We have previously shown that $AppCCl_2p$ is an inhibitor of the mitochondrial ADP/ATP translocator (ANT) [4]. Now we show that also ApppI inhibits ANT.

It is known that ANT inhibitors can be divided to two types depending in the conformation in which they lock the translocator. Atractyloside represents the pro-apoptotic and bongkrekate the anti-apoptotic inhibitors of ANT. Evidently, both $AppCCl_2p$ and ApppI are in their ANT effects reminiscent of pro-apoptotic ANT inhibitors.

Although the N-BPs inhibit isoprenylation of several small GTPases, signal molecules linked to cytoskeletal function, membrane ruffling, trafficking of intracellular vesicles and apoptosis, both N-BPs and non-N-BPs induce apoptosis by inhibiting ANT. It is significant that while the drugs of the

former class themselves are metabolized to an inhibitor, the latter class drugs act differently, causing formation of an inhibitor from physiological intracellular constituents.

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P2.5.14. MITOCHONDRIAL K^+/H^+ EXCHANGER IN MITOPHAGY AND APOPTOSIS

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The existence of the mitochondrial K^+/H^+ exchanger was postulated by Peter Mitchells and is a fundamental requirement for cell life. For the first time, we have molecularly identified *MKH1*, the yeast gene encoding an essential component of the K^+/H^+ exchange system in mitochondria. Mutants defective in K^+ extrusion from mitochondria have heavily swollen organelles with reduced membrane potential and show a break-down of normal mitochondrial network. In yeast these mitochondria get into close contact to the vacuole, which ingests and digests them (mitophagy). *MKH1* is conserved in all eukaryotes. In mammalian cells we find that apoptosis is starting in parallel and is the predominant effect induced by the overload of mitochondria with K^+.

P2.5.15. MITOCHONDRIA MEDIATE HOST AND PATHOGEN INDUCED APOPTOSIS

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Induction of cell death via apoptosis has been adapted by both, vertebrates and parasites during their co-evolution as one strategy for mutual defense and survival. The immune system of vertebrates employ apoptotic mechanisms to combat intracellular parasites such as viruses and tumours. The two principal cytolytic effector lymphocyte populations, natural killer (NK) and cytotoxic T cells (CTL) execute their function primarily via the granule exocytosis pathway mediated by perforin and the two granzymes (gzm) A and B[1-5]. By using *ex vivo* derived CTL from mice deficient in either gzmA and/or gzmB we have demonstrated, that gzmA and gzmB independently and differentially induce a number of mitochondria-dependent pro-apoptotic processes, including mitochondrial depolarization, ROS production, PS exposure at the plasma membrane. Moreover, only gzmB but not gzmA induces activation of caspases and, consequently, amplification of apoptosis. However, neither inhibition of ROS nor that of caspase activity abolished induction of gzm-mediated mitochondrial dysfunction and final cell death [6].

Pathogens have also evolved mechanisms to induce apoptosis of host immune cells as a mean of survival strategy. One case in point is the opportunistic human pathogen *Aspergillus fumigatus*[7]. It is known that one of its secondary metabolite, gliotoxin (GT), has immunosuppressive properties as a result of induction of apoptosis in relevant target cells[8]. By analysing the molecular mechanism(s) of GT-induced apoptosis we have confirmed previously findings that GT triggers apoptosis via mitochondrial depolarization and ROS production[9]. Furthermore, we have shown that gliotoxin-induced mitochondrial perturbation is independent of caspases and/or the pro-apoptotic protein Bid, and associated with cytochrome c, AIF release and cell death.

Thus the differences in the apoptotic pathways activated by the immune system on the one hand and *A. fumigatus* on the other may open new avenues for therapeutic treatment of the pathogen without interfering with the immune defense.

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P2.5.16. CYCLOSPORIN A-SENSITIVE SWELLING OF WINTER WHEAT MITOCHONDRIA IN THE PRESENCE OF Ca^{2+} IONS AND FATTY ACIDS

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Opening of high conductance permeability transition pores (PTP) induces the mitochondrial permeability transition, which is characterized by mitochondrial swelling, uncoupling and inner membrane permeabilization to solutes up to 1500 Da. The regulation of PTP in plant mitochondria remains poorly understood. The aim of the present investigation was to study the influence of Ca^{2+} ions, cyclosporin A (CsA) and saturated fatty acids on winter wheat mitochondria swelling. The isolated mitochondria of etiolated seedlings shoots of winter wheat (Triticum aestivum L, cv. Zalarinka) germinated on moist paper at 26 ^{0}C were used in this work. Seedlings were subjected to short-term (-1 ^{0}C, 1 h) cold stress ("stress") or were cold-hardened for 7 days at 4 ^{0}C ("hardening"). Mitochondrial swelling was estimated from the decrease in the absorbance of the mitochondrial suspension at 26 ^{0}C at 540 nm. Incubation medium contained 200 mM KCl and 20 mM MOPS (pH 7,4).

It was shown that the addition of 1 μM CsA to mitochondria from non-stressed seedlings ("control") caused the decrease of their swelling whereas the incubation of mitochondria with 4 mM Ca^{2+} ions induced a 3-fold the increase of this process (in 5 minutes of incubation). Ca^{2+}-induced swelling was fully sensitive to CsA. 50 μM palmitic acid caused a 4-fold the increase of swelling, which was sensitive to CsA addition. The cold stress and hardening induced the CsA-insensitive mitochondrial swelling. The incubation of mitochondria isolated from cold stress and hardening shoots with Ca^{2+} ions induced a 3-fold and a 2-fold induction of CsA-insensitive swelling, respectively. In the presence of palmitic acid the swelling of mitochondria from stressed and hardening seedlings was less expressed as compared to control.

It should be note that data obtained agree with our earlier results about increasing of oxygen consumption in state 4 by plant mitochondria in the presence of Ca^{2+} ions and inhibition of this consumption in the presence of CsA. As the mitochondrial swelling is one of event of PTP opening, can be concluded that the uncoupling of oxidative phosphorylation and swelling of winter wheat mitochondria are associated with opening of mitochondrial pore. At the same time cold stress and cold hardening decrease the sensitivity of mitochondria to CsA, that permit to think about function CsA-insensitive pore in this conditions.

The work has been performed, in part, with the support of the Russian Science Support Foundation and Siberian Division of Russian Academy of Sciences Youth Grant (project 115).

P2.5.17. CLONING OF THE HUMAN CARDIOLIPIN SYNTHASE AND EFFECTS OF ITS KNOWKDOWN ON CELL DEATH PROGRESSION : A SWITCH BETWEEN APOPTOSIS AND NECROSIS

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Cardiolipin (CL) is a unique phospholipid with dimeric structure, carrying four acyl groups and two positive charges. For decades, biomembrane function of cardiolipin has been unresolved, although it is widely believed that is function is related to its unique ability to interacts with proteins.

CL a lipid usually known for anchoring respiratory chains proteins, or gluing the respiratory complex together and also attaching cytochrome *c* to the outer surface of the inner mitochondrial membrane, as come in recent years to be recognize as playing a key role in the cascade of events that takes place when apoptotic cell death is induced. Caspase cleavage of Bid leads to the translocation of tBid to the mitochondrial contact sites, activation of a deleterious mitochondrial membrane changes (inhibition of the respiratory chain to an extent which include slight uncoupling and an important inhibition of the state 3 respiration associated with superoxide anion production) and then, subsequent Bax or Bak activation and oligomerization leading to cytochrome *c* release and activation of executioner caspases through the apoptosome. CL level drop in the initial phase of apoptosis via oxidative processes and generates free cytochrome *c* into the intermembrane space. So, this line of argument suggest that decreased level of CL should be pro-apoptotic because high level of freely releasable cytochrome *c*, provided the transduction of the initial signal is not altered.

The present work address studies of the mammalian CLS gene, the role of cardiolipin in cardiolipin defiencent HeLa cells and its impact in cell death signal transduction through the mitochondrial pathway. We describe here cloning of the human gene for CLS and analysis of its knockdown on cell death progression. We find that decreased level of CL accelerates the rate of apoptosis as induced by Fas and staurosporine, and that there is apoptotic events.

P2.5.18. ON THE ORIGIN OF GLUTAMATE-INDUCED NEURONAL Ca^{2+} OVERLOAD AND MITOCHONDRIAL DEPOLARIZATION

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Deregulation of neuronal calcium homeostasis (DCH) caused by a prolonged glutamate (Glu) challenge is known to manifests itself by a delayed (secondary) $[Ca^{2+}]_i$ elevation associated with a synchronous profound mitochondrial depolarization (MD) during Glu exposure. In order to clarify the origin of this DCH we studied the effects of various nerve cells treatments on the dynamics of $[Ca^{2+}]_i$ and mitochondrial potential changes caused by glutamate in cultured cerebellar granule cells coloaded with fluorescent probes fura 2FF and rhodamin 123. We found that an increase in external Ca^{2+} concentration from 2 to 5 or 10mM greatly reduced a delay of the secondary $[Ca^{2+}]_i$ elevation. In contrast a removal of external Ca^{2+} or blockade of NMDA channels by AP-5 or memantine in the beginning of DCH caused a fast and reversible decrease in both $[Ca^{2+}]_i$ and MD. Blockade of glycolysis by glucose replacement with 2-deoxy-D-glucose greatly accelerated the development of DCH and raised the fraction of cells exhibiting the post-glutamate plateau. Addition of pyruvate to glucose free medium prevented or effectively attenuated the effect of glucose deprivation. A treatment of nerve cell cultures with prooxidant menadione also caused dramatic acceleration DCH. Conclusion is drown that Ca^{2+} influx via NMDA channels plays a dominant role in the secondary $[Ca^{2+}]_i$ increase and mitochondrial Ca^{2+} overload underlying the collapse of mitochondrial potential. Reactive oxygen species promote the DCH exhibition.

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P2.5.19. ON CELLULAR EFFECTS OF PHOTOSENSITIZED DAMAGE BY CATIONIC DYES

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Light-excitation of photosensitizers localized to cellular membranes including mitochondrial ones induces apoptosis due to release of apoptogenic molecules from mitochondria to cytosol.

In this study we investigated the effects of Rhodamine-123 – mediated photodynamic treatment of murine hepatoma MH22 cells in vitro. Rhodamine-123 (Rho-123) is a cationic, lipophilic, water-soluble oxonium chloride salt with a high affinity for the inner subcompartments of mitochondria.

Cellular uptake of Rho-123 demonstrated a biphasic character: at concentrations up to 10 μg/ml in incubation medium, the uptake was independent of the Rho-123 concentration implying the predominant involvement of electrochemical gradient, which funneled Rho-123 to mitochondrial matrix. When Rho-123 concentration in incubation medium exceeded 20 μg/ml, the dark cytotoxicity of Rho-123 for MH22 cells was observed. For photosensitization experiments, we used 5 μg/ml of Rho-123. At this concentration, Rho-123 was not cytotoxic in the dark, but still induced the photosensitized damage to the cells. The biochemical parameters of mitochondria in Rho-123 photosensitized cells were compared with those in cells photosensitized by mTHPC localized to cellular membranes. Cell viability following the light exposure was approx. 60% in both cases.

Activity of oxidoreductases measured by MTT assay was decreased at a similar slight extent following the photodynamic treatment with either of the dyes. Meanwhile, the activity of citratsynthase, the enzyme of mitochondrial matrix, was significantly decreased in the cells treated by Rho-123 and light, contrary to mTHPC-mediated photodynamic treatment, which did not affect the activity of the enzyme.

It is known, that most clinically relevant photosensitizers induce apoptosis following release of cytochrome c from mitochondria to cytosol and activation of effector caspase-3. Neither of these hallmarks of apoptosis nor morphological changes characteristic to apoptosis were detected in the Rho-123-photosensitized cells contrary to mTHPC-photosensitized ones. These data indicate the significance of specific subcompartmental localization of cell damage to the following processes of cell death.

P2.5.20. THE VOLTAGE-DEPENDENT ANION CHANNEL CONTROLS LIFE AND DEATH OF THE CELL

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Mitochondria, the "power houses" of the cell, have also become recognized as the cell's "arsenal," reflecting their key role during programmed cell death - apoptosis. Apoptosis is a complex process regulated by different proteins, some of which interact with the mitochondrial protein - voltage-dependent anion channel (VDAC). VDAC transports anions, cations, ATP and various metabolites into and out of the mitochondria and may function as the channel through which the apoptogenic protein cytochrome c is released. Thus, along with being an important site for the regulation of cellular energy metabolism, VDAC also serves as a site for apoptotic signaling.

The role of VDAC in regulating cell life and death was investigated by silencing endogenous human VDAC1 (hVDAC1) expression using a short hairpin RNA (shRNA)-expressing vector, controlling native and mutated murine VDAC1 (mVDAC1) expression, over-expressing hexokinase-I (HK-I), and using VDAC channel inhibitors.

We found that down-expression of hVDAC1 led to inhibition of cell proliferation due to disrupted energy production in the cell. On the other hand, over-expression of native or mutated VDAC1 resulted in apoptotic cell death. We demonstrate that over-expression of HK-I protects against apoptosis and that a single mutation in VDAC1 prevents this HK-I protection against apoptosis, suggesting that the protective effects of HK-I rely on its binding to VDAC1. In addition, our results suggest that oligomeric VDAC1 forms a protein-conducting channel for the passage of cytochrome *c*. These results indicate that VDAC1 regulates both cell life and apoptosis independent of the apoptosis-inducing pathway and may function as a checkpoint for cell life and death.

Poster session 2.6. Mitochondrial dynamics

P2.6.1. IMPORTANCE OF MITOCHONDRIAL NETWORK ORGANIZATION IN THE REGULATION OF ENERGY PRODUCTION

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Recent advances in cell biology have helped to create a picture of mitochondrial organization whereby the organelle exists as a single dynamic network (mt-network), either forming a reticulum or a fragmented collection of vesicles. Several observations have revealed that its tridimensional organization is variable in human living cells, both under normal or pathological situations. However, little is known about the determinants and the bioenergetic consequences of these changes. Such analysis is complex since mitochondria participate in a multiplicity of cellular functions such as energetics, ROS production, calcium signaling or apoptosis. Changes in mt-network organization can be observed in association with different physiological situations including cell cycle, cytoskeletal trafficking, energy status, pathology or apoptosis. These changes could be mediated by fission and fusion proteins, but their regulation is poorly understood. The contribution of physicochemical transitions in mitochondrial lipid bi-layers also remains to be investigated. Our analysis focused on the relationships between the energy status of the cell and mitochondrial network organization. Using fluorescence microscopy and ratiometric GFP biosensors we observed specific and opposite transformations of mitochondrial overall structure and internal organization in response to *in situ* activation or inhibition of mitochondrial energy production, respectively. We present also novel results we obtained on a cell line where DRP1, a protein involved in fission of the mt-network was knocked out using the siRNA technology. In these cells, we observed important changes in mitochondrial membrame fluidity associated with the uncoupling of oxidative phosphorylation. Taken together, our observations point out toward a strong implication of mt-network organization in the regulating OXPHOS.

P2.6.2. COMPARATIVE BIOENEREGETICS OF ISOLATED CARDIOMYOCYTES AND HL-1 CELLS: MITOCHONDRIAL DYNAMICS, RESPIRATION REGULATION AND CREATINE KINASE EXPRESSION

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Recent data showed that mitochondria behave as if they were included into functional units (ICEU) with adjacent ADP producing systems, sarcoplasmic reticulum, and myofibrils. A basic pattern of unitary organisation of muscle energy metabolism ensures effective energy crosstalk between mitochondria and ATPases via multienzyme energy transfer networks: the CK and AK systems [1].

In this study, we compared two types of cells with different structures: the cardiomyocytes which were isolated from rat adult heart, and the HL-1 cardiac cells ancestry developed by W. Claycomb in USA (1998) and selected to obtain a stable non-beating cell line which were used as a cardiac cells model [2].

First, mitochondria arrangement was visualized by confocal microscopy and then, kinetics of respiration regulation were studied in these two types of permeabilized cells by oxygraphy and spectrophotometry methods.

Analysis of confocal images of non-fixed cardiomyocytes reveals that mitochondria which are situated between myofibrils are arranged in highly ordered pattern (crystal-like) [3]. A proteolytic treatment destroys this arrangement and decreases apparent Km for exogenous ADP (from 300 ± 35 μM to 100 ± 8 μM) determined after measurement of respiration rates by oxygraphy method. These results show that mitochondrial arrangement and sensitivity for ADP was controlled by the cytoskeleton proteins.

Contrary to cardiomyocytes, mitochondria inside the HL-1 cells move with no effect of a proteolytic treatment. The K_m^{app} for exogenous ADP measured by oxygraphy is 10 ± 6 μM. These results show that a diffusion restriction for ADP exists in cardiomyocytes because of specific structure, but not in the HL-1 cells because of the cell disorganisation.

By spectrophotometry method, a coupled enzymes system (Pyruvate Kinase / Lactate Deshydrogenase) was used to trap endogenous ADP produced by cytoplasmic ATPases (SERCA and Myosin-ATPase) with and without mitochondrial respiratory substrates. The results show that there is a channelling of endogenous ADP to the mitochondria in cardiomyocytes but this phenomenon does not exist in HL-1 cells. Moreover, very low creatin kinase activity was found in HL-1 cells.

In conclusion, Hl-1 cells are structurally and metabolically very different from the adult cardiac cells. The cellular mechanism of mitochondrial respiration regulation and mitochondrial dynamics are related to cell structure of adult cardiomyocytes.

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P2.6.3. THE CYTOSOLIC PHOSPHATASE CALCINEURIN (PP2B) REGULATES DRP-1 DEPENDENT MITOCHONDRIAL FISSION

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In certain cell types, mitochondria are organized in an extended network that undergoes regulated fission and fusion in response to cellular and mitochondrial stimuli. Dynamic changes of mitochondrial shape must therefore be finely tuned, but while their molecular mechanisms are being identified, the nature of the mitochondrial and cytosolic signals controlling mitochondrial fusion-fission equilibrium remains elusive. Here we show that the cytosolic Ca^{2+}-dependent phosphatase calcineurin controls recruitment of the fission protein DRP-1 to mitochondria following dysfunction of the organelle via Drp1 dephosphorylation. Pharmacologic or genetic inhibition of calcineurin blocks dephosphorylation of DRP-1 and its translocation to depolarized mitochondria, by anchoring DRP-1 to microtubules. Fragmentation of the mitochondrial network and apoptosis by intrinsic death stimuli is blocked by genetic inhibition of calcineurin. Calcineurin is an unexpected rheostat controlling mitochondrial involvement in apoptosis.

P2.6.4. MITOCHONDRIAL RHOMBOID PARL REGULATES CYTOCHROME C RELEASE DURING APOPTOSIS VIA OPA1 DEPENDENT CRISTAE REMODELING

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Rhomboids, evolutionarily conserved integral membrane proteases, participate in crucial signaling pathways. An inner mitochondrial membrane rhomboid is essential for mitochondrial fusion and function in yeast, while the role of its mammalian orthologue PARL is unknown. Mice lacking *Parl* display normal intrauterine development but display from the 4th week on progressive atrophy in multiple tissues leading to cachexia and death. Atrophy is sustained by increased apoptosis, both *in* and *ex vivo*. Unexpectedly *Parl*$^{-/-}$ cells display normal mitochondrial morphology, which becomes elongated upon OPA1 expression. They are however extraordinarily sensitive to intrinsic death stimuli and not longer protected by OPA1 expression. *Parl*$^{-/-}$ mitochondria lack a soluble isoform of OPA1 in their intermembrane space and during apoptosis undergo faster remodeling of mitochondrial cristae with increased mobilization and release of cytochrome *c*. Thus, rhomboids control the OPA1-dependent mitochondrial remodeling pathway of apoptosis, implicating regulated intramembrane proteolysis in control of cell death.

P2.6.5. ELECTRONEUTRAL K^+/H^+ EXCHANGE IN MITOCHONDRIAL MEMBRANE VESICLES INVOLVES MKH1/LETM1 PROTEINS

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Mkh1p in yeast and hLetm1 in humans are integral proteins of the inner mitochondrial membrane and their function is conserved from yeast to man. They are involved in mitochondrial K+ homeostasis, the maintenance of the mitochondrial membrane potential and in volume control. Using submitochondrial particles (SMPs) with entrapped K+- and H+-sensitive fluorescent dyes PBFI and BCECF, respectively, the kinetics of K+ and H+ transport across the yeast inner mitochondrial membrane was studied in detail. Wild-type SMPs exhibited rapid, reciprocal translocations of K+ and H+, driven by concentration gradients of either of them. K+ and H+ translocations have stoichiometries similar to those mediated by the exogenous K+/H+ exchanger nigericin, and they are shown to be essentially electroneutral and obligatorily coupled. These features, as well as the sensitivity of K+ and H+ fluxes to quinine and Mg2+, qualify these activities as K+/H+ exchange reactions. Both activities are abolished when the yeast Mkh1p protein is absent (mkh1Δ mutant SMPs), indicating that it has an essential role in this reaction. Consequently, apoptosis is induced in these mutant cells. The replacement of the yeast Mkh1p by the human Letm1 protein restores K+/H+ exchange activity confirming functional homology of the yeast and human proteins.

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P2.6.6. METABOLIC STATE-RELATED STRUCTURE OF ISOLATED CARDIAC MITOCHONDRIA AS SEEN BY HIGH RESOLUTION SCANNING ELECTRON MICROSCOPY (HRSEM)

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Osmium extraction of isolated cardiac mitochondria renders their membranes in stark relief when viewed by HRSEM. In our previous HRSEM study of cardiomyocytes in adult Sprague-Dawley rats it was found that subsarcolemmal mitochondria (SSM) have predominantly lamelliform cristae, whereas interfibrillar mitochondria (IFM) have mainly tubular cristae. When these respective populations are independently isolated, SSM are largely unchanged whereas the IFM have acquired a heterogenous (mixed) cristal structure. We asked if cardiac mitochondria morphologically reflected metabolic state as seen to be the case in hepatic mitochondria [Hackenbrock, J Cell Biol. 30:269, 1966]; to this end, we examined over 600 organelles by HRSEM. Under state 2, 60% of the SSM have lamelliform cristae; in state 3, 83% have tubular cristae; in state 4, 81% have lamelliform cristae. In the case of IFM, 72% of those in state 2 have tubular cristae; in state 3 69% have tubular cristae; in state 4, 77% have a mixture of cristae. In summary, with high oxidative rates and ATP production the cristae are predominantly tubular in both populations of cardiac mitochondria. When ADP becomes limiting with low oxidation and ATP production the cristae in SSM become lamelliform whereas in IFM there is a mixture of lamelliform and tubular cristae. We conclude that cristal architecture is closely associated with ATP production in cardiac mitochondria.
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P2.6.7. MITOCHONDRIAL FUSION AND FISSION IN MYOGENIC DIFFERENTIATION

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Mitochondrial dynamics is a highly regulated cellular process. In mammals, mitofusins and OPA1 are essential for mitochondrial fusion, whereas Drp1 and Fis1 act on mitochondrial fission. Recent studies demonstrate that mitochondrial fusion and fission processes are important in cell and tissue physiology. In addition, mutations in Mfn2 and OPA1 cause inherited neuropathies[1,2] and muscle Mfn2 expression is dysregulated in obese and type 2 diabetic subjects[3]. Here we show the expression pattern of mitochondrial dynamics players (Mfn2, OPA1, Fis1 and Drp1) during myogenic differentiation of C2C12 myoblasts. The expression of these proteins increased in total lysates during myogenesis, and this increase was higher than the one observed in mitochondrial markers such as porin. This suggests a specific increase in the components of the mitochondrial fusion and fission machinery during myogenic differentiation. Mfn2, OPA1 and FIS1 also increased their levels in mitochondrial enriched fractions during myogenesis. However, in enriched mitochondrial fractions, Drp1 abundance decreased at the onset of myogenic differentiation and, in fully diferentiated myotubes, Drp1 levels were similar than in myoblasts. The increase of Drp1 levels detected in total lysates was localized in the cytosolic and membrane enriched fractions. Our data suggest that the mitochondrial fusion and fission machinery play a relevant role in differentiated muscle cells. We also propose that cellular distribution of Drp1 is also regulated during myogenic differentiation and that the low Drp1 levels present in mitochondria may shift the mitochondrial dynamics towards fusion in differentiated muscle cells.

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P2.6.8. SELECTIVE ELIMINATION OF MITOCHONDRIA IN HELA CELLS DURING TREATMENT WITH MITOCHONDRIAL INHIBITORS

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We have studied the dynamics of mitochondrial in human carcinoma cells HeLa. It was shown that inhibitors of respiration (piericidin, antimycin, myxothiazol), ATP synthase (oligomycin, aurovertin) and uncouplers (DNP and FCCP) did not cause any loss in viability of these cells during 48 h in the presence of glucose. The inhibitors of respiration and uncouplers induced massive fragmentation of mitochondria unrelated with ATP depletion, activation of caspases or other apoptotic events. In the following experiment we have took the advantage of this model for investigation of the long-term consequences of mitochondrial fission. The most rapid fission of mitochondria was induced by combined treatment with respiratory inhibitors and uncouplers simultaneously. At the final steps of the treatment with uncouplers in combination with the respiratory inhibitors the fragmented mitochondria gathered near the nucleus and formed several aggregates. When HeLa cells were treated for 72h with FCCP or DNP in combination with antimycine or myxothiazole (inhibitors of Complex III), a fraction of the cells (60-70 %) died by apoptosis but the rest of population was viable, without any signs of apoptosis (normal nucleus, normal cytoskeleton, no annexin V staining). The total amount of mitochondrial material in these cells decreased significantly in the process described earlier as “mitoptosis” [1,2]. Cytochrome *c* was not released from mitochondria until the very last steps while the release of the other mitochondrial proteins could not be excluded. We did not observed co-localization of mitochondrial clusters with autophagosomes and the size of the clusters at the final steps strongly exceeded the capability of canonical autophagy. Probably mechanism of mitoptosis in HeLa cells during treatment with mitochondrial inhibitors is closer to the mechanism of elimination of organelles during the lens and erythroid differentiation, which is independent from major components of autophagic machinery. It could be suggested that this mechanism provide cells with selective advantages due to low content of pro-apoptotic mitochondrial proteins and elimination of the major source of ROS production and probably underlies the low mitochondria content observed in some tumors in vivo.

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P2.6.9. MITOFUSIN-2, MUTATED IN CHARCOT-MARIE-TOOTH TYPE IIA, LINKS ENDOPLASMIC RETICULUM TO MITOCHONDRIA

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Endoplasmic reticulum (ER) and mitochondria are in close juxtaposition to support their intercommunication essential in Ca^{2+} homeostasis and apoptosis. Molecular mechanisms controlling ER-mitochondria interactions are unknown. Mitofusin-2 (MFN2), a dynamin-related protein of the outer mitochondrial membrane mutated in Charcot-Marie-Tooth type IIa, specifically controls morphology not only of mitochondria, but also of ER. In cells lacking *Mfn2*, ER-mitochondria interactions are largely reduced. GTPase and RAS-binding domains of MFN2 proved essential to regulate ER morphology and interaction with mitochondria, consistent with an increase in phosphorylation of downstream RAS targets in *Mfn2*-/- cells. MFN2 is the first mitochondrial protein that regulates ER-mitochondria interaction.

P2.6.10. DETECTION OF NEW TYPE OF MEMBRANE STRUCTURE IN MITOCHONDRIA UNDER LOW-AMPLITUDE SWELLING BY SMALL ANGLE NEUTRON SCATTERING

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It is well known that changes in volume of cells, organelles, bacteria stimulate specific structural reorganization in them accompanied by alterations in function of their enzymatic systems. Earlier influence of low-amplitude matrix swelling on ultrastructure of mitochondrial membrane was studied. The ultrastructure is concerned with working of the membrane enzymes. Namely under hypotonic conditions appearance of so called “dried cristae” with small gap between ctista membranes was found out in rat liver mitochondria [1]. This structural changes are accompanied by rise of electron transport in respiratory chain, changes in pH-regulation of potassium transport and in kinetic parameters of oxidative phosphorylation [1,2,3].

We conducted small angle neutron scattering (SANS) experiments with intact rat heart mitochondria under isotonic and hypotonic conditions. The experiments was carried out on YuMO spectrometer placed on beam 4 of pulsed reactor IBR-2 of Joint Institute for Nuclear Research (Dubna, Moscow region, Russia) [4,5]. Mitochondria were placed in media containing 1 mM $MgSO_4 \cdot 7H_2O$, 1 mM KH_2PO_4, 20 mM *tris*, 10 mM *KCl*, 0.25 mM EDTA, 0.2 M sucrose (isotonic medium) and 0.058 M sucrose (hypotonic medium), pH=7.5. The temperature was 15 ^oC.

From SANS experiments it was shown that in case of isotonic conditions the inner mitochondrial membrane forms lamellae placing quasi parallel with each other at particular distance. So the SANS curves have the interference peak corresponding to a parameter equaled 195 ± 10 Å. In case of hypotonic medium the curves have two peaks with ratio 1 : 1.6 ± 0.1. That can point to presence of two types of lamellar structures or presence of non-lamellar structures (such as cubic and hexagonal) in the mitochondria [6,7]. Thus SANS experiments have shown that under the low-amplitude matrix swelling the qualitative structural reorganization in the mitochondrial membranes has a place.

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P2.6.11. INTRAMITOCHONDRIAL REACTIVE OXYGEN SPECIES ARE IMPORTANT FOR DYNAMICS OF MITOCHONDRIAL RETICULUM IN LIVING CELLS.

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A complex network of mitochondria in living cells (mitochondrial reticulum) is able to undergo the thread-grain transition. This process could be important for cell division, apoptosis and cell responses to different types of signals, including oxidative stress. We suggested that production of reactive oxygen species (ROS) by respiratory chain could initiate fission of mitochondria. We have found, that the inhibitors of respiratory chain piericidin (Complex I) and myxothiazol (Complex III) induced fission of mitochondria and subsequent gathering of them around nucleus in HeLa (human carcinoma) and CV-1 (monkey epithelium) cells. Added hydrogen peroxide caused the similar events. Oxidative stress and fission of mitochondria, induced by low doses of hydrogen peroxide were enhanced by myxothiazol or piericidin. The role of intramitochondrial ROS production was investigated using the mitochondria-targeted antioxidant, 10-(6'-ubiquinolyl)decyltriphenilphosphonium (MitoQ). This cationic compound is accumulated in mitochondrial matrix and its quinone residue is reduced by respiratory chain after ROS-dependent oxidation making this antioxidant renewable and very effective. Preincubation of the cells with MitoQ prevented fragmentation of mitochondria induced either by the inhibitors of respiration or by H_2O_2 and by combinations. The effect of MitoQ was eliminated by uncouplers (DNP, FCCP), which prevented accumulation of the antioxidant in the matrix. We found that even without stresses prolonged incubation of HeLa cells with MitoQ improved (increased the length of) mitochondrial reticullum. Functioning of mitochondrial network was investigated using local photodynamic damage (laser focused by confocal microscope) of rhodamin loaded tubular mitochondria. It was shown that the size of electrically-connected area of the network in HeLa cells strongly increased and reached 30-50% of total mitochondrial population after pretreatment with MitoQ. The similar effect was observed with human fibroblasts. Our data indicate that mitochondrial ROS production plays an important role in structural and functional dynamics of mitochondria both under stressful and normal conditions in the cell culture.

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P2.6.12. EFFECTS OF OPA1 MUTATIONS ON MITOCHONDRIAL MORPHOLOGY AND APOPTOSIS: RELEVANCE TO ADOA PATHOGENESIS

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To characterize the molecular links between type-1 autosomal dominant optic atrophy and OPA1 dysfunctions, the effects of pathogenic alleles of this dynamin on mitochondrial morphology and apoptosis were analyzed either in fibroblasts from affected individuals, or in HeLa cells transfected with similar mutants. The alleles were missense substitutions in the GTPase domain (OPA1G300E and OPA1R290Q) or deletion of the GTPase effector domain (OPA1Δ58). Fragmentation of mitochondria and apoptosis increased in OPA1R290Q fibroblasts and in OPA1G300E transfected HeLa cells. OPA1Δ58 did not influence mitochondrial morphology but increased the sensitivity to staurosporine of fibroblasts. In these cells, the amount of OPA1 protein was half that in control fibroblasts. We conclude that GTPase mutants exert a dominant negative effect by competing with wild-type alleles to integrate into fusioncompetent complexes whereas C-terminal truncated alleles act by haploinsufficiency. We present a model where antagonistic fusion and fission forces maintain the mitochondrial network within morphological limits that are compatible with cellular functions. In the retinal ganglion cells of patients suffering from type-1 autosomal dominant optic atrophy, OPA1-driven fusion cannot adequately oppose fission thereby rendering them more sensitive to apoptotic stimuli and eventually leading to optic nerve degeneration.

P2.6.13. MITOCHONDRIAL CONJUGATION

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Mitochondrial dysfunction is commonly attributed to a loss in fusion activity. Previous work has described that mitochondria undergo complete fusion to form mitochondrial networks that permit redistribution of their components. It is unclear whether mitochondria can also engage in transient interactions that would allow them to complement each other without losing their discrete entity. Visualizing mitochondrial fusion in real time we identified two classes of fusion events in mammalian cells. We observed complete fusion but more frequently, transient conjugation events, wherein two mitochondria came into close apposition, exchanged soluble intermembrane-space and matrix proteins, and reseparated without loss of identity. Both conjugation and fusion exhibited rapid kinetics of the sequential and separable mergers of the outer and inner membranes and astonishingly, the opening and resealing of the four membranes between the matrices of conjugating mitochondria required extremely short time (as little as 4s). Visualization of conjugation and fusion simultaneously with monitoring the mitochondrial inner membrane potential allowed us to show that in contrast to the current views, the inner membrane potential per se is not required for conjugation or fusion, nor is the potential lost during these events. Based on these data, conjugations represent a novel mechanism to exchange the contents necessary for proper functioning while maintaining a desired morphology of mitochondria.

P2.6.14. RESPONSES OF MITOCHONDRION IN IMMOBILIZED LYMPHOCYTE

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We have elaborated sensitive method to study responses of mitochondria without isolation from the cell, with preservation of native structure organization in network - mitochondrion (mitochondriom). Lymphocytes were used in freshly prepared smear of blood, dried in air for 30 sec. The experience of cytochemistry shows that this procedure keeps activity of dehydrogenases in mitochondria and other enzymes, apparently due to their immobilization on the glass within their native cellular environment. We studied activity of succinate dehydrogenase (SDH) by the reduction of nitro-blue tetrazolium (NBT) with registration of dye by computer videomicroscopy. Traditional conditions of cytochemical investigations were essentially modified to near to biochemical experiments. This allowed us to reveal new phenomena.

1. It was shown that activation of SDH in response to adrenaline administration to the animal can reach 200 - 400% instead of 20 - 40% that are observed in biochemical investigations in isolated mitochondria or by standard catechetical methods.

2. It was first observed that under real quiescent state of animal before and during euthanasia the complete switching off of succinate (SUC) oxidation occurs, that is switched on by adrenaline activation [1].

3. The signal action of a very low concentrations of SUC (micro molar) far below that are necessary to supply mitochondria with substrate, previously discovered *in vivo* [2-7], was first observed in isolated cells. Our view of signal action of SUC *in vivo* is supported by finding its receptor [8].

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Poster session 2.7. Therapeutic approach

P2.7.1. MITOCHONDRIAL STUDY AS THE IMPORTANT STEP IN THE SEACHING OF NEUROPROTECTORS

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The disturbances in synaptic transmission and calcium homeostasis, impairment of energy metabolism, increased generation of free radicals and death of neurons of selective population are the hallmark of all neurodegenerative diseases. Mitochondria play the key role in development of these disturbances. Now it is clear that the mitochondria and phenomenon of mitochondrial permeability transition (MPT) mark the point of no return in both necrotic and apoptotic cell death and increased oxidative stress may be the coactivator of the MPT. But the calcium handling ability of mitochondria and phenomenon of MPT is also important for normal nerve impulse transmission and especially for glutamate receptor functioning. Despite such a dualism in the physiological role, the MPT is considered now as a very promising target for the new effective neuroprotective drugs. It should be noted that the threshold for calcium-induced opening of MPT pores in brain mitochondria decreases with ageing. We suggest that for effective neuroprotection the compounds which decrease the vulnerability of mitochondria to inductors of MPT, prevent peroxidation process in mitochondria and increase their calcium capacity (but not inhibit the calcium entry into mitochondria) may be useful.

We have shown that some widely used neuroprotectors also exhibit the mitochondria protective properties. Some of them combine antioxidant potential with the ability to prevent the activation of MPT by neurotoxins. Melatonin precursor N-acethylserotonine (NAS), and extract of Gingko biloba, which exhibit the moderate neuroprotective effect, inhibit the lipid peroxidation in mitochondria and simultaneously prevent the MPP^+- and t-butylhydroxyperoxide - induced MPT, accordingly. Dimebon, which was patented as a new agent for the therapy of neurodegenerative disorders, also possess antioxidant properties and effectively increase of mitochondria threshold onset to different inductors of MPT. On the other hand, this compound demonstrated a pronounced anti-NMDA effect and at once blocks the glutamate-induced calcium influx into neuron and increase the calcium capacity of the mitochondria. Other low-affinity non-competitive antagonist of NMDA receptors memantine is approved for the clinical application for treatment Alzheimer's

disease in a number of European countries including Russia. We have shown that this compound increases the MPT threshold onset to different inductors and calcium capacity of mitochondria, but there is no any antioxidant effect or influence on mitochondrial respiration.

So it may be helpful to use mitochondria as the important stage in searching the neuroprotective agents, which can increase the resistance of neurons to apoptosis to target mitochondria and combine mitochondria protective potential with antioxidant properties and/or capacity to restore or to compensate damaged brain function due to affinity with any neuromediator system.

P2.7.2. ALTERNATIVE OXIDASE, A POTENTIAL THERAPY FOR MITOCHONDRIAL DISEASES

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In most plants and in a number of micro organisms but not in mammals, the alternative oxidase (AOX) provides a by-pass of the cytochrome segment of the mitochondrial respiratory chain. This enzyme which is located at the inner surface of the inner mitochondrial membrane is activated under specific metabolic conditions to facilitate respiration. These conditions include the over-reduction of the quinone pool and the accumulation of pyruvate and other organic acids.

Since these conditions commonly arise in cases of mitochondrial OXPHOS disease, we decided to test whether the expression of the recently discovered alternative oxidase from the ascidian *Ciona intestinalis* (1) can alleviate the deleterious consequences of a blockade of the respiratory chain in human cells. We therefore expressed *C. intestinalis* AOX, both epitope-tagged and untagged, in cultured human cells (HEK 293) using the inducible Flp-In™ T-REx expression system. AOX was efficiently expressed, targeted to mitochondria and had no detectable deleterious effects on cell growth. The respiration of these cells became insensitive to cyanide but the cyanide-insensitive respiration was abolished by n-propyl gallate, a specific inhibitor of the alternative oxidase. Furthermore the AOX involvement in human cell respiration was enhanced by pyruvate as in plants. Finally, the AOX expression alleviated oxidative stress upon inhibition of the OXPHOS system (2).

We are now in the process of stably expressing the AOX in COX-deficient human cells. On one hand we transfected the AOX construct in immortalized COX15-deficient fibroblasts. On the other hand, we used HEK 293 cells in which COX10 or SURF1 gene have been silenced by shRNA expressing vectors.

The successful expression of *C. intestinalis* AOX in human cells (ref) constitutes a promising tool to further study the consequences of an OXPHOS dysfunction because it offers a unique possibility to disconnect electron flow through most of the respiratory chain from the phosphorylation process. In a longer term, allotopic expression of AOX may provide an effective therapy for currently intractable RC diseases. The first step in this endeavour should be the expression of AOX in whole organism models, *e.g.* mouse or *Drosophila*, exhibiting OXPHOS deficiency.

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P2.7.3. SIGNAL, SYMPATHETIC ACTION OF SUCCINATE IN EXPERIMENTAL AND CLINICAL STUDIES

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The therapeutic effect of succinate was demonstrated in our group and elsewhere [1-5].

It was found that SUC in the organism controls physiological functions by concentrations far below that are necessary for supply mitochondria with substrate : micrograms in rats, 100, 50 mg and even lower doses once a day in patients. These data suggested signal action of SUC *in vivo.* We show below that signal action of SUC involves adrenaline/noradrenaline (A/NA) release.

1. Low doses of SUC (in the form of diammonium salt, (AS) were taken *per os* (1.5 mg SUC anion/kg) by sportsmen. In two hours after administration the increase in diuresis and A/NA release in urine.

2.A/NA administration to rats stimulates SUC oxidation in mitochondria selectively [6]. We found that SUC administration to rats mimicks effect of A/NA in mitochondria and this effect is abolished by β-adrenoblokator, obsidane [2,3].

3. Highly sensitive tests, inavaiable in experiments with animals were investigated in patients.These tests measure ratio of sympathetic/parasympathetic activities.

AS, food additive Enerlite, elaborted by the authors, was used as a source of SUC in low, signal doses: 0.5; 2.0; 3.0 mg of SUC anion / kg twice a day.

Investigations were carried out in patients with chronic diseases related to increase in vagotone, such as neuro-circulatory dystony, heart ischemia, climacteric syndrome, mental fatigue during examinations, and meteopathy. Investigations were controlled by placebo taking. In all patients with initial vagotony the sympathetic effect of AS was shown, while it was absent in persons with initial normal sympathetic/parasympathetic balance.

The conclusion that signal acton of SUC is linked with stimulation of A/NA formation is supported by the recent finding receptor to SUC [8]. The link between this receptor and angiotensin synthesis, controlling diuresis and A/NA formation probably underlies the described regulatory effects of SUC in the organism.

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P2.7.4. THE SINGLE SUBUNIT NADH DEHYDROGENASE REDUCES GENERATION OF REACTIVE OXYGEN SPECIES FROM COMPLEX I

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It is generally believed that major sites of reactive oxygen species (ROS) production in the cells are complexes I and III in mitochondrial respiratory chain. ROS overproduction has been reported in cells bearing mtDNA pathogenic mutations, especially when complex I is impaired. In addition, it was recently hypothesized that the ROS overproduction caused by complex I defects is involved in Parkinsonian symptoms of animal models [1]. Here, we propose to use the yeast Ndi1 enzyme as an agent to suppress the ROS overproduction by complex I. Ndi1 is the rotenone-insensitive NADH dehydrogenase that is found in yeast mitochondria. We had previously shown that the *NDI1* gene can be functionally expressed in mammalian cultured cells and indicated that the expressed Ndi1 acts as a member of the respiratory chain in host cells [2, 3]. In the present study, we used the rat PC12 and SK-N-MC human neuroblastoma cells and compared ROS production between the cells expressing the Ndi1 protein and those without Ndi1. Generation of ROS was assessed by analyzing DNA damage, lipid peroxidation and by direct measurement of H_2O_2 with fluorescent probes. Incubation of non-*NDI1*-transduced control cells in the presence of complex I inhibitor, rotenone, resulted in appearance of 8-oxo-deoxyguanosine primarily in mitochondria, indicating a damage in mtDNA but not in nuclear DNA. In the *NDI1*-transduced cells, the same treatment with rotenone did not have any appreciable effect. When antimycin A, a complex III inhibitor, was used, mtDNA damage was observed in both the *NDI1*-transduced and the non-transduced cells. Similarly, a significant amount of ROS (such as H_2O_2) was detected when rotenone or antimycin A was added to mitochondria prepared from the control cells as reported by dichloroflourescein or Amplex red. The rotenone-induced ROS generation was largely suppressed in the cells expressing Ndi1. The presence of Ndi1 did not lower the antimycin A-dependent ROS generation. These results suggest that the Ndi1 enzyme may protect the cell from oxidative stress caused by ROS from defective complex I.

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P2.7.5. THE MITOCHONDRIAL EFFECTS OF SMALL ORGANIC LIGANDS OF BCL-2 AT THE BH3 DOMAIN. SENSITIZATION OF BCL-2 OVEREXPRESSING CELLS TO APOPTOSIS WITHOUT MITOCHONDRIAL TOXICITY BY A PYRIMIDINE-2,4,6-TRIONE DERIVATIVE

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BCL-2 belongs to a family of proteins that regulate apoptosis, or programmed cell death.This family includes both antiapoptotic proteins such as BCL-2 and BCL-XL and proapoptotic proteins such as BID, BAD, BAK and BAX. A computer screening has identified HA14-1, a small organic ligand that is able to displace peptides modeled on the BCL-2 binding region of BAK, a proapoptotic member of the family. HA14-1 was able to cause cell death that was preceded by activation of caspase 9 and 3, and caused mitochondrial depolarization *in situ* (1). We have investigated the mitochondrial effects of BH3I-2', Chelerythrine and HA14-1. All compounds displayed a biphasic effect on mitochondrial respiration with uncoupling at low concentrations and respiratory inhibition at higher concentrations. At concentratios lower than required for uncoupling all compounds sensitized the PTP to opening both in isolated mitochondria and intact cells. BCL-2 overexpression did not sensitize but rather protected cells from the cytotoxic effects of BH3I-2', Chelerythrine and HA14-1. In order to assess whether the BCL-2-binding and PTP-inducing effects could be separated from the effects on respiration, we have tested a set of HA14-1 analogs from the Hoffmann-La Roche chemical library. We have identified a pyrimidine-2,4,6-trione derivative (EM20-25) as a molecule devoid of effects on respiration that is able to induce PTP opening, to disrupt the BCL-2/BAX interactions *in situ* and to activate caspase-9 in BCL-2-overexpressing cells. EM20-25 neutralized the antiapoptotic activity of overexpressed BCL-2 towards staurosporine, and sensitized BCL-2-expressing cells from leukemic patients to the killing effects of staurosporine, chlorambucil and fludarabine. These results provide a proof of principle that the potentially toxic effects of BCL-2 ligands on mitochondrial respiration are not essential for their

antiapoptotic activity, and represent a step forward in the development of tumor-selective drugs acting on BCL-2.

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P2.7.6. CORRELATION OF SPERM MOTILITY WITH CHANGES OF MITOCHONDRIAL MEMBRANE POTENTIAL

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The main source of chemical energy for sperm motility is obtained from ATP, which is produced by the sperm mitochondria during the process of oxidative phosphorylation. Mitochondrial function and sperm viability were quantified in samples of cryopreserved bovine spermatozoa from 10 bulls using fluorescence and computer techniques. The active mitochondria of the spermatozoa were fluorescent stained using two different fluorophores: 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) and MitoTracker Green FM (MITO). The resulting fluorescent measurements of mitochondrial function were compared with computer assisted optical assessments of sperm motility immediately after thawing, with motility after several times of incubation at 37°C, and with the fluorescent assessment of changes in mitochondrial membrane potential. Staining with MITO resulted in a single green population. In contrast, the JC-1 staining of mitochondria produced both green and red-orange populations of spermatozoa. The ability of JC-1 to discriminate between mitochondria exhibiting high membrane potential from those having low to medium membrane potential provided a more rigorous estimate of metabolic function than the other fluorescent stains.

Determining a possible correlation between changes of mitochondrial membrane potential and sperm motility is one of the main aims of the experiment.

3. Miscellaneous (Other topics)

P3.1. INCREASE IN OXIDATIVE PHOSPHORILATION SYSTEM ACTIVITIES IS RELATED TO MITOCHONDRIAL DIFFERENTIATION IN RAT EMBRYO DURING PLACENTATION

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Mitochondrial biogenesis is a complex event that includes both mitochondrial proliferation and differentiation. Research on this topic is of great interest since advances in this field would help to understand the pathophysiology of mitochondrial diseases. In this sense, mitochondria of rat embryo during placentation are a suitable model to further understand mitochondrial biogenesis due to the important oxidative metabolism activation that takes place at this stage of development [1]. In fact, we have previously reported important changes in the molecular pathways responsible for mitochondrial proliferation and differentiation throughout placentation period [2]. The aim of the present study was to investigate the expression of genes involved in the coordinated transcriptional regulation of both mitochondrial and nuclear genome (PGC-1, NRF-1, NRF-2 and TFAM) [3, 4], as well as genes related to mitochondrial replication (mtSSB) [5], and function (COX IV and COX I) in rat embryo throughout gestational days 11, 12 and 13, when the placentation takes place. Furthermore, we measured the oxidative phosphorilation system (OXPHOS) activities to determine if the changes in gene expression were translated into an activation of the mitochondrial respiratory capacity.

The mRNA levels encoding proteins implicated in mitochondrial biogenesis reflected an acute mitochondrial differentiation process that would face up to the important energy demands of the embryo at this developmental period. Regarding OXPHOS activities, our results showed a similar profile for all the enzymes, i.e., a statistically significant gestational day upward trend when they were expressed per both mitochondrial protein and mitochondrial DNA. These results reflected an increase in OXPHOS capacity per mitochondria that exceeded the rise observed in its protein content. Thus, we can conclude that mitochondria are actually enhancing their functionality, which would corroborate the idea that on gestational day 13 mitochondria are more differentiated

compared with the previous days studied. In addition, the increase in OXPHOS potential activities could be related to the rise seen in mitochondrial gene expression.

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P3.2. SUBSTRATE CROSSTALK IN E. COLI COMPLEX I MEDIATED BY LONG-RANGE CONFORMATIONAL CHANGES

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The proton-pumping NADH:ubiquinone oxidoreductase, the respiratory complex I, consists of a peripheral arm located in the aqueous milieu and a membrane arm buried within the lipid bilayer. The NADH binding site is located in the peripheral arm and it is discussed that the ubiquinone binding site is located at the interface between the two arms at a distance of at least 100 Å. We found that binding of NADH to the *Escherichia coli* complex I induces long-range conformational changes, which are required to give access to the ubiquinone reduction site. The addition of NADH but not NADPH induced conformational changes as revealed by electron microscopy and CD-spectroscopy. The enzymatic activity with NADPH is, in contrast to the activity with NADH, not sensitive to inhibitors of the quinone-site and not coupled with proton translocation. We conclude that NADPH is not a substrate for complex I. However, the cofactors of the complex, namely one FMN and several iron-sulfur clusters, are reduced by NADPH. We propose that binding of NADPH leads to a reduction of complex I but leaves the quinone reduction site in a closed state. The reaction with NADPH but not with NADH is coupled with an increased production of superoxide radicals.

P3.3. ACTIVATION OF MOLECULAR OXYGEN BY INFRARED LASER RADIATION IN PIGMENT-FREE AEROBIC SYSTEMS

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We have shown that infrared low intensity laser radiation causes oxygenation of the chemical traps of singlet oxygen (SO), dissolved in organic media and water saturated by air at the normal atmospheric pressure. The photooxygenation rate was directly proportional to the oxygen concentration and inhibited by the singlet oxygen quenchers. The maximum of the photo-oxygenation action spectrum coincided with the maximum of the oxygen absorption band at about 1270 nm. The efficiency of 1064 nm radiation was about 100 times less. The data provide an unambiguous evidence that photooxygenation is determined by the reactive singlet $^1\Delta_g$ state formed as a result of direct laser excitation of molecular oxygen. Using this photoreaction, we determined molar extinction coefficients (ε) for oxygen molecules at 1270 nm in air-saturated carbon tetrachloride. The relative values of ε in organic solvents, whose molecules do not contain hydroxyl groups, were shown to correlate with the relative values of the rates of radiative deactivation of singlet oxygen in the same media, which were obtained from phosphorescence measurements. In alcohol, water and deuterium oxide the values of ε and the radiative rates did not correlate that might be due to a hydrogen bond formation between singlet oxygen and OH-groups of solvent molecules. The data are of interest in connection with the studies of biological action of low energy laser radiation, and show that activation of oxygen caused by its direct photoexcitation may occur in the natural chemical and biological systems. This work was supported by the Russian Foundation for Basic Research and the International Science and Technology Center.

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P3.4. QUANTITATIVE ANALYSIS OF MITOCHONDRIAL PROTEIN LEVELS, POST-TRANSLATIONAL MODIFICATIONS AND ENZYME ACTIVITIES WITH RAPID IMMUNOASSAYS SUITABLE FOR BASIC RESEARCH, PHARMACEUTICAL DRUG SAFETY SCREENING AND DIAGNOSIS OF MITOCHONDRIAL DISEASE

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MitoSciences has developed a wide range of reagents, assays and kits (the MitoProfile™ technology) for the study of mitochondrial structure and function. MitoProfile™ tests use a large library of proprietary, specific monoclonal antibodies to identify, purify and analyze mitochondrial proteins from very small amounts of experimental samples or patient-derived tissues. These tests facilitate analysis of problems and questions that would otherwise be difficult to address using classical techniques. As a result, MitoProfile™ tests have proven useful and have gained wide acceptance as tools for basic research into mitochondrial structure/function. Importantly, the tests are also being used in pharmaceutical drug safety screening to reveal adverse mitotoxic effects of drug candidates and in clinical research to help diagnose and characterize mitochondrial disorders. Examples of each of these applications will be presented and discussed in detail. This will include a description of results of basic research applications in which each of the 5 OXPHOS enzyme complexes have been isolated in a single step and then used for both proteomic and functional analysis, for example to characterize the molecular pathology of Complex I alterations in Parkinson's Disease. We will also show how Pharmaceutical drug safety screening studies have analyzed hundreds of compounds using High-Throughput Screening (HTS) formats to quickly identify molecules that have adverse effects on individual OXPHOS enzymes and how these same HTS assays have been used to measure properties such as OXPHOS enzyme IC_{50} of many compounds. Finally, we will present examples of clinical research in which the clinical utility of various simple tests, such as point-of-care dipsticks that can measure protein levels in easily accessible samples such as cheek swabs, are being evaluated for diagnosis and monitoring of inherited mitochondrial disorders affecting the OXPHOS system, progressive neurodegenerative diseases such as Friedrich's Ataxia (Frataxin, see abstract in EBEC 2006 Colloquium 2.4) and the

adverse mitotoxic effects of therapeutic drugs such as Highly Active Anti-Retroviral Therapy (HAART) used to treat HIV/AIDS (Marusich et al., 2006, 13th Conference on Retroviruses and Opportunistic Infections, abstract N-1004).

P3.5. HEPATIC MITOCHONDRIA FROM NIMESULIDE-TREATED ANIMALS HAVE UNCHANGED OXIDATIVE PHOSPHORYLATION AND PERMEABILITY TRANSITION: RELEVANCE FOR NIMESULIDE HEPATOTOXICITY

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Nonsteroidal anti-inflammatory drugs have been associated with hepatoxicity in susceptible patients. One such example is nimesulide, a selective cyclo-oxygenase-2 inhibitor, widely used for the treatment of inflammatory and pain conditions. It was already suggested that nimesulide could exert its hepatoxicity by altering hepatic mitochondrial function. In fact, several published works have demonstrated that nimesulide is toxic for isolated liver mitochondria, including due to the induction of the mitochondrial permeability transition (MPT).

The objective of this work was to verify if liver mitochondria isolated from rats treated with doses of nimesulide well above the therapeutic levels possessed decreased calcium tolerance calcium and oxidative phosphorylation.

Male and female rats received nimesulide or its vehicle twice daily, during 5 days and were killed on the 7th day for the isolation of liver mitochondria. Mitochondrial respiration, the generation of the electric transmembrane potential and MPT induction with calcium and phosphate were characterized in all experimental groups.

Nimesulide had no effect on liver mitochondrial function. Not only indexes of mitochondrial integrity and oxidative phosphorylation efficiency were unchanged, as liver mitochondria isolated from treated and control animals showed the same behavior towards calcium, which demonstrates that liver mitochondria from nimesulide-treated animals were not more susceptible to the induction of the MPT.

The hypothesis that mitochondrial dysfunction is linked to nimesulide-induced hepatotoxicity may be only valid in some individuals with changed ability to metabolize the parent compound. In the animals tested, no evidence of degraded mitochondrial function due to nimesulide administration could be found.

P3.6. cAMP-DEPENDENT PROTEIN KINASE AND A-KINASE ANCHOR PROTEINS IN THE INNER COMPARTMENT OF MAMMALIAN HEART MITOCHONDRIA

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In mammalian cells cAMP is produced by the adenylyl cyclase of the plasma membrane, in response to extracellular signals, and by the bicarbonate activated soluble adenylyl cyclase localized to nucleus, mitochondria, microtubules and other intracellular structures (1) Production of cAMP by adenylyl cyclase and its hydrolysis by phosphodiesterases (2) can generate different pools of cAMP in various cellular compartments with activation of cAMP effectors. PKA is considered the main effector of cAMP action. The molecular basis for distinct subcellular localization of cAMP effectors is exemplified by the class of PKA anchoring proteins (AKAPs) (3). PKA has been detected in the outer and inner membrane/matrix fraction of mammalian mitochondria (4-6) where it phosphorylates various mitochondrial proteins (7).

In this work an immunochemical, radioactive labelling and activity analysis of the PKA/AKAP complex submitochondrial localization in rat heart is presented. The densitometric immunoblot analysis shown that the large majority (90%) of mitochondrial PKA is found in the mitoplast fraction (inner membrane/matrix fraction) when prepared both from isolated mitochondria or cardiomyocytes. The remaining amount of C-PKA and R-PKA, associated with the outer mitochondrial membrane is, thus relatively small. The same distribution pattern applies to AKAP121. This distribution pattern was further verified by measurement of the specific PKA activity. C-PKA, R-PKA and AKAP121 are largely resistant to trypsin digestion, unless mitochondria are not disrupted by Triton-X 100. R-PKA and with it C-PKA are released from mitoplasts by the Ht31 peptide, competitive inhibitor for the R-PKA-AKAP binding. Electron microscopy analysis of proteins labelled by gold-conjugated antibody shows that like C-PKA and R-PKA (4), also AKAP121 is present in the inner mitochondrial compartment apparently clustered on the inner mitochondrial membrane. Radiolabelling analysis of R-PKA by ^{32}P-cAMP and AKAP by ^{32}P-phosphorylated RII-PKA respectively, confirmed the presence of these proteins in the inner

mitochondrial compartment and revealed, at the same time, that in the inner mitochondrial compartment R-PKA and AKAP undergo proteolytic degradation by mitochondrial processing peptidase. Turnover of these proteins might contribute to mid-term plasticity of the cAMP-controlled protein phosphorylation system. A down-stream extension of the effect of cAMP in the inner mitochondrial compartment mediated by adenylyl cyclase, phosphodiesterases, PKA, protein phosphatase(s), EPAC proteins and G proteins localized in this subcellular compartment can have definite impact on cellular energy metabolism, apoptosis, cell growth, differentiation and transformation.

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