EBEC SHORT REPORTS
SUPPLEMENT VOLUME 13

13th EUROPEAN
BIOENERGETICS CONFERENCE
21 AUGUST–26 AUGUST 2004
PISA, ITALY

doi:10.1016/j.bbabio.2004.06.002

THIRTEENTH EUROPEAN BIOENERGETICS CONFERENCE

Pisa, Italy

21 August–26 August 2004

EBEC SHORT REPORTS, SUPPLEMENT Vol. 13

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USA mailing notice: *Biochimica et Biophysica Acta (Bioenergetics)* (ISSN 0005-2728) is published monthly by Elsevier B.V. (P.O. Box 211, 1000 AE Amsterdam, The Netherlands).
Annual subscription price in the USA US$ 2223.00 (valid in North, Central and South America), including air speed delivery. Periodical postage rate is paid at Jamaica, NY 11431.
USA POSTMASTER: Send address changes to *Biochimica et Biophysica Acta (Bioenergetics)*, Publications Expediting, Inc., 200 Meacham Avenue, Elmont, NY 11003.
AIRFREIGHT AND MAILING in the USA by Publications Expediting, Inc., 200 Meacham Avenue, Elmont, NY 11003.

Printed in The Netherlands
♾ The paper used in this publication meets the requirements of ANSI/NISO Z39.48-1992 (Permanence of Paper).

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FROM THE ORGANISERS

In a Meeting of the delegates of the different European Bioenergetics Groups in Göteborg, 1998, it was chosen Italy as the country hosting the 13th EBEC meeting in 2004. The Italian group of Bioenergetics and Biomembranes took the risk and the responsibility to organize the meeting and indicated Pisa as a place with excellent facilities.

Despite the occurring of several meetings in fields related with Bioenergetics in the same period, the cost of the registration fee, and the difficulty of finding sponsorships to support Conferences on fundamental research, the Conference in Pisa has recorded more than 400 participants at present (June 15th), indicating that the topics of Bioenergetics are still very attractive.

This book contains as many as 365 abstracts of contributing authors presenting as oral or as posters the results of their work. In this book the abstracts have been grouped and numbered strictly following the Scientific Program of the Conference: Plenary Lecture Abstracts first, Colloquium Abstracts followed by the 14 Poster Session Abstracts. **Please note that the printing order of all abstracts is related to the Scientific program and not to the actual timetable of the Conference.**

We could choose only a limited number of oral presentations among many more of high scientific impact, and besides the well known excellent colleagues, we also selected, in collaboration with the Colloquium Chairpersons, young researchers, who will continue the tradition of the highly recognized research in Bioenergetics.

It has been difficult for the organizers to set up this International Conference of the present magnitude and costs, but enthusiasm and help from a professional congress agency, that we thank, allowed to reach the goal. We apologize for possible inconveniences which might occur, and thank all members of the Committees for their valuable assistance and all colleagues who will contribute actively to make this 13th EBEC a successful scientific event.

Welcome to Pisa!
Andrea B. Melandri and Giancarlo Solaini

Contents

From The Organisers 7
Scientific Program 11
Plenary Sessions 33
Colloquia 55
I-A F-Type ATP Synthases 57
I-B Ion Motive Redox Systems 60
I-C Mitochondrial NADH-Ubiquinone Oxidoreductase 64
I-D Heme-Copper Oxygen Reductase 68
I-E ATP-Driven Transport Systems 72
I-F Quinol-Acceptor Oxidoreductases 75
II-A Mitochondrial Dynamics 78
II-B Channels in Coupling Membranes 81
II-C Secondary Active Transport Systems 84
II-D Physiology and Pathology of Energy Dissipation 87
II-E Reactive Nitrogen Species and Reactive Oxygen Species in Mitochondria 90
III-A Mitochondria in Aging and Degenerative Diseases 95
III-B Biogenesis of Energy Transducing Systems 99
Posters 103
I-A F-Type ATP Synthases 105
I-B Ion Motive Redox Systems 126
I-C Mitochondrial NADH-Ubiquinone Oxidoreductase 133
I-D Heme-Copper Oxygen Reductases 147
I-E ATP-Driven Transport Systems 162
I-F Quinol-Acceptor Oxidoreductases 163
II-A Mitochondrial Dynamics 177
II-B Channels in Coupling Membranes 185
II-C Secondary Active Transport Systems 189
II-D Physiology and Pathology of Energy Dissipation 202
II-E Reactive Nitrogen Species and Reactive Oxygen Species in Mitochondria 215
III-A Mitochondria in Aging and Degenerative Diseases 226
III-B Biogenesis of Energy Transducing Systems 236
Miscellaneous (Other topics) 243
Author Index 265

Scientific Program

MITCHELL MEDAL LECTURE

S-1
Hartmut Michel
Max Planck Inst. Für Biophysik, Abt. Molekulare Membranbiologie, Frankfurt, Germany
Cytochrome c Oxidase: Controversies and Contradictions, but what Do We Really Know ?

PLENARY SESSIONS

AREA I - ENERGY TRANSDUCING SYSTEMS

Session I-A: Electron and Proton Transfer Coupling

I-A pl-1
So Iwata
Department of Biological Sciences, Imperial College, London, UK
Structural Basis of Proton Motive Force Generation and Utilization

I-A pl-2
Sergio Papa
Dept. of Medical Biochemistry and Biology, Univ. of Bari, Italy,
Protonmotive Cooperativity in Cytochrome c Oxidase

I-A pl-3
A.V. Xavier
Inst. de Tecnologia Química e Biológica, Univ. Nova de Lisboa, Lisboa, Portugal
Thermodynamic and Choreographic Constraints for Energy Transduction by Cytochrome c Oxidase

Session I-B: Proton Conduction Pathways

I-B pl-1
Hideki Kandori
Dept. of Materials Science and Engineering, Nagoya Institute of Technology, Nagoya, Japan
Role of Internal Water Molecules in the Proton Conduction of Bacteriorhodopsin

I-B pl-2
Melvin Okamura
Dept. of Physics, Univ. of California in San Diego,La Jolla, USA
Pathway for Proton Transfer in Bacterial Reaction Centers

I-B pl-3
Armen Mulkidjanian, D. Cherepanov, B. Feniouk, O. Gopta, S. Klishin, M. Kozlova, D. Knorre, L. Baciou, P. Sebban & W. Junge
Abt. Biophysik, Univ. Osnabrück, Osnabrück, Germany and Belozersky Inst. of Physico-Chemical Biology, Moscow State Univ., Moscow, Russia, Lab. de Chimie Physique, University Paris-IX, Orsay, France.
Proton Transfer Dynamics in Photosynthetic Membranes

Session I-C: Transport ATPases

I-B pl-1
John E. Walker
Medical Research Council, Dunn Human Nutrition Unit, Cambridge, UK
The Rotary Mechanism of ATP Synthase

I-B pl-2
David H. MacLennan
Banting and Best Dept. of Medical Research, Univ. of Toronto, Toronto, Canada
The Regulation of SERCA Type Pumps by Phospholamban and Sarcolipin

I-B pl-3
Misamitsu Futai, G-H Sun-Wada and Y. Wada
Div. of Biological Sciences, ISIR, Osaka Univ. and CREST (JST), Osaka, Japan
Diverse Roles of Mammalian V-Type ATPases: Toward the Physiological Understanding of Inside Acidic Compartments

Session I-D: Photoactivated Systems

I-D pl-1
Petra Fromme, H. Yu, Y. Bukman, C. Yolley, D.K. Chauhan, A. Melkozernov and I. Grotjohann,
Dept. of Chemistry and Biochemistry, Arizona State University, Tempe, U.S.A.
Structure and Function of Photosystem I and II

I-D pl-2
Janos. K. Lanyi,
Dept. Physiology and Biophysics, University of California, Irvine, U.S.A.
Mechanism of Proton Transport from Crystallographic Structures of the Nine States of the Bacteriorhodopsin Photocycle.

I-D pl-3
Giovanni Venturoli, F. Francia, G. Palazzo, A. Mallardi, L. Cordone
Dept. of Biology, Univ. of Bologna, Bologna, Italy
The Coupling between Electron Transfer and Protein Dynamics in the Bacterial Photosynthetic Reaction Center: Trapping of Conformational Substates in Room Temperature Amorphous Matrices.

AREA II—CELLULAR BIOENERGETICS

Session II-A: Bioenergetics and Apoptosis

II-A pl-1
Richard J. Youle
Biochemistry Section, SNB, NINDS, NIH, Bethesda, USA
Mitochondrial Morphogenesis and Apoptosis

II-A pl-2
Pierluigi Nicotera
MRC Toxicology Unit, Univ. of Leicester, Leicester, U.K.
The Role of Mitochondrial Calcium Overload and Mitochondrial Function in Ischemic Cell Death and Apoptosis

II-A pl-3
Fabio Di Lisa
Dip. di Chimica Biologica, Univ. di Padova, Padova, Italy
Mitochondria and Ischemic Heart

Session II-B: Secondary Active Transport in Cell Biology

II-B pl-1
Padan, E., Tzubery, T., Herz, K., Kozachkov, L., Rimon, A. and Galili, L.
Institute of Life Sciences, Hebrew University of Jerusalem, erusalem, Israel
Nhaa of Escherichia coli, as a Model of a pH Regulated Na^+/H^+ Antiporter

II-B pl-2
Ferdinando Palmieri
Dept. of Pharmaco-Biology, Univ. of Bari, Italy
Identification of New Mitochondrial Carriers: from Gene to Function

II-B pl-3
Reinhard Krämer and Susanne Morbach
Inst. of Biochemistry, Univ. Köln, Köln, Germany
BetP, a Transporter with Three Different Functions: Betaine Transport, Osmosensing, and Osmoregulation.

Session II-C: Molecular Mechanic Energy Transducers

II-C pl-1
Wolfgang Junge
Abt. Biophysik, Univ. Osnabrück, Osnabrück, Germany
Torque-Generation in and -Transmission between F_o and F_1

II-C pl-2
Günther Woehlke, M. Schliwa
Adolf-Butenandt-Inst., Cell Biology, Ludwig-Maximilians-University, Munich, Germany
Comparative Aspects of Kinesins, Dyneins and Myosins

II-C pl-3
Dale B. Wigley
Molecular Enzymology Lab., Cancer Research UK, Clare Hall Laboratories, London Research Institute, Potters Bar, U.K.
Structure of Rec BCD: A Machine for Processing Breaks in DNA

Session II-D: Oxygen Radicals and Nitrogen Monoxide

II-D pl-1
Michael T. Wilson
Dept. Biological Sciences, Univ. of Essex, Colchester, UK
Radicals and Radical Chemistry in the Mitochondrion.

II-D pl-2
Cecilia Giulivi, Nathaniel Traaseth, Sarah Elfering, Joseph Solien and Virginia Haynes
Dept. of Chemistry, Univ. of Minnesota, Duluth, U.S.A.
Regulation of Mitochondrial Nitric-Oxide Synthase

II-D pl-3
Guy C. Brown
Dept. of Biochemistry, Univ. of Cambridge, Cambridge, U.K.
How Nitric Oxide Zaps Mitochondria

AREA III—BIOGENESIS AND PATHOLOGY

Session III-A: Organelle Bioegenesis

III-A pl-1
Douglas C. Wallace
Center for Molecular and Mitochondrial Medicine and Genetics, Univ. of California, Irvine, USA
Ancient Origins—Modern Disease: the Mitochondrial Connection

III-A pl-2
Nikolaus Pfanner, Chris Meisinger, Nils Wiedemann, Agnieszka Chacinska, Peter Rehling
Inst. für Biochemie und Molekularbiologie, Univ. Freiburg, Freiburg, Germany
Import and Assembly of Mitochondrial Proteins

III-A pl-3
Francis-André Wollman, Drapier D., Eberhard S., Rimbault B., Vallon O., Choquet Y.
UPR-CNRS assP6- 1261, IBPC, Paris, France
Regulation of atpA and atpB Gene Expression Illustrates Specific Traits of Chloroplast Gene Expression and Chloroplast Protein Assembly

Session III-B: Mitochondrial Bioenergetics and Diseases

III-B pl-1
Salvatore Di Mauro
Dept. of Neurology, College of Physicians and Surgeons, Columbia Univ., New York, USA
Mitochondrial DNA and Pathology.

III-B pl-2
Giorgio Lenaz, Alessandra Baracca, Valerio Carelli, Marilena D'Aurelio, Gianluca Sgarbi and Giancarlo Solaini
Dept. of Biochemistry, Univ. of Bologna, Bologna, Italy
Bioenergetics of Mitochondrial Diseases Associated with mtDNA Mutations

III-B pl-3
Joseph Houstek
Inst. of Physiology, Acad. of Sciences of the Czech Republic, Prague, Czech Republic
Nuclear DNA Mutations and Energy Transduction Impairment

SPECIAL EVENING SESSION

S-2

G. Brandolin, C. Dahout, R. Kahn, V. Trézéguet, G.J.M. Lauquin and E. Pebay-Peyroula
DRDC/BBSI, CEA-Grenoble, France, IBS Grenoble, France LPMC-IBGC, Bordeaux, France.

Structure and Function of the Mitochondrial ADP/ATP Carrier

COLLOQUIA

AREA I—ENERGY TRANSDUCING SYSTEM

Colloquium I-A: F type ATP Synthases.
Chairpersons: Robert H. Fillingame and Giorgio Lenaz

I-A c-1
Robert H. Fillingame, Oleg Dmitriev, Brian Schwem, Christine Angevine and Karen Freedman
Dept. of Biomolecular Chemistry, Univ. of Wisconsin Medical School, Madison, USA
Helix–Helix Interactions and Aqueous Access Channels in Subunit a of E. coli ATP Synthase

I-A c-2
Peter Gräber, M. Diez, B. Zimmermann, S. Steigmiller, J. Petersen, M. Börsch
Inst. für Physikalische Chemie, Albert Ludwigs Univ., Freiburg, Germany
Subunit Movements in H-ATP Synthases during Catalysis in Single Molecules

I-A c-3
Masasuke Yoshida
Tokyo Inst. of Technology, Yokohama, Japan
Engine and Brakes of ATP Synthase

I-A c-4
Peter Dimroth
Inst. for Microbiology, ETH, Zürich, Switzerland
Molecular Insights into the operation of the F_o Motor of the Na^+-F-ATP synthase

I-A c-5
F. Di Pancrazio[(1)], **G. Lippe**[(1)], G. Losano[(2)], I. Mavelli[(1)] and D.A. Harris[(3)]
[(1)]University of Udine, [(2)]University of Torino, [(3)]University of Oxford
Active Regulation of ATP Synthase by the Inhibitor Protein IF1 in Heart.

I-A c-6
P. Turina, D. Giovannini, F. Gubellini, B. A. Melandri
Dept. of Biology, Lab. of Biochemistry and Biophysics, Univ. of Bologna, Bologna, Italy
The Physiological Ligands ADP and P_i Modulate the Degree of Intrinsic Coupling in the ATP Synthase of the Photosynthetic Bacterium Rhodobacter capsulatus.

Colloquium I-B: Ion Motive Redox Systems.
Chairpersons: Günther Schaefer and Davide Zannoni

I-B c-1
Günter Schaefer
Inst. für Biochemie, Univ. zu Lübeck, Lübeck
Simplicity and Complexity of Ion Motive Redox Systems in Archaea

I-B c-2
A. Pedersen, T. Johansson, J. Rydstrom, B.G. Karlsson
Dept. of Chemistry and Bioscience, Goteborg Univ and Chalmers Tech. Univ, Goteborg, Sweden
Transhydrogenase DIII Solution Structure Reveals a Redox-Dependent Conformational Change—A Mechanistic Key to Enzyme Turnover?

I-B c-3
T.H.C. Brondijk, G.I. van Boxel, S.A. White and J.B. Jackson
School of Biosciences, Univ. of Birmingham, Edgbaston, Birmingham, U. K.
Hydride Transfer Site of Proton-Translocating Transhydrogenase of Rhodospirillum rubrum

I-B c-4
Reiner Hedderich, L. Forzi, B. Soboh and A. Stojanowic
Max-Planck-Institute for Terrestrial Microbiology, Marburg, Germany
Energy-Converting [NiFe] Hydrogenases from Archaea and Bacteria: Ancestors of Complex I?

I-B c-5
Karine Bagramyan[(1)], Rajat Sapra [(2)], Michael W. Adams[(2)]
[(1)]Yerevan State University, 375049 Yerevan, Armenia; [(2)]University of Georgia, Athens, GA 30602, USA
Hydrogenases—A Mere Electron Sink or Something More?

I-B c-6
A.G. McEwan, P.V. Bernhardt, N. Creevey, *G.R. Hanson, C.A. McDevitt
School of Molecular and Microbial Sciences and *Centre for Magnetic Resonance, The Univ. of Queensland, Brisbane, Australia
Ion Motive Electron Transfer Pathways Involving Type II Molybdoenzymes of the DMSO Reductase Family

Colloquium I-C: Mitochondrial NADH – Ubiquinone Oxidoreductase.
Chairpersons: Ulrich Brandt and Sergio Papa

I-C c-1
Ulrich Brandt
Inst. für Biochemie I, Fachb. Medizin der Univ. Frankfurt, Frankfurt am Main, Germany
Mitochondrial Complex I (NADH–Ubiquinone Oxidoreductase)—Old Questions, Unexpected Answers and New Approaches

I-C c-2
Judy Hirst
Dunn Human Nutrition Unit, Medical Research Council, Cambridge, U.K.
Coupled Electron Transfer and Catalysis in Mitochondrial Complex I

I-C c-3
Leonid A. Sazanov, Ekaterina A. Baranova, Peter J. Holt, Aygun A. Mamedova and David J. Morgan
Medical Research Council, Dunn Human Nutrition Unit, Cambridge, UK
Conformational Changes as Part of the Mechanism of E. Coli Complex I

I-C c-4
M. Kervinen, J. Pätsi,(1) M. Finel, I.E. Hassinen
Dept. of Medical Biochemistry and Molecular Biology, Univ. of Oulu, and (1)University of Helsinki, Finland.
Positional Constraints of a Pair of Membrane-Embedded Acidic Residues in the nuok Subunit (ND4L Subunit Counterpart) of Escherichia coli NDH-1 for High Ubiquinone Reductase Activity and Growth.

I-C c-5
Andrej D. Vinogradov, I.S. Gostimskaya, M.V. Loskovich, and V.G. Grivennikova
Dept. of Biochemistry, School of Biology, Moscow State University, Moscow, Russia
Possible Mechanisms of Regulation of the Mitochondrial Complex I as Related to its Slow Active/Inactive Transition

I-C c-6
S. Scacco(1), A.M. Sardanelli(1), V. Petruzzella(1), R. Vergari(1), A. Signorile(1) and S. Papa(1,2)
(1)Dep. Medical Biochem., University of Bari; (2)Inst. of Biomembr. and Bioenerg.,CNR; Bari-Italy
Expression Products and Function of the Nuclear Ndufs4 Gene of Complex I (NADH–Ubiquinone Oxidoreductase)

Colloquium I-D: Heme-Copper Oxygen Reductase:
Chairpersons: Mårten Wikström and Nazzareno Capitanio

I-D c-1
Mårten Wikström
Helsinki Bioenergetics Group, Inst. of Biotechnology, Univ.of Helsinki, Helsinki, Finland
The Proton Transfer Pathways in Cytochrome Oxidase

I-D c-2
Shinya Yoshikawa[(1)], Hideo Shimada[(2)], and Tomitake Tsukihara[(3)]
[(1)]Dept. of Life Science, Univ. of Hyogo, [(2)]Dept. Biochemistry, School of Medicine, Keio Univ., [(3)]Dept. for Protein Research, Osaka University, Japan
The Low Spin Heme of Cytochrome c Oxidase as the Driving Element of the Proton Pumping Process

I-D c-3
Bernd Ludwig
Biozentrum, Univ. of Frankfurt, Frankfurt; Germany
Electron and Proton Access to the Paracoccus denitrificans Cytochrome c Oxidase.

I-D c-4
J. Heberle, R.M. Nyquist, D. Heitbrink, C. Bolwien, R.B. Gennis
Forschungszentrum Jülich, IBI-2: Structural Biology, Jülich, Germany
Direct Observation of Protonation Reactions During the Catalytic Cycle of Cytochrome c Oxidase: Controversies on the Role of E_{286}.

I-D c-5
G. Larsson, A. Namslauer, M. Brändén and P. Brzezinski
Dept. of Biochemistry and Biophysics, Stockholm Univ., Arrhenius Lab. for Natural Sciences, Stockholm, Sweden
Electron and Proton Transfer during the F to O Transition in Cytochrome c Oxidase

I-D c-6
A. Giuffre', E. Forte, F. M. Scandurra, O.H. Richter[(1)], E. D'Itri, P. Sarti, B. Ludwig[(1)] and M. Brunori
Dept. of Biochemical Sciences and CNR Inst. of Molecular Biology and Pathology, Univ. of Rome 'La Sapienza', Rome, Italy. [(1)]Inst. of Biochemistry, Molecular Genetics, Univ. of Frankfurt, Biozentrum, Frankfurt, Germany
Proton Uptake upon Anaerobic Reduction of the Paracoccus denitrificans Cytochrome c Oxidase

Colloquium I-E: ATP – Driven Transport Systems
Chairpersons: André Goffeau and Sabina Passamonti

I-E c-1
André Goffeau, Chair
Institut Sciences Vie, Univ. de Louvain, Louvain La Neuve, Belgium
Comments on the Structure and Catalytic Mechanism of the Yeast Multidrug Efflux ABC Transporter PDR5P.

I-E c-2
Amy Davidson, M. Austermuhle, C. Klug
Baylor College of Medicine, Houston, TX; Medical College of Wisconsin, Milwaukee, WI, USA
Mechanism of Action of a Bacterial ATP-Binding Cassette Transporter

I-E c-3
Enrico Martinoia, Markus Klein, Markus Geisler
Inst. für Pflanzenbiologie, Univ. Zürich, Zürich, Switzerland
Plant ABC Transporters? Detoxifiers, Stomata Regulators, and Hormone Transporters

I-E c-4
L. Balakrishnan, S. Velamakanni, A. Kleijn and H. W. van Veen
Dept. of Pharmacology, Cambridge Univ., Cambridge, UK.
Reversed Transport by the ABC Multidrug Efflux Pump LmrA Couples Drug Uptake to ATP Synthesis.

I-E c-5
A. Armbrüster[(1)], S. Juliano[(1)], Ü. Coskun[(1)], D.I. Svergun[(2)], M. Börsch[(3)], and G. Grüber[(1)]
[(1)]Univ. des Saarlandes, Fachrichtung Biophysik, Homburg, [(2)]European Molecular Biology Laboratory, Hamburg Outstation, Hamburg, [(3)] Physikalisches Institut, Universität Stuttgart, Stuttgart, Germany
Structural And Functional Characterization of Vma5p (Subunit C) of the Yeast Vacuolar ATPase

I-E c-6
M. Iwata, H. Imamura, E. Stambouli, M. Tamakoshi, H. Makyio, J. Barber, M. Yoshida, K. Yokoyama, S. Iwata
ATP System Project, ERATO, Japan Science and Technology Corporation, Yokohama, Japan,
Crystal Structure of a Central Stalk Subunit C and Reversible Association/Dissociation of V-ATPase.

Colloquium I-F: Quinol – Acceptor Oxidoreductases
Chairpersons: Peter R. Rich and Bruno A. Melandri

I-F c-1
Peter R Rich
Dept. of Biology, University College, London, UK
The Quinone Chemistry of bc_1 Complexes

I-F c-2
Carola Hunte, T. Wenz, S. Solmaz, F. MacMillan, P. Hellwig, S. Richers, H. Palsdottir
Max-Planck-Inst. für Biophysik, Abt. Molekulare Membranbiologie, Frankfurt, Germany
Structure–Function Relationship in the Cytochrome bc_1 Complex

I-F c-3
Daniel Picot
CNRS UMR 7099 Institut de Biologie Physico-Chimique Paris, France
The Structure of the Cytochrome b_6f Complex from a Green Alga

I-F c-4
B.L. Trumpower, R. Covian, and E.B. Gutierrez-Cirlos
Dept. of Biochemistry, Dartmouth Medical School, Hanover, NH, USA
Half-of-the-Sites Reactivity of the Yeast Cytochrome bc_1 Complex

I-F c-5
M.S. Albury and A.L. Moore.
Biochemistry Dept., School of Life Sciences, Univ. of Sussex, Falmer, Brighton, UK,
Structure–Function Studies of the Trypanosome Alternative Oxidase

I-F c-6
F. Macmillan, C. Lange, K. Redding, and C. Hunte
Inst. of Physical and Theoretical Chemistry, J. W. Goethe University, Frankfurt, Germany
Resolving Protein–Quinone Interactions Using Pulsed EPR Spectroscopy

AREA II—CELLULAR BIOENERGETICS

Colloquium II-A: Mitochondrial Dynamics
Chairpersons: Jean Claude Martinou and Luca Scorrano

II-A c-1
Benedikt Westerman
Inst. Zellbiologie, Univ. Bayreuth, Bayreuth, Germany
Molecular Machinery of Mitochondrial Motility, Fusion and Fission

II-A c-2
Yasushi Matsui, Takashi Itoh, Shunsuke Kondo, Akio Toh-e, Akiko Watabe, and Rie Matsui
Dept. of Biological Sciences, Univ. of Tokyo, Hongo, Tokyo, Japan
Class V Myosin-Dependent Systems for Mitochondrial Distribution in the Budding Yeast.

II-A c-3
Manuel Rojo, Frédéric Legros, Florence Malka, Paule Frachon and Anne Lombès
INSERM U582-Institut de Myologie, 75651 Paris Cedex 13-France
Organization and Dynamics of Human Mitochondrial DNA

II-A c-4
R. Rossignol, R. Gilkerson, R. Aggeler, K., Yamagata, S.J Remington and R.A. Capaldi
Inst. of Molecular Biology, Univ. of Oregon, Eugene, OR, USA
Energy Substrate Modulates Mitochondrial Structure and Oxidative Capacity in Cancer Cells.

II-A c-5
S. Cipolat, O. Martins de Brito, B. Dal Zilio and L. Scorrano
Dulbecco Telethon Inst., Venetian Inst. of Molecular Medicine, Padova, Italy
Opa1 Requires Mitofusin-1 to Promote Mitochondrial Fusion.

II-A c-6
Jean-Claude Martinou, Philippe Parone, Sandrine Da Cruz and Yves Mattenberger
Dept of Cell Biology, Sciences III, Univ. of Geneva, Geneva, Switzerland.
Mitochondrial Fission: what for?

Colloquium II-B: Channels in Coupling Membranes
Chairperson: Mario Zoratti

II-B c-1
Javier Alvarez,
Inst. of Biology and Molecular Genetics, Univ. of Valladolid, Valladolid, Spain
Modulation of the Mitochondrial Ca^{2+} Uniporter

II-B c-2
Adam Szewczyk
Nencki Institute of Experimental Biology, Warsaw, Poland
Mitochondrial ATP-Regulated Potassium Channel

II-B c-3
Varda Shoshan-Barmatz, Ran Zalk, Adrian Israelson and Heftsi Azoulay-Zohar
Department of Life Sciences, Ben-Gurion University, Beer-Sheva, Israel
A Tetrameric Voltage-Dependent Anion Channel Mediates Cytochrome c Release from Mitochondria

II-B c-4
D. Siemen, I. Sayeed, S. Andrabi, S. Parvez, G. Seitz, T. Horn, P. Schoenfeld
Depts. of Neurol., Medical Neurobiol. and Biochem., Univ. of Magdeburg, Magdeburg, Germany
Evidence from Patch-Clamp Experiments for Cell-Protective Effect of Permeability Transition Pore Blockade

II-B c-5
Emy Basso[(1)], Paolo Bernardi[(1)] and Michael Forte[(2)]
Dept. of Biomedical Sciences, Univ. of Padova, Italy[(1)], and The Vollum Inst., Oregon Health and Sciences Univ., Portland, Oregon, USA[(2)]
Cyclophilin D, Cyclosporin A and the Modulation of the Mitochondrial Permeability Transition in Cyclophilin D-Null Mice.

II-B c-6
Szabo, I. (EMBO Young Investigator), Adams, C., Jekle, A., Bock, J., Lang, F., Zoratti, M. and Gulbins, E.
Dep. of Biology and Inst. of Neuroscience, Padova; Italy, Physiology Inst. Tuebingen, Dep. of Molecular Biology, Essen, Germany.
Role of a Novel Mitochondrial Potassium Channel in Apoptosis

Colloquium II-C: Secondary Active Transport Systems
Chairpersons: Ulf-Ingo Flügge and Cesare Indiveri

II-C c-1
U.I. Flügge (Chair), R. Kunze, R. Schwacke, A. Schneider, E. van der Graaf
Univ. zu Köln, Botanisches Inst., Köln, Germany
Functional Genomics of Plant Membrane Proteins

II-C c-2
M. Joanne Lemieux[(1)], Yafei Huang, Jinmei Song, Manfred Auer[(2)] and Da-Neng WangSkirball
Inst. of Biomolecular Medicine, New York Univ. School of Medicine, New York, USA;
[(1)]Present address: Dept. of Biochemistry, Univ. of Alberta, Edmonton, Canada, [(2)]Lawrence Berkeley National Laboratory, Berkeley
Structural Basis for Substrate Translocation of the Escherichia coli Glycerol-3-Phosphate Transporter, GlpT

II-C c-3
Eckhard Boles
Inst. für Mikrobiologie, Goethe-Univ. Frankfurt, Frankfurt am Main, Germany
Yeast as a Model System for Studying Glucose Transport and Sensing.

II-C c-4
Hartmut Wohlrab
Boston Biomedical Research Inst. and Harvard Medical School, Watertown,
Novel Inter- and Intrasubunit Transport-Relevant Contact Sites between Residues of the Mitochondrial Phosphate Transport Protein.

II-C c-5
Nathan Nelson, Adiel Cohen
Dept. of Biochemistry, Tel Aviv Univ., Ramat Aviv, Tel Aviv, Israel
The Function of Two Novel Families of Metal-Ion Transporters Chaperons

II-C c-6
C. Trötschel, A. Burkovski, R. Krämer
Univ. of Cologne, Inst. of Biochemistry, Cologne, Germany
Characterization of L-Methionine Transport in Corynebacterium glutamicum: Identification of the First Bacterial Methionine Excretion System

Colloquium II-D: Physiology and Pathology of Energy Dissipation.
Chairpersons: Eduardo Rial and Paolo Bernardi

II-D c-1
Xavier Leverve, C. Batandier, B. Garait, B. Sibille, R. Favier, E. Fontaine
LBFA-Inserm E0221, Université J. Fourier, Grenoble, France
Central Role of the Respiratory Chain Complex 1 in the Tuning of Oxidative Phosphorylation Efficiency.

II-D c-2
Abdul G. Dulloo
Dept. of Medicine, Div. of Physiology, Univ. of Fribourg, Fribourg, Switzerland;
The Search for Mechanisms Underlying Thermogenesis in Skeletal Muscle: beyond Uncoupling Proteins

II-D c-3
Rachel Navet, [(1)]Ange Mouithys-Mickalad, Pierre Douette, Claudine Sluse-Goffart, and Francis Sluse.
Lab. of Bioenergetics, and [(1)] Lab. of Oxygen Biochemistry, Centre of Oxygen Research and Development, Inst. of Chemistry, Univ. of Liège, Belgium.
Uncoupling Protein Activation Decreases the Superoxide Anion Production in Muscle Mitochondria Submitted to Anoxia/ Reoxygenation in vitro.

II-D c-4
Eduardo Rial, P. Tomás, J. Jiménez-Jiménez
Centro de Investigaciones Biológicas CSIC, Madrid, Spain;
Fatty Acid Uncoupling and Mitochondrial Carrier Function

II-D c-5
Michel Rigoulet, Beauvoit Bertrand, Bunoust Odile, Chevtzoff Cyrille, Dejean Laurent, Noubhani Majid, Schaeffer Jacques & Devin Anne
IBGC du CNRS, 1 rue Camille Saint Saëns, Bordeaux, France
Growth Yield Homeostasis in Yeast under Respiratory Conditions: Role of the cAMP Pathway

II-D c-6
S. Uribe, V. Pérez-Vázquez
Inst. de Fisiología Celular, Univ. Nacional Autónoma de México. México City, Mexico
The Yeast Mitochondrial Unspecific Channel as a Possible Regulator of Oxidative Phosphorylation.

Colloquium II-E: Reactive Nitrogen Species and Reactive Oxygen Species in Mitochondria.
Chairpersons: Vladimir Skulachev and Paolo Sarti

II-E c-1
Juan P. Bolaños and Angeles Almeida
Dept. de Bioquimica y Biologia Molecular, Univ. de Salamanca, Salamanca, Spain
Cellular Metabolic Responses to NO-Mediated Mitochondrial Inhibition.

II-E c-2
Vladimir P. Skulachev
Belozersky Inst. of Physico-Chemical Biology, Moscow State Univ., Russia, Moscow
Mitochondria and Reactive Oxygen Species in Programmed Death Phenomena

II-E c-3
Hans Nohl[(1)], Katrin Staniek[(1)] and Andrey Kozlov[(2)]
[(1)]Res. Inst.of Pharmacology and Toxicology, [(2)]Inst. for Experimental and Clinical Traumatology, Veterinary Univ. of Vienna, Vienna, Austria
The Existence of a Mitochondrial Nitrite-Reductase

II-E c-4
Paolo. Sarti, A. Giuffrè, E. Forte, M. Arese, D. Mastronicola[(1)], A. Bacchi, F.M. Scandurra, and M. Brunori.
Dept. of Biochemical Sciences and CNR Inst. of Molecular Biology and Pathology, Univ. of Rome ''La Sapienza'', Rome, Italy,
[(1)]IFO Cancer Institute Regina Elena I00100- Rome, Italy
Nitric Oxide And Cytochrome c Oxidase

II-E c-5
Izyumov D.S., Pletjushkina O. Yu., Sakharov D.V., Wirtz K.W., **Chernyak B.V.**
Belozersky Inst. of Physico-Chemical Biology, Moscow State Univ., Moscow, Russia
Induction of Reactive Oxygen Species Production in Complex I of Mitochodrial Respiratory Chain by Photodynamic Treatment of HeLa Cells

II-E c-6
M. Giorgio, E. Migliaccio, D. Paolucci, F. Orsini, C. Contursi, M. Moroni, M. Marcaccio, F. Paolucci and P.G. Pelicci
European Inst. of Oncology, FIRC institute, Congenia srl, Milan and Univ. of Bologna, Bologna, Italy
P66Shc is a Signal Transduction Red-Ox Enzyme

II-E c-7
P. Ježek[(1)], S. Miyamoto[(2)], P. Di Mascio[(2)], K.D.Garlid[(3)], M. Jabùrek[(1)]
[(1)]Inst. Physiology, Prague Czech Republic; [(2)]Sao Paulo University Brazil; [(3)]Dept. Biology, Portland State Univ., Portland, USA.
Feedback Regulation of Oxidative Stress: Fatty Acid Hydroperoxides are Cycling Substrates of Mitochondrial Uncoupling Protein UCP2

AREA III—BIOGENESIS AND PATHOLOGY

Colloquium III-A: Mitochondria in Aging and Degenerative Diseases
Chairpersons: Nils-Göran Larsson and Giancarlo Solaini

III-A c-1
Nils-Göran Larsson
Dept. of Medical Nutrition and Biosciences, Karolinska Inst., Stockholm, Sweden
Testing the Mitochondrial Theory of Ageing

III-A c-2
Valerio Carelli, Michela Rugolo, Gianluca Sgarbi, Anna Ghelli, Claudia Zanna, Alessandra Baracca, Giorgio Lenaz, Eleonora Napoli, Andrea Martinuzzi, Giancarlo Solaini, Bologna,
Dip. di Scienze Neurologiche, Univ. di Bologna, Bologna, Italy
Bioenergetics Shapes Cellular Death Pathways in Leber's Hereditary Optic Neuropathy (LHON): a Model of Mitochondrial Neurodegeneration.

III-A c-3
Frank N. Gellerich, Zemfira Gizatullina, Ying Chen, Katrin S. Lindenberg, Bernhard G. Landwehrmeyer and Stephan Zierz
Muskellabor der Neurologischen Universitätsklinik, Martin-Luther-Universität Halle-Wittenberg und Abt. für Neurologie der Univ. Ulm
Calcium Sensitivity of Complex I-Dependent Respiration and of Permeability Transition in Skeletal Muscle Mitochondria of Huntington Disease Mices

III-A c-4
A. Matsuno-Yagi, B.B. Seo, E. Nakamaru-Ogiso, P. Cruz, T.R. Flotte, T. Yagi
Div. Biochem, Dept. MEM, The Scripps Res. Inst., La Jolla, CA, USA
The Yeast NADH Dehydrogenase as a Therapeutic Agent for Complex I Defects

III-A c-5
Wolfram S. Kunz
Dept. of Epileptology, Univ. Bonn Medical Center, Bonn, Germany
Alterations of Mitochondrial Function in Epilepsy and Neurodegenerative Diseases

III-A c-6
Natascha Bergamin[(1)], Tania Tiepolo[(1)], Alessia Angelin[(2)], William Irwin[(1,2)], Patrizia Sabatelli[(3)], Carlo Reggiani[(4)], Luciano Merlini[(5)], Nadir Maraldi[(3)], Giorgio Bressan[(1)], Paolo Bernardi[(2)] and **Paolo Bonaldo**[(1)]
Depts. of [(1)] Medical Biotechnologies, [(2)]Biomedical Sciences and [(4)]Anatomy and Physiology, University of Padova, and [(3)]ITOI-CNR and [(5)]Istituto Ortopedico Rizzoli, Bologna
Collagen VI Muscular Dystrophies from Animal Models to Human Therapy: (I) Mitochondrial Mechanisms of Apoptosis in Myoblast Cultures from Ullrich Patients

Colloquium III-B: Biogenesis of Energy Transducing Systems.
Chairpersons: Piotr Slonimski and Maria Nicola Gadaleta

III-B c-1
Fevzi Daldal
Dept. of Biology, Univ. of Pennsylvania, Philadelphia; U.S.A.
Structure, Function and Biogenesis of the Cytochrome bc_1 Complex in Bacteria

III-B c-2
G. Dujardin, N. Bonnefoy, C. Lemaire, P. Hamel, Y. Saint-Georges.
Centre de Génétique Moléculaire, CNRS, Gif sur Yvette France,
Role of Oxa1p in the Assembly of Mitochondrial Respiratory Complexes

III-B c-3
Leo Nijtmans, Cristina Ugalde, Rutger Vogel, Richard Huijbens, Bert Van Den Heuvel, Jan Smeitink
Nijmegen Center for Mitochondrial Disorders, Nijmegen, The Netherlands
Complex I Assembly in Human Cells in Health and Disease

III-B c-4
E Hervouet, J Demont, H Simonnet and C Godinot.
Centre de Génétique Moléculaire et Cellulaire-CNRS-Univ. Claude Bernard de Lyon 1, Villeurbanne, France
Upregulation of Mitochondrial DNA Synthesis and of Oxidative Phosphorylation Complex Biogenesis by the Von Hippel Lindau Factor

III-B c-5
S. Horan and B. Meunier
Wolfson Inst. for Biomedical Research, Univ. College London, London, UK
Cytochrome Oxidase Subunit 2 Mutations and their Effect on Copper Transfer

III-B-c-6
A. Devin., C. Chevztoff, J. Vallortigara and M. Rigoulet
IBGC du CNRS, Bordeaux, France
Yeast Mitochondrial Biogenesis is Regulated via the camp Protein Kinase TPK3

POSTER SESSIONS

I-A F-Type ATP Synthases
I-B Ion Motive Redox Systems
I-C Mitochondrial NADH–Ubiquinone Oxidoreductases
I-D Heme-Copper Oxygen Reductases
I-E ATP-Driven Transport Systems
I-F Quinol Acceptor Oxidoreductases
II-A Mitochondrial Dynamics
II-B Channels in Coupling Membranes
II-C Secondary Active Transport Systems
II-D Physiology and Pathology of Energy Dissipation
II-E NO and Oxygen Radicals in Mitochondria
III-A Mitochondria in Aging and Degenerative Diseases
III-B Biogenesis of Energy Transducing Systems
Miscellaneous

Plenary Sessions

I-A: Electron and Proton Transfer Systems

I-A pl-1. Structural basis of proton motive force generation and utilisation

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A wide range of membrane proteins, including ATP-synthase and secondary transporters, are driven by the proton motive force across biomembranes, which is ubiquitous energy conservation system for many biological systems. The proton motive force is generated by various respiratory enzymes, where proton translocation is driven by the energy released from the electron transport chain. Since 1995, structures of various respiratory enzymes in the aerobic and anaerobic respiratory systems, have been revealed. The results clearly demonstrated that different enzymes utilized totally different strategies for the proton motive force generation. In the lecture, I would summarize the membrane protein structures, which have been solved in my laboratory, and compare the strategies of these enzymes on how to generate proton motive force and how to utilize it from the point of view of structural biology.

I-A pl-2. Protonmotive cooperativity in cytochrome *c* oxidase

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Cooperative linkage of ion, electron and solute interaction at separate sites in allosteric proteins is an important functional attribute of soluble and membrane bound hemoproteins. A paradigm of cooperative/allosteric interactions is offered by hemoglobin. Cooperative electron/electron and proton/electron linkage appears to be widely present in membrane bound protonmotive redox enzymes.

Analysis of cooperative proton/electron linkage at the low potential heme a in cytochrome *c* oxidase is presented. The cooperative interaction of heme a with CuA and CuB is also analysed in the CO-liganded and CN-liganded oxidase. A survey of structural and functional data showing an essential role of cooperative proton/electron linkage at heme a in the proton pump of cytochrome *c* oxidase is presented. On the basis of this, and related functional and structural information, variants for cooperative mechanisms in the proton pump of cytochrome *c* oxidase are examined.

I-A pl-3. Thermodynamic and choreographic constraints for energy transduction by cytochrome *c* oxidase

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Cooperative effects are fundamental for redox/protonic energy transduction processes. However, the primary cooperative mechanism used by transmembrane protein transducer (e.g., cytochrome *c* oxidase, CcO) is still a matter of controversy.

To understand cooperative processes fully it is necessary to obtain the compulsory microscopic cooperativity parameters (thermodynamics) for the functional centres, and relate them to the relevant structural features, including conformational changes (choreographics). Thus, this is a difficult task to achieve for large proteins. One approach is to explore how this may be done by extrapolation from mechanisms used by simpler transducing proteins.

Detailed studies of small, soluble transducing cytochromes show how they can use anti-electrostatic effects to control the synchronous movement of charges. These include negative e-/H^+ cooperativities (redox-Bohr effect, rB). This capacity is the basis of a mechanistic model consistent with the available experimental data for the energy transduction process performed by CcO [1 and references therein]. The key feature of this model is a T/R conformational switch governed by a negative rB, rB-, coupled to the Fea of CcO, which is consistent with the deceptive thermodynamic data available for the redox chemistry of Fea, when combined with a rB^+ linked to another protonic centre. This combination of rBs linked to haem a can adequately explain the small pH dependence of its Em and contributes to explain the previously inversion of the redox potentials of haems a and a3 that governs the electron transfer through these haems.

Orchestrated transfer of electrons and protons, as well as induced conformational changes are essential requirements to ensure the proper order of steps (directionality) and accomplish energy transduction, avoiding unwanted (short-circuiting) reactions. Uncoupling of these processes can have drastic consequences, such as generation of dangerous reactive oxygen species and proton leak back (energy dissipation). This can be achieved by a process governed by anti-electroneutral e-/H^+ cooperative effects. This mechanism does not involve a metastable state and explains how protons can be translocated (ejected) when haem a is reduced, the pulsing/resting switch, as well as the variability observed for the number of protons activated per redox cycle of CcO [2 and references therein].

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I-B: Proton Conduction Pathways

I-B pl-1. Role of internal water molecules in the proton conduction of bacteriorhodopsin

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Rhodopsins possess a positively charged retinal chromophore and negatively charged counterion(s). Internal water molecules have been presumed to play an important role to stabilize the ion-pair state inside protein. In addition, they would assist the transport of proton and chloride ion in bacteriorhodopsin (BR) and halorhodopsin (HR), respectively. Fourier-transform infrared (FTIR) spectroscopy has observed hydrogen-bonding alterations of internal water molecules in archaeal and visual rhodopsins [1, 2]. Together with the recent structural determinations of rhodopsins, water structural changes in their functional processes can be (should be) now discussed in atomic detail.

Although the frequency region was limited for the water bands under weak hydrogen bonds in the previous FTIR spectroscopy [1], newly developed frequency region for X-H and X-D stretching vibrations in D2O provided direct information on hydrogen-bonding alterations that involve water molecules under strong hydrogen bonds [3]. Subsequent isotope labeled studies for BR have revealed how functional groups change their hydrogen bonds; such as the Schiff base N–D group [4], threonine sidechains [5], arginine sidechains [6], and internal water molecules [1, 7, 8]. Similar approaches have been also applied to *pharaonis* phoborhodopsin [9] and bovine rhodopsin [10], which convert light into signal.

In my talk, the recent results of our low-temperature FTIR spectroscopy will be presented to observe internal water molecules of archaeal rhodopsins during their functional processes. In particular, I like to discuss the mechanism of the primary proton transfer reaction in BR, which is closely related to the vectoriality of pump. On the basis of the difference IR spectra for the late photointermediates, we proposed a hydration switch model for the mechanism of proton transfer from the Schiff base to Asp85 [7]. Recent results on HR will be also presented [11].

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I-B pl-2. Pathway for proton transfer in bacterial reaction centers

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The primary energy conversion process in bacterial photosynthesis involves light induced electron and proton transfer processes in the Reaction Center (RC). The absorbed photon leads to the oxidation of a Bacteriochlorophyll dimer and the reduction of a bound quinone molecule coupled with the uptake of two protons from the cytoplasmic side of the membrane.

$$QB + 2e - + 2H + - > QBH2$$

Following this reduction, the reduced quinone is oxidized by the cytochrome bc_1 complex. The electrons cyle back to the RC to rereduce the oxidized BChl and the protons are released on the periplamic side of the membrane giving rise to the proton gradient that drives ATP synthesis.

The binding site for QB lies in the interior of the protein separated from the protein surface by a region containing acidic residues and bound water molecules. The pathway for proton transfer to the QB site has been determined by site directed mutagenesis of acidic residues near the QB site. Proton transfer was blocked by single mutations of acidic groups close to the QB site or by double mutations of acidic groups farther away from the QB site closer to the surface. The involvement of two surface-His residues in the proton transfer pathway were shown to be involved, first by the discovery that metal ion ($Zn2^+$ or $Cd2^+$) binding inhibits proton transfer, then by site directed mutation and chemical rescue.

A puzzling feature of the proton transfer in RCs is the earlier finding that mutants with blocked proton transfer could have proton transfer restored by second site mutations to residues located at some distance away from the original mutation. Recent X-ray crystal structures of two such mutant RCs revealed the structural changes responsible for this long distance effect on proton transfer.

I-B pl-3. Proton transfer dynamics in photosynthetic membranes

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Using native membrane vesicles (chromatophores) of purple phototrophic bacteria *Rhodobacter sphaeroides* and *Rhodobacter capsulatus*, we studied the dynamics of flash-induced proton transfer between the buried catalytic centers of several energy-converting enzymes and the bulk water phase. Generally, these reactions proceed in two steps, namely the proton exchange between the buried group and the membrane surface is followed by the proton transfer across the membrane/water interface.

Tracing proton displacements in response to the reduction of the secondary quinone Qb in the photosynthetic reaction center (RC) has revealed that proton transfer from the bulk water to the membrane surface was delayed as compared to the faster proton equilibration between the Qb site and the membrane surface [Gopta et al., PNAS 1999, 96:13159–13164]. The concurrent kinetic analysis of these data and of the earlier congruent results of Heberle et al. on retarded proton release from the bacteriorhodopsin-containing membranes [Nature, 1994, 370:379–382] yielded a consensus estimate of about 120 meV for the height of the interfacial potential barrier for H^+/OH^- ions [Cherepanov et al. Biophys. J. 2004, 86:665–680]. Due to such a barrier, the proton concentration at the surface (e.g., of bacterial cells) could be higher than the bulk proton concentration and independent of the latter in vivo. The possible nature of the interfacial potential barrier and its consequences (i) for the mechanism of bioenergetic coupling, (ii) for the problem of the seemingly insufficient protonmotive force in alkaliphilic bacteria, and (iii) for the architecture of membrane proteins will be discussed.

Proton transfer from the surface to a membrane-buried catalytic group is, generally, unfavourable because of large solvation penalty. The possible means to compensate this penalty will be considered in relation to the recent data (i) on reduction and protonation of the Qb quinone in the RC of *Rb. sphaeroides* [see the abstract of Kozlova et al. in this volume], (ii) on the electron/proton coupling in the cytochrome bc complex of *Rb. capsulatus* [see the abstract of Klishin and Mulkidjanian in this volume], and (iii) on the proton transfer through the ATP synthase of *Rb. capsulatus* [see the abstract of Junge et al. in this volume].

I-C: Transport ATPases

I-C pl-1. The rotary mechanism of ATP synthase

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The ATP synthase from mitochondria, bacteria and chloroplasts has two major domains Fo is buried in the membrane, and the globular domain, F_1, is outside the membrane and connected to Fo by a central stalk. The proton motive force across the membrane provides the energy for the rotation of an ensemble of the central stalk (subunits γ, δ and ε) and an associated ring of c-subunits in the membrane. The central stalk penetrates through F_1 and is surrounded by a spherical assembly of 3α and 3β subunits arranged in alternation around the central stalk. The three catalytic sites lie mainly in β-subunits at interfaces with α-subunits. The rotation deforms these sites and takes them through states that are ready (the empty state) to move to a substrate accepting state, which then closes to entrap ADP and phosphate (the tight state) allowing ATP to form (the loose state) and then be released as the empty state reforms. These three states (and three intermediate states) have been defined by structural analysis.

Issues to be discussed in the lecture are first, structural changes during catalysis and the role of a peripheral stalk that connects subunit a in Fo to the top of F_1, second to determine whether, as suggested by molecular dynamics, the structure of the β-subunit changes during catalysis, third, how the regulatory protein IF_1 binds to the F_1 domain to inhibit ATP hydrolysis and fourth how the rotation is generated in the Fo domain.

I-C pl-2. The regulation of SERCA type pumps by phospholamban and sarcolipin

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The activity of sarco(endo)plasmic reticulum Ca^{2+} ATPase isoform 2 (SERCA2a) determines the rate of relaxation of the heart and influences contractility by its effect on the size of the lumenal Ca^{2+} store that is available for release in the next beat. The activity of SERCA2a is regulated by phospholamban (PLN) and, potentially by sarcolipin (SLN), a PLN homologue. With many colleagues, we have used mutagenesis and modeling to predict the structure of the PLN/SERCA1a inhibitory interaction. Our model shows that PLN fits into a transmembrane groove in the calcium-free E2 conformation of SERCA1a, but calcium binding closes the groove, accounting for the ability of calcium to disrupt PLN/SERCA interactions. SLN fits into the same groove in SERCA1a. SLN interacts strongly with PLN to form a stable binary complex, which interacts with SERCA to form a superinhibited PLN/SLN/SERCA complex. Modeling predicts that the regulator binding cavity in the E2 conformation of SERCA1a can accommodate both SLN and PLN, but not two PLN helices. With a number of colleagues, we associated overexpression of superinhibitory PLN mutants with depressed SERCA2a function, decreased myocyte calcium kinetics and mechanics, and cardiac remodeling, which led to heart failure in some cases. In collaborative studies, we have studied the role of PLN mutants in human cardiomyopathy. In one family, refractory congestive heart failure was found to be caused by the PLN mutation, Arg-9 to Cys. This dominant mutant did not inhibit SERCA2a directly, but trapped protein kinase A (PKA) and blocked PKA-mediated phosphorylation of wild-type PLN. The resulting dysregulation of calcium homeostasis initiated heart failure. In a second study, a premature stop codon in PLN (L39stop) could be compared to a PLN-null genotype. Heterozygous individuals exhibited hypertrophy without diminished contractile performance, but homozygous individuals developed dilated cardiomyopathy and heart failure. In contrast to reported benefits of PLN ablation in mouse heart failure, humans lacking PLN develop lethal dilated cardiomyopathy. The role of the superinhibitory PLN/SLN complex was evaluated in a mouse model in which SLN was overexpressed in the atrium and ventricle. The complex superinhibited SERCA2a and impaired cardiac contractility, but did not progress to heart failure. Supported by CIHR, HSFO, CGDN.

I-C pl-3. Diverse roles of mammalian V-type ATPases: toward the physiological understanding of inside acidic compartments

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The vacuolar-type H^+-ATPases (V-type or V- ATPase) are ubiquitous eukaryotic proton pummps. They are present in endomembrane organelles such as vacuoles, lysosomes, endosomes, Golgi apparatus, chromaffin granules and coated vesicles, and also in the plasma membranes of specialized cells including osteoclasts and kidney epithelial cells. They pump protons into corresponding compartments.

V-ATPase consists of a membrane periphereal V1 catalytic domain and an integral Vo proton pathway. V-ATPase has some similarities with F-ATPase including rotational mechanism for catalysis and proton translocation, but different in composition of subunits and their unique isoforms.

The Vo ‘‘subunit a’’ has a1, a2, and a3 isoforms showing different subcellular localization, although they are expressed ubiquitously. V-ATPase with a4 is specifically expressed in plasma membrane of renal intercalated cells, and that with a3 in osteoclase plasma membrane. The endosomes/lysosomes having V-ATPase with a3 are transported to the cell periphery and assembled into the plasma membrane of osteoclasts during differentiation. Subunit isoforms are also found in the V1 sector: kidney (d2, G3, and C2-b), testis (E1), brain (G2), lung lamellar bodies (C2-a). The different isoforms affect energy coupling and ATPase catalysis. Thus, unique V-ATPases with different isoforms are involved in bone resorption, renal ion homeostasis, fertilization, transmitter uptake and surfactant excretion.

These results indicate that diverse physiological roles of V-ATPase are established through utilization of specific subunit isoforms, the basic subunit structure and enzyme mechanism being maintained.

I-D: Photoactivated Systems

I-D pl-1. Structure and function of photosystems I and II

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Oxygenic photosynthesis is the main biological process on earth that converts the light energy from the sun into chemical energy.The primary step in this energy conversion, the light induced charge separation, is catalyzed by two distinct, membrane intrinsic protein complexes, photosystems I and II.Photosystem I of cyanobacteria consists of 12 protein subunits, to which more than 100 cofactors are noncovalently bound: one functional unit of Photosystem I contains 96 Chlorophyll a molecules, 22 carotenoids, 3 Fe4S4-clusters and 2 phylloquinones that perform the complex function of light harvesting and charge separation.

The X-ray structure of photosystem I at a resolution of 2.5 Å [1] showed the location of the individual subunits and cofactors and provided new information on the protein-cofactor interactions. Recently, the structural model of plant PS I was determined by BenShem at al [2] shining ligth into the similarities and differences between the plant and cyanobacterial Photosystem I. In the talk, biochemical data and results of biophysical investigations are discussed with respect to the X-ray crystallographic structures of PS I in order to discuss the following open problems in Photosystem I:

(a) Interaction of Photosystem I with the soluble electron carriers ferredoxin and cytochrome c6/plastocyanin

(b) Function of the antenna system in Photosystem I

(c) Interaction of Photosystem I with its peripheral antenna systems

Increasing knowledge on the structure and function of Photosystem II has been accumulated during tha last 3 years by X-ray structure analysis of cyanobacterial Photosystem II at 3.8Å [3], 3.7 Å [4], 3.6 [5] and 3.5 [Å] resolution. The structures will be compared and discussed in respect to the function of Photosystem II with special focus on the oxygen-evolving complex. In the last part of the talk, the structures of Photosystem I and II will be compared and discussed in respect to the evolution of the Photosystem I and II.

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I-D pl-2. Mechanism of proton transport from crystallographic structures of the nine states of the bacteriorhodopsin photocycle

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In the last few years we have (6) intermediates of the bacteriorhodopsin photocycle in crystabeen able to trap the K (1), L (2), M1 (3), M2 (4), M2′ (5) and N′ ls, and determine their structures by X-ray diffraction to 1.43–1.62 Å resolutions. With models proposed earlier for N and O from crystallography of nonilluminated mutants (7, 8), structures are now available for the initial bacteriorhodopsin state (1, 9) as well as its eight intermediate states. They reveal the structural reasons for protonation of the retinal Schiff base by Asp85, proton release to the extracellular membrane surface, the switch event that allows reprotonation of the Schiff base from the cytoplasmic side, side-chain and main-chain motions initiated in the cytoplasmic region, formation of a single-file chain of hydrogen-bonded water molecules that conducts the proton of Asp96 to the Schiff base, and reprotonation of Asp96 from the cytoplasmic surface. The structural models describe in atomic detail how the transformations of the photoisomerized retinal change its interaction with wat402, Asp85, and Trp182, and how the displacements of main-chain and functional residues, and the water molecules sequestered in the extracellular and cytoplasmic regions facilitate the transfer of a proton from one membrane surface to the other. A detailed atomic-level model for the transport will be given, that describes it as the gradual relaxation of the distorted retinal that causes a cascade of displacements of water and protein atoms that spreads to the rest of the protein and results in vectorial proton transfers to and from the Schiff base. Such local-global coupling of conformational changes may be the general principle for how ion pumps and receptors function (10).

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I-D pl-3. The coupling between electron transfer and protein dynamics in the bacterial photosynthetic reaction center: trapping of conformational substates in room temperature amorphous matrices

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The photosynthetic reaction center (RC) from purple bacteria is becoming a prototype in exploring the coupling between internal protein motions and long-range electron transfer (ET). This interplay has been extensively investigated by hampering RC substate interconversion and relaxations at low temperatures (McMahon et al., 1998, Biophys. J. 74, 2567). As a complementary approach for limiting RC dynamics at room temperature we embedded the protein within a dehydrated trehalose matrix. Spectroscopic studies and molecular dynamics simulations performed on myoglobin/trehalose/water systems have shown that the nonharmonic contributions to the protein motions (attributed to thermal fluctuations among conformational substates) are greatly reduced in a dry trehalose matrix (Librizzi et al., 2002, J. Chem. Phys. 116, 1193) when the protein is confined within a network of hydrogen bonds connecting protein groups, residual water and trehalose molecules (Cottone et al., 2002, J. Chem. Phys. 117, 9862). These properties can be put in relation with the unique efficacy of trehalose in the preservation of biostructures. We analysed the effect of progressive dehydration of RC-trehalose-water matrices on two light-induced reactions: (a) ET from the primary (QA-) to the secondary (QB) quinone acceptor; (b) recombination of QA- with the photoxidized primary donor P^+. Reaction (a) is slowed by more than four orders of magnitude in relatively wet glasses (Francia et al., 2003, Biophys. J. 85, 2760), consistently with a conformational gate mechanism. When the amount of residual water in the matrix is further reduced (to approximately 0.5 water per trehalose molecule), the kinetics of reaction (b) at room temperature becomes broadly distributed and accelerated as observed in glycerol-water mixtures only at cryogenic temperatures. These effects have been taken to reflect a drastic inhibition of the RC relaxation from the dark-adapted to the light-adapted conformations as well as of the interconversion between conformational substates (Palazzo et al., 2002, Biophys. J. 82, 558). Comparison of the effects of matrix dehydration on the two ET reactions examined points to a highly selective correlation (slaving) between the specific internal motions governing different ET processes and the structure and dynamics of the external medium at the protein surface.

II-A: Bioenergetics and Apoptosis

II-A pl-1. Mitochondrial morphogenesis and apoptosis

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Mitochondria undergo dramatic morphologic remodeling during apoptosis independently of caspase activation. The mitochondria utilize the physiological fission and fusion machinery including the dynamin related protein 1 (Drp1) to fragment into more numerous and smaller units. Interestingly, two proapoptotic members of the Bcl-2 family, Bax and Bak, colocalize with Drp1 at mitochondrial fission sites during the early phases of apoptosis in contrast to two BH3—only members of the Bcl-2 family Bid and Bad that continually circumscribe the outer mitochondrial membrane. How different members of the mitochondrial fission and fusion machinery, Drp1, Fis1, Mfn1 and 2 and Opa1, participate in apoptosis will be discuss.

II-A pl-2. The role of mitochondrial calcium overload and mitochondrial function in ischemic cell death and apoptosis

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Excitotoxicity (overstimulation of glutamate receptors) is the main mechanism leading to neuronal Ca overload in brain ischemia. Whereas excessive Ca entry can be mediated by influx through other channels, it is still unclear what mediates the irreversible Ca^{2+} overload that preludes neuronal death. Excitotoxic death may occur by necrosis or apoptosis depending on the intensity of the insult and the energy level in the injured neurons. The mechanism for neuronal demise following an uncontrolled Ca^{2+} overload remains unclear although mitochondrial Ca^{2+} release has been implicated. Here we show that following NMDA-mediated Ca influx calpains cleave and inactivate the plasma membrane Na/Ca exchanger (NCXs), the activity of which is essential to extrude Ca^{2+} in excitable cells, Overexpresion of the calpain endogenous inhibitor calpastain in CGN, prevents NCX cleavage, the secondary Ca^{2+} overload and neuronal death. Our data exclude hat mitochondria can participate to the secondary Ca^{2+} overload that leads to excitotoxic demise of neurons.

II-A pl-3. Mitochondria and ischemic heart

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The viability of the ischemic myocardium is jeopardized by alterations, such as ATP decrease and elevation in intracellular $[Ca^{2+}]$, that are related to derangements in mitochondrial function. Besides these established notions, the elucidation of the apoptotic cascade and the availability of novel methodologies for in situ studies prompted a renovated interest in mitochondria. The characterization of mitochondrial channels provided a contribution that is particularly relevant to cardiovascular research.

Here we focus on the role of the permeability transition pore in ischemia–reperfusion injury by analyzing (i) the methodological requirements for its characterization in isolated cells and intact organs; (ii) the consequences of its opening highlighting the derangements in NAD^+ metabolism; (iii) the contribution to necrosis and apoptosis; (iv) the possible relationship with the formation or reactive oxygen species.

II-B: Secondary Active Transport in Cell Biology

II-B pl-1. NhaA of *escherichia coli*, as a model of a PH regulated NA^+/H^+ antiporter

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NhaA of *Escherichia coli*, as a model of a pH regulated Na^+/H^+ antiporterE. PADAN, T. Tzubery, K. Herz, L. Kozachkov, A. Rimon, and L. GaliliInstitute of Life Sciences, Hebrew University of Jerusalem, 91904 Jerusalem, IsraelThroughout the biological kingdom, many of the Na^+/H^+ antiporters are regulated by pH. This characteristic property of the antiporters corroborates their primary role in pH homeostasis. The pH regulation of NhaA, as of other, both eukaryotic and prokaryotic Na^+/H^+ antiporters, involves pH sensors and conformational changes in different parts of the protein that transduce the pH signal into a change in activity (1). Hence, to understand the mechanism underlying the pH regulation of the antiporters, it is essential to identify the amino acid residues and domains involved, and to elucidate the pH induced conformational changes.

It is already clear that residues that affect the pH response of NhaA, as of other antiporters, are not necessarily identical with those that determine the Km for ions of the translocation process (2). They may even cluster at different domains along the antiporter molecules. Thus, based on cross-linking data, helix-packing model of NhaA suggests that in the three dimensional structure of NhaA, residues that affect the pH response may be in close proximity, forming a single pH sensitive domain (3,4). Therefore, we suggest that despite considerable differences in the primary structure of the antiporters from the bacterial NhaA to the mammalian NHEs, it is highly possible that their three-dimensional architecture is conserved. Test of this possibility awaits the atomic resolution of the 3D structure of the antiporters.

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II-B pl-2. Identification of new mitochondrial carriers: from gene to function

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The mitochondrial carriers (MC) are a family of transport proteins that, with a few exceptions, are found in the inner membranes of mitochondria. They shuttle metabolites, nucleotides and cofactors through this membrane, and thereby connect and/or regulate cytoplasm and matrix functions. Family members have three tandemly repeated sequences of about 100 residues, each containing two hydrophobic stretches and a sequence motif. The genome of *S. cerevisiae* encodes 35 putative MC, that of *A. thaliana* 58, and at least 50 putative MC have been found in humans. The function of most of them is unknown. To identify the function of the genes encoding MC of unknown function, we expressed them in *E. coli* and/or yeast, and identified the gene products from their transport properties after purification and functional reconstitution into liposomes. Using this strategy, in the last 7–8 years we identified the genes and the amino acid sequences of 13 new MC in yeast, four in plants and 12 in man (including isoforms). The more recently identified MC are:

(a) The yeast thiamine pyrophosphate carrier that transports the essential coenzyme TPP from the cytosol, where it is synthesized, into the mitochondria. Yeast cells lacking the *tpc1* gene exhibit reduced levels of TPP and decreased activities of TPP-requiring enzymes in their mitochondria, and auxotrophy for thiamine on fermentative carbon sources.
(b) The yeast and human carriers for S-adenosylmethionine (SAM) which catalyze the import of SAM in mitochondria (where it is required for DNA, RNA and protein methylation) in exchange for mt S-adenosylhomocysteine;
(c) the yeast GTP/GDP carrier, which transports GTP and GDP by an electroneutral exchange. In yeast GTP must be imported for mt nucleic acid and protein synthesis;
(d) the human aspartate/glutamate carrier (2 isoforms) that catalyzes the electrophoretic exchange of aspartate for glutamate + H^+ and is important in the urea cycle and the aspartate/malate NADH shuttle;
(e) the human glutamate carrier (2 isoforms) that catalyzes the entry of glutamate into the mitochondrial matrix with a H^+ and plays important roles in amino acid degradation, nitrogen metabolism and urea synthesis;
(f) the human adenine nucleotide/phosphate carrier (three isoforms) that exchanges ATP-Mg with phosphate and is responsible for the net uptake or efflux of adenine nucleotides into or from the mitochondria; and
(g) the second ornithine carrier (ORC2) in man, which has a broader specificity than ORC1. The main function of ORC is to exchange cytosolic ornithine for mt citrulline, an important step in urea synthesis. Mutations in ORC1 cause the HHH syndrome (OMIM 238970). In these patients ORC2 is unmodified and partially compensates for ORC1.

The discovery of new MC has made it possible to (a) gain insight into the physiological role of MC in several metabolic pathways, and (b) identify the molecular defects responsible for various human inherited diseases.

II-B pl-3. BetP, a transporter with three different functions: betaine transport, osmosensing, and osmoregulation

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In order to circumvent deleterious effects of hypo- and hyperosmotic conditions in its environment, *Corynebacterium glutamicum* has developed a number of mechanisms to counteract osmotic stress. The first response to an osmotic upshift is the activation of uptake mechanisms for the compatible solutes betaine, proline, or ectoine, namely BetP, EctP, ProP, LcoP and PutP. BetP, the most important uptake system responds to osmotic stress by regulation at the level of both protein activity and gene expression. BetP was shown to harbour three different properties, i.e., catalytic activity (betaine transport), sensing of appropriate stimuli (osmosensing) and signal transduction to the catalytic part of the carrier protein which adapts its activity to the extent of osmotic stress (osmoregulation). BetP is a homotrimeric protein, each monomer comprises 12 transmembrane segments and carries N- and C-terminal domains, which are involved in osmosensing and/or osmoregulation. The primary stimulus for activation of BetP in response to hyperosmotic stress was shown to be a rise in the internal K^+ concentration, thus the process of osmosensing is converted into a chemosensory event. Recent results on molecular properties of the C-terminal domain of BetP indicate the significance in the sensory and regulatory events of particular amino acids within the terminal 25 amino acids of this domain by interaction.

II-C: Molecular Mechanic Energy Transducers

II-C pl-1. Rotary ATP synthase: torque generation and transmission, simmetry of stepping and proton conduction by F_O

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ATP synthase is composed from two rotary motor/generators, the ATPase (F_1) and the ion translocator (F_O). Both are rotary steppers. An elastic power transmission would account for its robust operation with symmetry mismatch and in drastically modified constructs. We gauged the angular torque profile at F_O as generated by ATP hydrolysis in F_1. The curvature of fluorescent actin filaments, attached to the rotating c ring of F_O, was used as a spring balance. The mean torque was 56 pNnm and the angular torque variations were small. It implied a soft elastic power transmission between F_1 and F_O. Elastic transmission is considered as essential, not only for the robust operation of this ubiquitous enzyme under symmetry mismatch, but also for a high turnover rate under load.

The high torque of this nanomachine, 56 pNnm, calls for high binding strength between subunits, as established for two parts of the eccentric stator, subunits δ and b, to the $(\alpha\beta)$3-hexagon (Kd < 1 nM). We asked whether or not the tightness affects the symmetry of the stepped rotary progression of the central shaft in F_1. We investigated stepping, for the first time, in the complete enzyme, F_OF_1, in two different ways, as driven (i) by the intrinsic hydrolysis activity of the enzyme and (ii) by an externally applied and rotating magnetic field. Both sets revealed a surprisingly symmetrical stepping of the central rotor.

Proton conduction by the exposed F_O was studied in isolated chromatophores of the photosynthetic bacterium *Rhodobacter capsulatus*. Owing to small size, only a small fraction of vesicles (25%) contained one copy of F_O. The discharge of a light-induced voltage jump was monitored by electrochromic absorption transients. The current–voltage relationship of F_O was linear from 7 to 70 mV. The current was extremely proton specific ($>10^7$) and varied only slightly ($\sim$ 3-fold) from pH 6 to 10. The unitary conductance of F_O was $\sim$ 10 fS at pH 8, equivalent to 6240 $H^+ s^{-1}$ at 100 mV driving force, which is an order of magnitude greater than that of F_OF_1. There was no voltage-gating of F_O even at low voltage, and proton translocation could be driven by ΔpH alone, without voltage. The reported voltage gating in F_OF_1 is thus attributable to the interaction of F_O with F_1 but not to F_O proper. We simulated proton conduction by a minimal rotary model including the rotating c-ring and two relay groups mediating proton exchange between the ring and the respective membrane surface. The fit to data attributed pK-values of $\sim$ 6 and $\sim$ 10 to these relays, and placed them close to the membrane/electrolyte interface.

II-C pl-2. Comparative aspects of kinesins, dyneins and myosins

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Eukaryotic cells control their complex structure and structural dynamics to a large part by the cytoskeleton and associated proteins. Among them are molecular motors that move along F-actin or microtubules at the expense of ATP. Depending on the cellular function, they use different strategies for the generation of movements: one type of motors (such as muscle myosin) is adapted to work in large ensembles and generates maximum velocities collaboratively, whereas others can operate as single molecules and are able to move along filaments for many micrometers.

Three superfamilies of filament-dependent motor proteins are known. Myosins move along F-actin, kinesins and dynein along microtubules. Interestingly, myosins and kinesins share common structural features such as a P-loop, and appear to be related more closely to each other than kinesins and dyneins. The crystal structures of myosins and kinesins show homology to G-proteins, especially in the vicinity of the nucleotide binding pocket. Both motor proteins couple their nucleotide affinity and hydrolysis rate with the affinity to their respective filament in a similar manner as G-proteins are stimulated by their effector proteins or exchange factors. As in G-proteins, so-called switch regions in the proximity of the gamma-phosphate group are thought to ‘distinguish between’ the presence of ADP or ATP, which leads to larger conformational changes that regulate the filament binding.

Desite the similarities in the ATPase mechanism, the force generating steps are different in each class of molecular motors. Myosins seem to use a lever arm mechanism that shifts the motor with respect to the actin filament. The primary event in kinesin motors may be a conformational change in a short stretch of amino acids that can either be in a flexible conformation, or in an ordered state associated with the core motor domain.

Dynein is an ATPase of the AAA-type, and thus is expected to function differently. As no crystal structures are available the mechanism remains much more obscure. Different approaches, however, suggest that the ATP hydrolysis may lead to a small shift among the six AAA subdomains that may cause motility.

II-C pl-3. Structure of Rec BCD: a machine for processing breaks in DNA

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Double strand breaks in DNA are potentially lethal to cells. In *E.coli* these breaks are processed by the RecBCD enzyme. The DNA is digested away by the combined action of two helicase motors of opposite polarity and two nuclease activities. Upon reaching a defined sequence, the enzyme pauses and loads the RecA protein onto a 3′-tail that is produced by the enzyme. We have determined the crystal structure of the entire RecBCD complex in a complex with DNA. The structure reveals how the enzyme processes double strand breaks and prepares them for recombinational repair.

II-D: Oxygen Radicals and Nitrogen Monoxide and Mitochondria

II-D pl-1. Radicals and radical chemistry in the mitochondrion

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It has become clear that the chemistry of free radicals is central to an ever-expanding range of reactions having important biological implications. Free radicals are molecules containing unpaired electrons and are therefore generally unstable, having the potential to react with molecules within the cell and also to generate other reactive species and in some cases thereby initiating chain reactions. It is for this reason that uncontrolled production of free radicals can lead to cellular damage that is fundamental to some pathological conditions. In addition to this role in cell pathology it is now apparent that radicals, appropriately generated, can also act as control or signaling molecules. This presentation will consider the radicals that are formed and/or found within the mitochondrion, including superoxide and nitric oxide, their reactivities and their roles. These include the possible roles in control of cell respiration, in cell damage and in the apoptotic process and in signaling. The chemistry of radicals, and associated reactive species, formed by the redox chemistry of oxygen, and from nitric oxide will be considered together with organic radicals and their reaction products.

II-D pl-2. Regulation of mitochondrial nitric-oxide synthase

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An apparent discrepancy rises about the role of calcium on the rates of oxygen consumption by mitochondria: mitochondrial calcium increases the rate of oxygen consumption because of the activation of calcium-activated dehydrogenases, and by activating mitochondrial nitric-oxide synthase (mtNOS), decreases the rates of oxygen consumption because nitric oxide is a competitive inhibitor of cytochrome oxidase. To this end, the rates of oxygen consumption and nitric oxide production were followed in isolated rat liver mitochondria in the presence of either L-Arg (to sustain a mtNOS' activity) or NG-monomethyl-L-Arg (a competitive inhibitor of mtNOS) under State 3 conditions. In the presence of NMMA, the rates of State 3 oxygen consumption exhibited a K0.5 of 0.16 μM intramitochondrial free calcium, agreeing with those required for the activation of the Krebs' cycle. By plotting the difference between the rates of oxygen consumption in State 3 with L-Arg and with NMMA at various calcium concentrations, a K0.5 of 1.2 μM intramitochondrial free calcium was obtained, similar to the K0.5 (0.9 μM) of the dependence of the rate of nitric oxide production on calcium concentrations. The activation of dehydrogenases, followed by the activation of mtNOS would lead to the modulation of the Krebs' cycle activity by the modulation of nitric oxide on the respiratory rates. This would ensue in changes in the NADH/NAD and ATP/ADP ratios which would influence the rate of the cycle and the oxygen diffusion.

II-D pl-3. How nitric oxide zaps mitochondria

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Nitric oxide (NO) mediates cell death in a wide range of inflammatory, ischaemic and neurodegenerative pathologies. NO has three relevant actions on mitochondria: (1) inhibition of mitochondrial respiration due to rapid and reversible inhibition of cytochrome *c* oxidase by NO, and inactivation of complex I by peroxynitrite and S-nitrosothiols, (2) stimulation of mitochondrial production of oxidants (superoxide, hydrogen peroxide and peroxynitrite), and (3) induction of permeability transition by peroxynitrite and S-nitrosothiols. We find that NO induces cell death by three corresponding mechanisms: (a) NO inhibition of mitochondrial respiration leading to energy depletion-induced necrosis (preventable by active glycolysis) or excitotoxicity of neurons (preventable by MK801), (b) NO-induced oxidant production from mitochondria causing apoptosis (preventable by antioxidants or caspase inhibitors), or (c) peroxynitrite or S-nitrosothiol-induced mitochondrial permeability transition (preventable by cyclosporin A). The mechanism of cell death varies with cell type and conditions. The NO inhibition of cytochrome *c* oxidase is in competition with oxygen, and consequently sensitises tissues to hypoxic damage. This may be important in inflamed tissues where high levels of NO are produced from iNOS.

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Grant support: BBSRC, MRC, BHF, Royal Society, and Wellcome Trust.

III-A: Organelle Biogenesis

III-A pl-1. Ancient origins—modern disease: the mitochondrial connection

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The mitochondrial genome encompasses approximately 1500 genes, only 37 of which are retained in the human mitochondrial DNA (mtDNA). Since all of the genes of the mtDNA encode either essential subunits of oxidative phosphorylation (OXPHOS) or the structural RNAs for their expression, any mtDNA mutation will affect OXPHOS. OXPHOS makes three major contributions to the pathobiology of disease: production of cellular energy, generation of toxic reactive oxygen species (ROS), and contribution of subunits to the mitochondrial permeability transition pore (mtPTP) which regulates apoptosis. The mtDNA is maternally inherited, has a high mutation rate and is present in thousands of copies per cell. New mutations result in intracellular mixtures of mutant and normal mtDNAs, a state known as heteroplasmy. Heteroplasmic mtDNA mutations can segregate during cell division resulting in different percentages of mutant mtDNAs, variable biochemical defects, and a diversity of clinical manifestations. The organ systems most affected by mtDNA mutations are the brain, heart, muscle, renal and endocrine systems. In addition to new mtDNA mutations, the mtDNAs from different populations harbor population-specific polymorphisms. These ancient variants became fixed, as our ancestors migrated out of tropical Africa and into temperate Eurasia and arctic Siberia, because they partially uncouple OXPHOS and thus increasing mitochondrial heat production and permitting adaptation to colder latitudes. Today these mtDNA polymorphisms can increase individual predisposed to energy deficiency diseases, presumably because they limit cellular ATP production; but they are also associated with increased longevity and protection against Alzheimer and Parkinson disease, presumably because they reduce ROS by keeping the mitochondrial respiratory chain oxidized. Mitochondrial diseases generally show a delayed onset and progressive course, a common feature of degenerative diseases and aging. This results from the accumulation of somatic mtDNA mutations in postmitotic tissues which then exacerbate inherited mitochondrial defects. Mouse mutants in mitochondrial genes have confirmed the importance of mitochondrial energy deficiency in heart and muscle disease and mitochondrial ROS toxicity in neurodegenerative disease. They have also provided insights into the structure and function of the mtPTP and permitted the development of new therapeutics for the treatment of mitochondrial diseases.

III-A pl-2. Import and assembly of mitochondrial proteins

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Mitochondria import most of their proteins from the cytosol. The mitochondrial membranes contain specific machineries for the recognition, translocation and assembly of proteins. The mitochondrial outer membrane contains the TOM complex (translocase of outer membrane) with specific receptors and a general import pore for translocation of precursor proteins. Recently, a second outer membrane complex has been identified that is essential for sorting and assembly of outer membrane proteins (SAM complex). The precursors of outer membrane beta-barrel proteins are first imported via the TOM complex and are then transferred to the SAM complex for integration into the outer membrane.

The mitochondrial inner membrane contains two translocases for different classes of preproteins. The presequence translocase (TIM23 complex) and the associated Hsp70 motor (PAM) cooperate in the transport of presequence-carrying proteins into the mitochondrial matrix in a manner that requires the inner membrane potential and ATP. The carrier translocase (TIM22 complex) promotes the insertion of hydrophobic carrier proteins into the inner membrane. The carrier translocase cooperates with small Tim proteins of the intermembrane space and uses the energy of the membrane potential for insertion of membrane proteins.

III-A pl-3. Regulation of ATPA and ATPB gene expression, illustrates specific traits of chloroplast gene expression and chloroplast protein assembly

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In mitochondria and chloroplasts, multimeric proteins of the energy transducing membranes have a dual genetic origin. The organellar genes display a copy number that can be higher by as much as 4 orders of magnitude than their nuclear counterparts although the protein gene products are most often present in a 1:1 stoichiometry. Using the atpA and atpB chloroplast genes as markers of chloroplast gene expression we will show how the overcoding capacity of the chloroplast is overridden by the nucleus through the targeted action of two types of nuclear protein factors that tightly control the fate of chloroplast mRNAs. Then we will describe an original assembly-dependent autoregulation of translation, the CES process, that allows a fine-tuning of the production of protein subunits that contributes to their stoichiometric assembly. Finally an assembly pathway for the catalytic moiety of the chloroplast ATP-synthase (CF1) will be presented.

III-B: Mitochondrial Bioenergetics and Diseases

III-B pl-1. Mitochondrial mutations and pathology

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By convention, the term ‘‘mitochondrial diseases’’ refers to disorders of the mitochondrial respiratory chain, which is the only metabolic pathway in the cell that is under the dual control of the mitochondrial genome (mtDNA) and the nuclear genome (nDNA). Therefore, a genetic classification of the mitochondrial diseases distinguishes disorders due to mutations in mtDNA, which are governed by the relatively lax rules of mitochondrial genetics, and disorders due to mutations in nDNA, which are governed by the stricter rules of Mendelian genetics.Mutations in mtDNA can be divided into those that impair mitochondrial protein synthesis in toto and those that affect any one of the 13 respiratory chain subunits encoded by mtDNA. Essential clinical features for each group of diseases are reviewed. Disorders due to mutations in nDNA are more abundant not only because most respiratory chain subunits are nucleus-encoded but also because correct assembly and functioning of the respiratory chain require numerous steps, all of which are under the control of nDNA. These steps (and related diseases) include: (i) synthesis of assembly proteins; (ii) intergenomic signaling; (iii) mitochondrial importation of nDNA-encoded proteins; (iv) synthesis of inner mitochondrial membrane phospholipids; (v) mitochondrial motility and fission.

Keywords: Mitochondrial diseases; Respiratory chain; mtDNA mutations; nDNA mutations

III-B pl-2. Bioenergetics of mitochondrial diseases associated with mtDNA mutations

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This lecture summarizes our present view of the biochemical alterations associated with mitochondrial DNA (mtDNA) point mutations. Mitochondrial cytopathies caused by mutations of mitochondrial DNA are well known genetic and clinical entities, but the biochemical pathogenic mechanisms are often obscure.

Leber’s Hereditary Optic Neuropathy (LHON) is due to three main mutations in genes for Complex I subunits. Even if the catalytic activity of Complex I is maintained except in cells carrying the 3460/ND1 mutation, in all cases there is a change in sensitivity to Complex I inhibitors and an impairment of mitochondrial respiration, eliciting the possibility of generation of reactive oxygen species by the Complex.

Neurogenic muscle weakness, Ataxia and Retinitis Pigmentosa (NARP) is due to a mutation in the ATP6 gene. In NARP patients ATP synthesis is strongly depressed to an extent proportional to the mutation load; nevertheless ATP hydrolysis and ATP-driven proton translocation are not affected. It is suggested that the NARP mutation affects the ability of the enzyme to couple proton transport to ATP synthesis.

A point mutation in subunit III of cytochrome *c* oxidase is accompanied by a syndrome resembling MELAS: however no major biochemical defect is found, if we except an enhanced production of reactive oxygen species. The mechanism of such enhancement is at present unknown. In this review we draw attention to a few examples in which the overproduction of ROS might represent a common step in the induction of clinical phenotypes and/or in the progression of several human pathologies associated with mtDNA point mutations.

III-B pl-3. Nuclear DNA mutations and energy transduction impairment

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During the last decade increasing number of mutations in nuclear genes has been recognised as a cause of mitochondrial diseases that are generally untreatable, often very severe with early onset and affect especially paediatric population. These mutations can impair all respiratory chain complexes and mitochondrial FoF_1-ATPase. They are found in genes encoding structural components of oxidative phosphorylation complexes, proteins essential for their assembly as well as factors that are involved in replication and expression of mitochondrial DNA. Among the most frequent mitochondrial diseases of nuclear origin belong the selective defects of cytochrome *c* oxidase with Leigh syndrome phenotype due to mutations in SURF1 gene for specific assembly protein; disturbed assembly process is also found in selective defects of mitochondrial FoF_1-ATPase. Their pathogenic mechanism at cellular and biochemical levels is, however, still poorly understood. For better understanding of genotype–phenotype relationship, studies in patient fibroblasts and application of sensitive functional techniques, such as high resolution oxygraphy, cytofluorometry and confocal microscopy in combination with proteomic analysis represent valuable approach to elucidate how these disorders affect in vivo ATP synthesis and mitochondrial ROS production—two major components of the molecular pathogenic mechanism of mitochondrial encephalo-cardio-myopathie.

Special Evening Session

S-2. Structure and function of the mitochondrial ADP/ATP carrier

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Specific transport of metabolites across the inner mitochondrial membrane is achieved by nuclear encoded carriers which form the mitochondrial carrier family (MCF). All members of the MCF share sequence similarities and display three tandem repeats of about 100 amino acids. Under physiological conditions the adenine nucleotide carrier catalyses the exchange of cytosolic ADP for mitochondrial ATP generated by oxidative phosphorylation. The ADP/ATP carrier has been extensively studied as a paradigm of the mitochondrial carriers because it is one of the most abundant membrane proteins in mitochondria. Its functional characterization was also largely facilitated by the occurrence of very specific inhibitors of the ADP/ATP transport, particularly carboxyatractyloside (CATR) and bongkrekic acid (BA). Both inhibitors bind with high affinity to the carrier and trap it under the form of stable CATR– and BA–carrier complexes which exhibit distinct conformational states.

We have solved the bovine ADP/ATP carrier structure in complex with carboxyatractyloside at a resolution of 2.2 Å by X-ray crystallography. The structure consists of six tilted ?—helices that form a compact transmembrane bundle. The odd-numbered helices are kinked due to the presence of proline residues. At the surface oriented towards the intermembrane space, the protein has a deep depression. Three pairs of charged residues of the MCF signature, the PX(D/E)XX(K/R), strengthen a closed conformation of the cavity toward the matrix. The RRRMMM motif, which is the signature of the mitochondrial ADP/ATP carriers, is also located at the bottom of the pit and spans over the thinnest part of the protein. Our structure, together with earlier biochemical results, suggests that transport substrates bind to the bottom of the cavity and that translocation results from a transient transition from a pit to a channel conformation.

Colloquia

I-A: F-Type ATP Synthases

I-A c-1. Helix–helix interactions and aqueous access channels in subunit a of *E. coli* ATP synthase

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ATP synthesis by *E. coli* F1Fo ATP synthase is driven by rotation of an oligomeric ring of ten c-subunits, with c-ring rotation being coupled to proton transport. Rotation occurs in steps with the alternate binding and release of protons from cAsp61 to each of the 10 c-subunits in the c-ring. Subunit a in the transmembrane Fo sector of ATP synthase is thought to function by providing gated, aqueous channels for protons to cAsp61 from the two sides of the membrane. Subunit a is a polytopic tranmembrane protein composed of five transmembrane helices (TMHs) and is proposed to form a part of the "stator" of the enzyme against which the c-ring rotates. aArg210 in the cytoplasmic half of aTMH4 is proposed to sequentially interact with the rotating c-subunits to lower the pKa of cAsp61 and promote proton release. We have mapped aqueous access pathways to a cytoplasmic face of aTMH4 and to the periplasmic sides of aTMH 2, 3, 4 and 5 based upon the Ag^{+} accessibility of Cys residues. The gating of the access channels from the two sides of the membrane is proposed to be mediated by rotation of aTMH4, the rotation of which also physically drives c-ring rotation. The packing of TMHs 2–5 at the center and the periplasmic side of the membrane has been defined by Cys–Cys disulfide cross-linking with Cys introduced by site directed mutagenesis. A model for the packing of helices will be proposed based upon these distance constraints and the silver access pathway. Experiments will also be presented indicating that subunit a folds with a similar structure in a monophasic chloroform–methanol–water (4:4:1) solvent, and initial NMR experiments on helical interactions between aTMH4 and aTMH5 presented.

I-A c-2. Subunit movements in H-ATP synthases during catalysis in single molecules

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Energy transduction in H-ATP synthases is assumed to be achieved by proton transport driven rotation of the membrane embedded ring of c-subunits. The attached gamma and epsilon subunit transmit the energy to the three catalytic sites on the beta subunits. To observe these movements in single molecules we use fluorescence resonance energy transfer (FRET) in combination with a confocal microscope. The b-subunit was labelled with a fluorescence acceptor, the gamma subunit with a fluorescence donor. Fluorescence labelling does not impair catalysis and FRET was measured during ATP hydrolysis and ATP synthesis. During catalysis three different states with different donor acceptor distances are observed. During ATP hydrolysis and ATP synthesis the same states are observed, however the sequential order of these states is opposite in both cases (Diez et al., 2004, NSMB 11, 135–141). A detailed analysis of the data indicates substeps of the three main steps during catalysis and in addition, differences in the FRET states between the active and the inactive states of the enzyme are observed.

I-A c-3. Engine and brakes of ATP synthase

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The ATP synthase can be thought of as a complex of two motors—the ATP-driven F1 motor and the proton-driven Fo motor—that rotate in opposite directions. Our recent findings are follows:

1. Mechanical synthsesis of ATP: The reverse rotation of F1 (alfa–beta–gamma subcomplex) by an external force brings about synthesis of ATP. Mechanical synthsesis of ATP.
2. Direct observation of reaction sequence: Single molecule imaging of F1 motor has provided direct evidence that all of the three catalytic subunits play a role in sequence during a 120° rotation; ATP binding (~ 80° substep), ATP hydrolysis (1ms), and, probably release of Pi or ADP (1ms, ~ 40° substep). The order of the second and third event is not decided yet.
3. Brakes: At least two kinds of brakes of ATPase activity of ATP synthase have been known; ADPMg inhibition and epsilon inhibition. ADPMg inhibition stops the rotation at an ~ 80° substep once in every 30 s, in average, and resumes rotation that continues next 30 s.
4. Mechanical regulation: If the rotation is pushed by an external force, the ADPMg-inhibited F1 ''wakes up'' and starts rotation.
5. Down–up transition of epsilon: Our crystal structure of F1 shows that the epsilon inhibition is dependent on the drastic down-to-up conformational transition of C-terminal helices of epsilon that may cause physical prevention of rotation. This transition is dependent on ATP/ADP balance and proton motive force.
6. Epsilon binds ATP: The epsilon subunit is an ATP-binding protein which may monitor the cellular ATP concentration.
7. Unidirectional inhibition: Interestingly (and puzzlingly), ATP synthesis is not inhibited by ADPMg and epsilon.
8. A single polypeptide c-ring of fused10monomers: The number of copies in the c subunits in the Fo-c ring is exclusively10in functional ATP synthase from a thermophile strain PS3. This means that flow of one proton may cause 36° step rotation. How can 120° step rotation of F1 coordinate with 36° step rotation of Fo-c ring?

I-A c-4. Molecular insights into the operation of the fo motor of the Na^+-F-ATP synthase

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F-ATP synthase is the ubiquitous molecular machine of living cells that transforms energy of an electrochemical gradient of H^+ or Na^+ into the chemical bond energy of ATP. The enzyme consists of two rotary motors, Fo and F1, which are connected by a common shaft to exchange energy with one another. Upon ion translocation the Fo motor generates torque causing the shaft to rotate and to enforce conformational changes at the catalytic sites which lead to ATP synthesis. The Na^+ F_o motor consists of an undecameric c ring (rotor) that is abutted laterally by the stator subunits a and b2. The c ring is built from a tightly packed inner ring comprising the N-terminal helices and a more loosely packed outer ring comprising the C-terminal helices. The binding sites for the coupling ions within the middle of the membrane are contributed by Q32 and S66 of one subunit and E65 of the neighboring subunit. These binding sites are readily accessible from the periplasmic reservoir through the subunit a inlet channel and they are accessible to the cytoplasmic reservoir through the rotor-intrinsic outlet channels. During ATP synthesis, Na^+ diffuses from the periplasm through the inlet channel onto the binding site at the subunit a/c interface. Following rotation the ion dissociates from the site out of the a/c interface and diffuses into the cytoplasm through its rotor-intrinsic outlet channel. This dissociation is enforced when the site approaches aR227 upon reentry of the site into the interface with subunit a. Two different operation modes of the motor have been recognized. Without external energy source, the motor is in an idling mode where it performs exchange of Na^+ across the membrane. Upon applying voltage but not with a Na^+ concentration gradient, the motor switches into the torque-generating mode and is then able to support ATP synthesis. ATP synthesis is coupled to the unidirectional flux of Na^+ ions through the membrane and is only observed at membrane potentials exceeding 40 mV. Single molecule studies have shown that the rotation is likewise dependent on the membrane potential. These results are corroborated by ion flux measurements through the isolated Fo motor: Na^+ flux is only observed above a threshold potential of 40 mV. These results unison identify the membrane potential as kinetically indispensable driving force for ATP synthesis and exclude the possibility that the chemical ion gradient by itself is kinetically competent to drive ATP synthesis.

I-A c-5. Active regulation of ATP synthase by the inhibitor protein IF1 in heart

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Active regulation of the mitochondrial ATP synthase has been shown to occur in response to cellular energy demand in different experimental models. A potential modulator is the inhibitor protein IF_1. IF_1 binding has been proposed to be responsible for the beneficial downregulation of ATP synthase during ischemia. In addition, IF_1 has been suggested to mediate enzyme inhibition during ischemic preconditioning. IF_1 release has also been suggested to upregulate ATP synthase in response to increased ATP demand [1]. Until now, the data presented were largely based on studies of ATPase activity in tissue extracts.

The aim of this study was to quantify IF_1 bound to the enzyme in heart tissue while concurrently monitoring ATP synthase capacity. Conditions were chosen to assure minimal perturbation of the IF_1/F_1 interaction and no disruption of cardiac function. Different animal models were used and IF1 content was correlated with ATP synthase capacity under different physiological conditions. Tissue fragments obtained from rat (a fast heart-rate animal) and goat (a slow heart-rate animal) heart were incubated in vitro under anoxic conditions, which downregulated the ATP synthase, or with high-salt or high-pH buffers, which upregulated the enzyme. IF_1 content was determined by detergent extraction followed by blue native gel electrophoresis, second dimensional SDS-PAGE and immunoblotting with anti-IF_1 antibodies [2]. ATP synthase capacity was determined from maximal mitochondrial ATP hydrolysis rate. The same methodological approaches were performed on biopsies from Langerdorff-perfused rat heart subjected ex vivo to 30 min of total ischemia followed by 120 min of reperfusion. In addition, biopsies were obtained from anaesthetized open-chest goat heart subjected to ischemic preconditioning (which decreased ATP synthase capacity), and to sudden increases of the coronary blood flow, which upregulated the enzyme. Over all physiological conditions tested we obtained a close inverse correlation between ATP synthase capacity and IF_1 quantity bound to the enzyme. This clearly indicates that ATP synthase is regulated by IF_1 in the different animal models. This is at variance with the suggestion by Rouslin that rat heart lacks a functional complement of IF_1.

Acknowledgements: Regione F.V.G. Misura D4 and MURST Cofin 2002.

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I-A c-6. The physiological ligands adp and Pi modulate the degree of intrinsic coupling in the ATP synthase of the photosynthetic bacterium *Rhodobacter capsulatus*

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In ATP synthases from several sources an uncoupling between ATP hydrolysis and proton pumping has been occasionally reported as a result of chemical modifications or mutagenesis.In the work to be presented, an intrinsic uncoupling has been found in the native, wild-type enzyme. The proton pumping and the ATP hydrolysis activities of the ATP synthase of *Rb. capsulatus* have been compared under the same experimental conditions, as a function of ADP and Pi concentration. The ADP concentrations were clamped in the steady state by coupling the ATP hydrolysis to the pyruvate kinase reaction. The proton pumping was measured either with the pH difference probe ACMA or with the electric potential difference probe oxonol VI, obtaining consistent results. The comparison indicates that an intrinsic uncoupling of ATP synthase is induced when the concentration of either ligand is decreased. The half-maximal effect was found in the submicromolar range for ADP and at about 70 μM for Pi (at pH 8). These data indicate that a switching from a partially uncoupled state of ATP synthase to the coupled state is induced by the simultaneous binding of ADP and Pi.

It is proposed that this switching could correspond to the position switching of the C-terminal alpha-helix hairpin of the epsilon subunit from F_O(“down-state”) to F_1 to (“up-state”).

I-B: Ion-Motive Redox Systems

I-B c-1. Simplicity and complexity of ion motive redox systems in archaea

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Ionmotive redox systems of aerobic archaea will be discussed in comparison to those of higher organisms. All respiratory systems contain ion pumps but their number and efficiency differ widely. Ionmotive NADH-dehydrogenases are modular systems of high complexity and are apparently not functioning in archaea. Equivalents to the respiratory complex-III are present but deviate in structure and composition. This relates to the use of archaetypical thiophenoquinones instead of ubiquinone as well as the replacement of c-type cytochromes by other electron carriers like small copper proteins. Also a novel type of Rieske/Cytochrome-*b* complex has been detected which may be considered to resemble an evolutionary precursor of higher b/c_1-complexes. The obvious coevolution of Rieske proteins and b-type cytochromes may delineate a pathway from very simple to rather complex structures when simple archaeal systems are compared to highly developed mitochondrial complexes. The terminal oxidases which were shown to act as proton pumps are either minimal systems simply consisting of a quinol oxidase directly linked to a specific metabolic process, or—surprisingly—can be organized as supercomplexes representing a structural fusion of several functions of a whole respiratory chain within one multisubunit assembly. For an extreme thermophile a novel regulatory mechanism of its terminal oxidases is proposed, based on the unique features of a CuA-center to respond reversibly to environmental pH changes and thus simultaneously to function as a pH sensor.

I-B c-2. Transhydrogenase DIII solution structure reveals a redox-dependent conformational change—a mechanistic key to enzyme turnover?

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Proton-translocating transhydrogenase is a 103 kDa integral membrane enzyme responsible for maintaining a high NADPH to $NADP^+$ ratio in the cell by catalyzing reversible hydride ion transfer between the NAD(H) and NADP(H) pools, utilizing the proton gradient as energy source. The catalytic process occurring between the soluble domains I and III is tightly coupled to proton translocation through the membrane-spanning domain II so that one catalytic cycle is completed per translocated proton. This coupling involves domain II and III, and is believed to be dependent entirely on conformational changes.

E. coli domain III (ecIII) is a 186 aa, 20.4 kDa protein that binds NADP(H). Sequential assignment and a global fold of ecIII have previously been reported [1–3]. In the present investigation, the solution structures of ecIII-$NADP^+$ and ecIII-NADPH have been determined on uniformly ^{13}C- and ^{15}N-labeled protein samples with ^{13}C- and ^{15}N-edited NOESY-experiments. Each of the two forms (ecIII-NADP, ecIII-NADPH) exhibits a well defined conformation with a global RMSD of 0.7 Å and a good Ramachandran distribution. The substrate is poorly defined in both forms, mainly due to lack of restraints between the protein and the pyrophosphate-region of NADP(H). Pronounced structural changes are found predominantly in the loop connecting beta strand 2 with helix 3, helix 3 and loops D and E at the C-terminal edge of the central beta-sheet, regions in direct contact with the substrate and domain I. This is in contrast to the recently reported crystal structures, where no structural changes were observed between the NADP and NADPH forms, but two different conformations in each case [4]. Furthermore, a 2D-HSQC NMR-based pH titration between pH 5.5–pH 12, monitoring each residue, suggests that neither His345 nor Asp392 are involved in protonation/deprotonation events coupled to enzyme turnover in the isolated dI–dIII system, and raises questions of the roles of these residues in the intact enzyme.

Keywords: Transhydrogenase; NADP; NMR; Solution structure; Conformational change

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I-B c-3. The hydride transfer site of proton-translocating transhydrogenase of *Rhodospirillum rubrum*

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Proton-translocating transhydrogenases (TH) are found in the inner mitochondrial membrane of eukaryotes and in the bacterial plasma membrane. They couple hydride transfer between NAD(H) and NADP(H) to proton translocation across the membrane. All TH's consist of three structural components; dI, dII and dIII. dI and dIII are peripheral to the membrane and bind NAD(H) and NADP(H), respectively. dII is an integral membrane protein and is thought to contain the proton translocating pathway. *Rhodospirillum rubrum* consists of three polypeptides, with dI expressed as a single polypeptide, making the enzyme attractive study.

As NAD(H) and NADP(H) are highly reactive molecules, it is of vital importance that the nicotinamide rings are kept apart until the right moment during turnover. Our present research focuses on the hydride transfer site. Residues R127, Q132, D135 and S138 are conserved among >80 different TH's. They are located in the so-called RQD loop of dI in to the nucleotide binding pocket. Mutation of R127, Q132, D135 or S138 causes a severely decreased transhydrogenation activity. In all four mutants, the inhibition activity is not caused by a decrease in affinity between the dI and dIII domains. The Q132N, D135N and S138A mutations do not have a significant effect on nucleotide binding. The R127A and R127M mutants, however, have a decreased binding affinity for NADH (Kd ~ 800 μM compared to 20 μM for the wild type). The NAD^+ binding affinity of the R127 mutants is much less affected: Kd ~ 1mM, compared to 400 μM for the wild type. Unlike the R127 mutants the Q132N, D153N and S138A mutants still have measurable hydride transfer rates albeit 100–10,000 × less than the wild type enzyme. The electron density for the nicotinamide ring in crystal structures of mutant dI2dIII complexes is reduced or absent in dIB, whereas the density for the nucleotide in dIA's of the mutants is good. The nucleotide in the dIB domain is close to the NADP(H) molecule in dIII. The reduced density for the nicotinamide rings suggests a higher mobility of this part of the nucleotide. Based on our observations we propose that R127, Q132, D135 and S138 have an important role in positioning the nucleotide for hydride transfer. Nucleotide conformational changes could play a vital role in the coupling of hydride transfer to H^+ translocation.

I-B c-4. Energy-converting [NiFe] hydrogenases from archaea and bacteria: ancestors of complex I?

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[NiFe] hydrogenases are well-characterized enzymes that have a key function in the H_2 metabolism of various microorganisms. In the recent years, a subfamily of [NiFe] hydrogenases with unique properties has emerged (for a recent review see: R. Hedderich, J. Bioenerg. Biomembr. 2004, 36, 65–75). The members of this family form multisubunit membrane-bound enzyme complexes composed of at least four hydrophilic- and two integral membrane proteins. These six conserved subunits, which build the core of these hydrogenases, have closely related counterparts in energy-conserving NADH:quinone oxidoreductases (complex I). However, the reactions catalyzed by these hydrogenases differ significantly from that of complex I. For some of these hydrogenases, the physiological role is to catalyze the reduction of H^+ with electrons derived from reduced ferredoxins or polyferredoxins. This exergonic process is coupled to energy conservation by means of electron-transport phosphorylation. Other members of this hydrogenase family mainly function to provide the cell with reduced ferredoxin using H_2 as an electron donor. Under physiological H_2-concentrations the latter reaction is endergonic and is driven by reverse electron transport. These hydrogenases have therefore been designated as energy-converting [NiFe] hydrogenases. Since energy-converting hydrogenases are found in organisms exhibiting an ancient metabolism, such as growth on CO under anaerobic conditions, it is tempting to speculate that these ion pumps first developed in these organisms. Complex I may have evolved from these ion-pumping hydrogenases by the addition of an alternative electron-input domain, the replacement of the [NiFe] center by a quinone-binding site and the addition of further membrane subunits.

I-B c-5. Hydrogenases—a mere electron sink or something more?

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Virtually all life forms obtain energy by coupling membrane-associated electron transfer reactions to ATP synthesis via a proton motive force. The hyperthemophilic archaeon, *Pyrococcus furiousus*, a strictly anaerobic organism (optimal growth temperature ~ 100 °C) was thought to have a purely fermentative metabolism containing a modified Embden–Meyerhof pathway (Adams M.W., Kletzin A. (1996) Adv. Protein Chem. 48, 101180). However, this was brought into question with the discovery of [NiFe]-hydrogenases of the *E. coli* hydrogenase 3-type in Archaea, first in *Methanosarcina barkeri* (Künkel A., Vorholt J.A., Thauer R.K.and Hedderich R. (1998) Eur. J. Biochem., 252, 467–476) and *M. thermoautotrophicum* (Teerstegen A. and Hedderich R. (1999) Eur. J. Biochem., 264, 930–943) and *P. furiousus* H2-evolving, membrane-bound hydrogenase (MBH) (Sapra R., Verhagen M.F., Adams M.W. (2000) J. Bacteriol., 182, 3423–3428; Silva P.J., van den Ban E.C., Wassink H., Haaker H., et al. (2000) Eur. J. Biochem., 267, 6541–6551). These multimeric membrane-bound hydrogenase complexes are remarkable by their similarities with subunits of Complex I and in particular with transmembrane subunits involved in proton pumping and energy coupling.

We have demonstrated that H_2 production catalyzed by the MBH is coupled directly to ATP synthesis. Carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP) inhibits ATP synthesis but stimulates H_2 production. If H_2 production (but not ATP hydrolysis) is prevented by the addition of CuC_{l2}, an electrochemical gradient is not formed when reduced ferredoxin (Fd) is added. The ATP-induced gradient does not form if DCCD is present, although DCCD has little effect on the Fd-induced gradient. The ratio of the H_2 evolution to H_2 oxidation activity catalyzed by the MBH is over 2000 (Sapra R., Verhagen M.F., Adams M.W. (2000) J. Bacteriol., 182, 3423–3428); the low H_2 uptake activity of inverted vesicles is unaffected by ADP and Pi but increases fourfold in the presence of ATP perhaps due to hydrolysis of ATP, which should stimulate H_2 oxidation by proton-driven reverse electron transport.

Results show that it is the hydrogenase system itself that is responsible for energy conservation. This simplicity suggests that such a system could be the precursor of complex respiratory chains of the type now seen in higher organisms, an idea consistent with the proposed evolutionary position of hyperthermophilic organisms such as *P. furiousus*.

I-B c-6. Ion motive electron transfer pathways involving type II molybdoenzymes of the DMSO reductase family

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Enzymes containing molybdenum play a central role as terminal reductases in anaerobic respiration in prokaryotes, and can also act as primary dehydrogenases in electron transfer pathways associated with chemolithotrophy and photolithotrophy. The above enzymes represent a distinct family (known as the DMSO reductase family) within the superfamily of molybdenum-containing enzymes. Enzymes of the DMSO reductase have a common tertiary structure as defined by the crystal structure of six distinct enzymes and they contain a distinctive form of the molybdenum cofactor composed of a Mo ion coordinated by two (ene)-dithiolate residues provided by two pyranopterin guanosine dinucleotide moieties. The square pyramidal coordination sphere is completed by an oxo or hydroxo or aqua group and a ligand provided by an amino acid side chain. Protein phylogeny has revealed that distinct clades within the DMSO family reflect differences in the amino acid ligand.

Recently, the crystal structure of the nitrate reductase from the Type II clade (NarGHI) was shown to coordinate the Mo via a carboxylate from an aspartate residue. This aspartate is conserved in other Type II enzymes including, dimethylsulfide dehydrogenase (Ddh) from *Rhodovulum sulfidophilum*, selenate reductase (Ser) from *Thauera selenatis* and ethylbenzene dehydrogenase (Ebd) from *Azoarcus* spp. All enzymes of the Type II clade also possess a polyferredoxin subunit (NarH in the case of nitrate reductase) but differ in their connection to the electron transfer chain. In nitrate reductase the NarGH unit is attached to the cytoplasmic face of the membrane by a trans-membrane di-haem NarI. This acts as a quinol-nitrate oxidoreductase and its topology allows energy conservation via a Mitchellian loop mechanism. In contrast, the DdhAB unit forms a complex with a high potential monomeric b-type haem (DdhC) and is located in the periplasm. Electrons from dimethylsulfide are transferred via cytochrome c_2 to the photochemical reaction centre and so energy is conserved via light-driven reactions only. Similarly, selenate reductase is located in the periplasm and accepts electrons from cytochrome *c550*. Although SerABC catalyses a scalar reaction, energy is conserved since it is connected to the cytochrome bc_1 complex via cytochrome *c550*. In contrast, ethylbenzene dehydrogenase is linked via cytochrome *c* to the periplasmic enzymes of denitrification and thus electron transfer conserves no energy.

I-C: Mitochondrial NADH–Ubiquinone Oxidoreductases

I-C c-1. Mitochondrial complex I (NADH–ubiquinone oxidoreductase)—old questions, unexpected answers and new approaches

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Some 50 years after its first purification, solving the structure and function of mitochondrial complex I remains a big challenge. This is in stark contrast to the growing interest to study this large and complicated membrane protein complex that is driven by its involvement in mitochondrial disease and ROS production. Mechanistic concepts and structural models are challenged and have to be revised continuously based on emerging evidence. This process is driven by a number of new approaches developed and by careful studies performed in recent years by several laboratories.Our laboratory has developed the strictly aerobic yeast *Yarrowia lipolytica* as a model to study complex I. Using this model, we could explore functionally important domains of the complex by site directed mutagenesis and obtain information on the localization of several subunits within the complex. Implications of these results for the mechanism of proton translocation will be discussed.

I-C c-2. Coupled electron transfer and catalysis in mitochondrial complex I

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Complex I (NADH:ubiquinone oxidoreductase), from the inner membrane of bovine heart mitochondria, contains 46 different subunits. NADH is oxidised by a flavin mononucleotide (FMN), and the electrons are then carried through the protein, by a number of iron–sulphur (FeS) clusters, to reduce ubiquinone. Each NADH oxidised causes four protons to be pumped across the membrane. At present, our knowledge of the molecular mechanism of complex I catalysis is limited, and defining the mechanism is difficult because FeS clusters and flavins (in contrast to haem centres, for example) are poor chromophores.

In Cambridge, one of the methods which we are applying to study the mechanism of complex I, is protein-film voltammetry. Here, a redox-active protein or enzyme is applied to the surface of a carbon electrode, and then the redox centres are repeatedly oxidised and reduced by the application of an electrochemical potential- the driving force. The flow of electrons is monitored via the current. For an individual FeS cluster, such as the [2Fe–2S] cluster in the 24 kDa subunit of complex I, the redox potential can be accurately and quickly defined under a wide range of conditions (pH, ionic strength, temperature), allowing a complete picture of the behaviour of the cluster to be constructed. For the Ilambda subcomplex of mitochondrial complex I (which contains all the redox cofactors except ubiquinone), the interconversion of NADH and NAD^+ can be observed by varying the driving force and pushing the reaction either in an oxidative or reductive direction. Thus, high-quality information on the catalytic mechanism of the enzyme can be derived.

In this talk, information about the mechanism of complex I which has been gained from protein-film voltammetry, and supporting techniques, will be discussed.

I-C c-3. Conformational changes as part of the mechanism of *E. coli* complex I

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Several independent lines of research in our laboratory indicate that conformational changes are likely to play a role in the mechanism of bacterial complex I. Firstly, fragmentation studies using different detergents have shown that two large subunits of the membrane domain, NuoL and NuoM, are located at a significant distance from the peripheral arm, which contains all the redox centres of the enzyme (Holt, Morgan and Sazanov, (2003) J. Biol. Chem. 278, 43114-20). Subunits NuoL, M and N are homologous to the antiporter family and thus are likely to participate in proton pumping. Distal locations for NuoL and NuoM would be consistent with a conformational link between electron transfer, which takes place in the peripheral arm, and proton translocation. Secondly, projection maps of two-dimensional crystals of the membrane domain of the complex are consistent with the proposed location of subunits NuoL and NuoM. Thirdly, our cross-linking and limited proteolysis studies indicated that upon NADH (but not NAD^+) binding, the enzyme adopts a more open conformation, with increased distances between many hydrophilic subunits. Lastly, we have shown by electron microscopy the nature of this conformational change. The enzyme retains its L-shape in the presence of NADH, but exhibits a significantly more open or expanded structure both in the peripheral arm and, unexpectedly, in the membrane domain (Mamedova, Holt, Carroll and Sazanov, (2004) J. Biol. Chem. in the press). Our latest findings in these areas and their implications will be discussed.

I-C c-4. Positional constraints of a pair of membrane-embedded acidic residues in the NuoK subunit (ND4L subunit counterpart) of *Escherichia coli* NDH-1 for high ubiquinone reductase activity and growth

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The ND4L subunit of mitochondrial NADH:ubiquinone oxidoreductase (complex I) is an integral membrane protein that contains two highly conserved glutamates in two putative trans-membrane helices. We employed complex I from *Escherichia coli* (NDH-1) and its NuoK subunit, a homologue of mitochondrial ND4L, as a model to study by site-directed mutagenesis the role of residues E36 and E72 in enzyme function. These two glutamate residues were together moved about one helix turn towards the periplasmic side of the membrane and the effects of this modification tested. In a cytochrome bo deficient strain growth of the cells on malate as the main carbon source was found a practicable screening method for complex I function. The rate of reduction of ubiquinone analogues by deamino-NADH was measured to evaluate the electron transfer function of NDH-1. The presence of two acidic residues in transmembrane helices was found to be indispensable, but, surprisingly, they did not have to be on adjacent helices as in the wild type subunit. Vicinal location of the acidic residues in one and the same helix, either helix 2 or 3, at an interval of three amino acids (about one helix turn) yielded high electron transfer activity and good-growth phenotypes. Nevertheless, shifting only one of the residues, either E36 or E72, toward the periplasmic side by about one helix turn severely hampered both enzyme activity and growth, whereas moving both acidic residues together to this direction stimulated the ubiquinone reductase activity of the enzyme, although the growth on malate remained poor. This suggests that energy conservation by NDH-1 in this mutant is impaired.

I-C c-5. Possible mechanisms of regulation of the mitochondrial complex I as related to its slow active/inactive transition

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The membrane permeant (*N*-ethylmaleimide, NEM) and membrane nonpermeant (5,5-dithiobis-(2-nitrobenzoate), DTNB) SH-reagents were employed as the specific inhibitors for the mitochondrial Complex I in intact mitochondria and inside-out submitochondrial particles (SMP) in order to locate the SH-group involved in slow interconversion between the active (A-) and inactive (D-) forms of the enzyme. Both NEM and DTNB rapidly inactivate the D-form of Complex I in SMP. In intact mitochondria NEM inhibits the D-form whereas the enzyme is not susceptible to inhibition by DTNB. The sensitivity of D-form in mitochondria to DTNB is induced by channel-forming antibiotic alamethicin. The reactivity of the A/D-transition-sensitive SH-group in SMP towards dianionic DTNB and neutral 2,2-dithiobis-(5-nitropyridine), DTNP were compared. The rate constant of the enzyme-DTNP interaction is 14-times higher than that for DTNB if the efficiency of either reagent in the thiol disulfide exchange reaction is normalized to their reactivity towards neutral dithiothreitol. The pH-profile of the pseudo-first order rate constant for the inhibition by DTNB indicate abnormally high pKa of the SH-group (>10) which makes the D-form unsusceptible for nonenzymatic modification by glutathione. These results show that the A/D-transition-sensitive SH-group is located in the mitochondrial matrix within negatively charged environment.

Three types of the specific inhibitory effects of free fatty acids on Complex I in SMP are found. (i) Palmitate inhibits reversibly the steady-state NADH oxidase and ubiquinone reductase activity with the half-maximal efficiency, which is strongly dependent on the protein (phospholipid) content. (ii) At high protein content palmitate prevents the D- to A-form transition with the significantly higher half-maximal efficiency than that for the inhibitory effect on the steady-state NADH oxidation. (iii) Low concentrations of palmitate (3–10 mkM) cause slow strongly temperature-dependent irreversible inactivation of Complex I.

Supported by the U.S. NIH Fogarty International grant (1R03TW006041-01), Russian Foundation for Fundamental Studies (grants 02-04-48679 and 03-04-48202), and by the program «Leading Schools in Science» (grant 596.2003.4).

I-C c-6. Expression products and function of the nuclear NDUFS4 gene of complex I (NADH–ubiquinone oxidoreductase)

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NADH–ubiquinone oxidoreductase (E.C. 1.6.5.3; complex I) of the respiratory chain in the inner mitochondrial membrane catalyses the oxidation of NADH by ubiquinone and conserves the energy thus made available as transmembrane proton motive force. Forty-six subunits are present in mammalian complex I which are arranged respectively in a membrane and a peripheral matrix arm. Seven subunits are encoded by the mitochondrial genome, the others by nuclear genes. The nuclear NDUFS4 gene encodes for an 18 kDa subunit. In higher eukaryotes this subunit has a conserved canonical phosphorylation site which, in cell cultures ''in vivo'', is reversibly phosphorylated by cAMP-dependent protein kinase (PKA). Phosphorylation of the NDUFS4 protein is associated with potent stimulation of the activity of complex I (1) and suppression of ROS production. Mutations of the human NDUFS4 gene have been found in children affected by lethal neurological syndrome, which are associated with disappearence of the 18 kDa protein product, defective assembly of the complex, suppression of its activity and stimulation by cAMP (2–4). Western blot analysis with specific antibodies raised against the N-terminus and the nonphosphorylated and phosphorylated C-terminus of the 18 kDa protein, which exhibits a >80% homology in high eukaryotes, reveales that the human protein is about 1500 Da s heavier that the mouse and the bovine proteins. In human cells the antibody against the non phosphorylated C-terminus reveals a protein of lower m.w. which at the difference of the heavier protein is not assembled in complex I. A PEST sequence search shows the existence of two proteolytic consensus sites in the presequence. Cleavage of the site closer to the N-terminus could explain the presence in human cells of the heavier mature protein. Two other subunits of about 18 and 10 kDa identified in bovine complex I, ESSS and MWFE respectively, encoded by the nuclear genes have phosphorylation consensus sites which can be phosphorylated by PKA (5). In contrast the NDUFS4 protein appears to be already phosphorylated at its consensus site in the isolated complex I. The phosphorylation site of the ESSS 18 kDa protein is, however, not conserved in the human and murine protein.

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I-D: Heme-Copper Oxygen Reductases

I-D c-1. The proton transfer pathways in cytochrome oxidase

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Cytochrome oxidase is responsible for cell respiration in eukaryotic mitochondria and in many aerobic bacteria. This membrane-bound enzyme is an intriguing energy transducer that converts much of the energy liberated by reduction of O_2 to water into a proton electrochemical gradient. The linkage of electron transfer to proton translocation in the catalytic cycle has recently been clarified, and occurs at each of the four reaction steps where an electron is transferred from the low-spin haem a into the binuclear haem a3-CuB site, and where a ''substrate proton'' is taken up, eventually to yield water. The so-called D- and K-pathways of proton transfer across the protein structure have been characterised by X-ray crystallography and by site-directed mutagenesis experiments. It appears that while the D-pathway is used for transfer of all four pumped protons and of two of the four ''substrate protons'', the K-pathway is used for uptake of the remaining two ''substrate protons'' that accompany the two-electron reduction of the ferrric/cupric binuclear site before it binds O_2. This curious division of labour between the two pathways will be discussed in the light of a proposed ''water-gated'' mechanism of proton translocation [1] that a priori only requires the D-pathway. It is suggested that the K-pathway may be necessary for two major reasons: (i) Reduction of the low redox potential ferric/cupric haem a3/CuB centre may require internal protonation by the adjacent tyrosine residue. (ii) Reprotonation of the tyrosine via the D-pathway may fail because in this phase of the catalytic cycle CuB has lost its hydroxide ligand that otherwise serves to anchor an array of water molecules that transfers the proton from the D-pathway to the binuclear centre [1]. Consequently, the conserved tyrosine in the binuclear site is proposed to have two key roles, viz. aiding O–O bond scission by donating an H atom, and assisting reduction of the ferric/cupric site by transiently donating a proton.

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I-D c-2. The low spin heme of cytochrome *c* oxidase as the driving element of the proton pumping process

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The low spin heme of cytochrome *c* oxidase as the driving element of the proton pumping process. S. YOSHIKAWA (University of Hyogo), H. Shimada (Keio University) and T. Tsukihara (Osaka University).

Redox-coupled structural changes of Asp51, in subunit I of bovine heart cytochrome *c* oxidase, located near the intermembrane surface in the X-ray structures at 2.3/2.35 Å resolution in the oxidized/reduced states are suggestive of a role for this residue in redox-driven proton pumping (1). The residue in the oxidized state is connected with the matrix surface via a hydrogen-bond network and a water channel located in tandem (H-pathway). However, neither clear evidence for the redox-coupled protonation state change in Asp51 nor structure which could drive the active proton transport are detectable in the X-ray structures.

Improved X-ray structures (at 1.8/1.9 Å resolution in the fully oxidized/reduced states) show redox-coupled hydrogen-bonding structural changes in Asp51, indicating deprotonation of its carboxyl group to eject protons to the intermembrane space upon the enzyme reduction (2). The conformation of the formyl group of heme a, which is located in porphyrin plane of heme a and strongly hydrogen bonded to Arg38 at one end of the hydrogen bond network of H-pathway, indicates that the net positive charge created upon oxidation of heme a in the six-coordinated low spin state in both oxidation states drives the active proton transport from Arg38 to Asp51 through the hydrogen bond network(2). A peptide bond in the hydrogen-bond network critically inhibits reverse proton transfer through the network. A redox-coupled change in the capacity of the water channel, induced by the hydroxyfarnesylethyl group of heme a, suggests that the channel functions as an effective proton collecting region for protonation of Arg38 deprotonated by heme a oxidation. The reductive titration of 1584 cm^{-1} band (COO^{-} of Asp51) indicates that the conformation of Asp51 is controlled only by the oxidation state of heme a (2). Mutational analysis for Asp51 (conserved only in animal kingdom) and the peptide bond in the H-pathway using a bovine-enzyme gene expression system showed critical roles of Asp51 and the peptide bond in the proton pumping. These results indicate that heme a drives the proton pumping process.

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I-D c-3. Electron and proton access to the *Paracoccus denitrificans* cytochrome *c* oxidase

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Oxidase mediates the electron transfer from its reduced donor, cytochrome *c*, via its internal redox centers to oxygen, and couples the free energy of this reaction to the generation of a transmembrane proton gradient. Using site-directed mutagenesis on bacterial model systems, the interaction of the two redox partner proteins, and fragments thereof, is followed both structurally and kinetically.Two different proton pathways, the K and the D channel, have been deduced from 3-D structures and from early mutant work. Recent results addressing the possible entry point of the K channel, and a cluster of asn residues in the D pathway leading to complete uncoupling in mutant oxidases, will be discussed.

I-D c-4. Direct observation of protonation reactions during the catalytic cycle of cytochrome *c* oxidase: controversies on the role of E286

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Cytochrome *c* oxidase, the terminal protein in the respiratory chain, converts oxygen into water and helps generate the electrochemical gradient used in the synthesis of adenosine 5′–triphosphate (ATP). The catalytic action of cytochrome *c* oxidase involves electron transfer, proton transfer, and O2 reduction. These events trigger specific molecular changes at the active site, which, in turn, influence changes throughout the protein, including alterations of amino acid side chain orientations, hydrogen bond patterns, and protonation states. We have employed infrared (IR) difference spectroscopy to investigate such modulations for the functional intermediate states E, R2, Pm, and F in cytochrome *c* oxidase from *Rhodobacter sphaeroides* [1]. These spectra reveal deprotonation of its key glutamic acid E286 in the E and in the Pm states. However, these vibrational changes have not been observed in the oxidase from *Paracoccus denitrificans* [2]. The consecutive deprotonation and reprotonation of E286 twice within one catalytic turnover illustrates the role of this residue as a proton shuttle. In addition, our spectra point towards deprotonation of a redox-active tyrosine, plausibly Y288, in the F intermediate. Structural insights into the molecular mechanism of catalysis based on the subtle molecular changes observed with IR difference spectroscopy are discussed. Moreover, avenues to follow these reactions in a time-resolved manner will be presented [3].

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I-D c-5. Electron and proton transfer during the F to O transition in cytochrome *c* oxidase

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The membrane bound cytochrome *c* oxidase (CcO) is the terminal enzyme of the respiratory chain, in mitochondria as well as in many bacteria. It catalyses the reduction of molecular oxygen to water, and in the process maintains a proton gradient across the membrane, which is used to drive ATP synthesis. In spite of extensive research using a variety of techniques, the coupling between the oxygen chemistry and the proton pumping by CcO is not yet fully understood.

In the P to F transition of the reaction cycle, the rate of proton transfer to the catalytic site is dependent on the extent of protonated E(I-286), the internal proton donor. Thus, the pH-dependence of this transition reflects the apparent pKa of E(I-286), shown to be ~ 9.4 [Namslauer et al. (2003) Biochemistry; 42 1488]. The following step, the F to O transition, has been studied in more detail. The formation rate of O is believed to depend on the proton transfer to the catalytic site times the fraction of the F species reduced with another electron. This transition shows a more complicated pH-behaviour that cannot be assigned to the titration of a single group. In addition to E(I-286), the protonation state of another protonatable group with a lower pKa also determines the rate of the F to O transition. To identify this group, we are investigating the RK(I-481) mutant enzyme, where R(I-481) is situated above the two haem groups and is hydrogen-bonded to the propionate of haem a [Mills and Ferguson–Miller (2002) Biochim. Biophys. Acta; 1555 96]. Preliminary results indicate that the mutation influences the electron–proton transfer coupling in the F to O transition. The involvement of heam a in proton translocation will be discussed.

I-D c-6. Proton uptake upon anaerobic reduction of the *Paracoccus denitrificans* cytochrome *c* oxidase

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The kinetics and the stoichiometry of the redox-linked protonation of the soluble *Paracoccus denitrificans* cytochrome *c* oxidase were investigated at pH = 7.2–7.5 by multiwavelength stopped-flow spectroscopy, using the pH indicator phenol red. We compared the wild-type enzyme with the K354M and the D124N subunit I mutants, in which the K- and D-proton-conducting pathways are impaired, respectively. Upon anaerobic reduction by the colorless reductant Ru-II hexamine, the 'as prepared' oxidized wild-type enzyme binds 3.3 ± 0.6 H^+/aa3, i.e., approximately 1 H^+ in excess over beef heart oxidase, as measured in a previous study carried out under similar conditions (1). The different H^+/enzyme stoichiometry suggests intrinsic differences between the *P. denitrificans* and the beef heart cytochrome *c* oxidase. The D124N mutation does not significantly affect the apparent H^+/enzyme stoichiometry in the *Paracoccus* oxidase, 3.2 ± 0.5 H^+/aa3. In contrast, in the K354M mutant, in which the reduction of heme a3-CuB is severely impaired, ~ 0.8 H^+ is promptly bound synchronously with the reduction of heme a, followed by a much slower protonation associated with the retarded reduction of the heme a3-CuB site. These results suggest that the complete reduction of heme a (and CuA) is coupled to a proton uptake (0.7–0.9 H^+ in the *Paracoccus* and 0.6–0.7 H^+ in the beef heart enzyme) that involves yet unidentified groups not belonging to either the K- or the D-pathway. The subsequent reduction of the heme a3-CuB site is associated with the binding of protons (~ 2.5 H^+ in the bacterial and ~ 1.7 H^+ in the mammalian enzyme), which are transferred at least partially through the K-pathway.

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I-E: ATP-Driven Transport Systems

I-E c-1. Comments on the structure and catalytic mechanism of the yeast multidrug efflux ABC transporter Pdr5p

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The yeast ABC transporter multidrug efflux pump Pdr5p (see reference 1) has been solubilized by dodecylmaltoside, purified through Ni-NTA chromatography using an N-terminal hexahistidine tag and reconstituted into a lipid bilayer. Controlled detergent removal produced square particles which by electron microscopy were shown to be made of two full-size Pdr5p polypeptide Under these conditions the four Nucleotide Binding Domains (NBD) were in contact while preserving an open V-shaped structure of the stalk. These structures revealed a structural asymmetric of neighbouring NBDs that face each other at a 90 angle. This raises the possibility of rotational movements in which the NBDs move around the major axes of the stalks during the catalytic site. On the other hand the binding of a nucleotide has been shown to involve the motifs Walker A and Walker B from one NBD and the Signature C from the adjacent NBD. Moreover the two NBDs of a Pdr5p transporter are asymmetric in their sequence and function and one of the WalkerA motifs may be nonfunctional. Implication of these constrains on a putative two-cylinder engine catalytic model will be discussed.

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I-E c-2. Mechanism of action of a bacterial ATP-binding cassette transporter

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The maltose transport complex of *E. coli*, a member of the ATP-binding cassette (ABC) superfamily, mediates the high affinity uptake of maltose at the expense of ATP. The membrane-associated transporter consists of two transmembrane subunits, MalF and MalG, and two copies of the cytoplasmic ABC subunit, MalK. Maltose-binding protein (MBP), a soluble periplasmic protein, plays a key role in the coupling of transport because it functions both to deliver maltose to the MalFGK2 transporter and to stimulate ATP hydrolysis by the transporter. The transporter passes through a conformational intermediate that can be stabilized by the nucleotide transition state analogue MgADP-vanadate. In this intermediate, the normally periplasmic MBP is tightly bound to the membrane transporter MalFGK2 indicating that it may stimulate hydrolysis by stabilizing the catalytic transition state of ATP in the transporter complex. MBP undergoes a conformational change upon binding maltose, from an open form that has lower affinity for maltose to a closed form that binds maltose tightly. We suggest that MBP is initially closed, but opens as it becomes tightly bound to MalFGK2 so that efficient transfer of maltose from MBP to MalFGK2 would occur concomitant with ATP hydrolysis. To test this hypothesis we employ site directed spin labeling (SDSL) and electron paramagnetic resonance spectroscopy (EPR) to monitor binding of MBP to MalFGK2 and conformational changes in MBP as it interacts with MalFGK2 at different points in the translocation cycle. We find that opening of MBP coincides with closure of the dimer interface between the nucleotide-binding MalK subunits when ATP is present.

I-E c-3. Plant ABC transporters – detoxifiers, stomata regulators, and hormone transporters

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An overview on the work performed in our laboratory on plant ABC transporters will be presented. In a first part I will summarize which substrate is transported by ABC-type mechanisms into plant vacuoles.

In a second part I will present data on MRP-like Arabidopsis ABC transporters affecting stomata regulation. Guard cells of AtMRP5 deletion mutants do not respond to glibenclamide, a sulfonylurea acting as stomata opener. Detailed investigations on stomata function showed that stomata from knock out plants do no more respond to the classical modulators of stomata movement. On the whole plant level knock-out plants exhibit a drought resistant phenotype. This result strongly suggests that AtMRP5 may either function as an ion channel regulator or is implicated in signal transduction. Recently we identified two other mutants affected in stomata regulation, one acts similarly to AtMRP5, the second exhibits a drought sensitive phenotype.In the third part I will show that a Pgp-like ABC type transporter is a phytohormone (auxin) transporter, which has a very specific expression pattern and exhibits a polar localization, which fits with its presumed function. Interestingly, this ABC transporter interacts with a regulatory protein, the immunophilin TWISTED DWARF (TWD).

I-E c-4. Reversed transport by the ABC multidrug efflux pump LmrA couples drug uptake to ATP synthesis

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A common feature of all ATP-binding cassette (ABC) transporters is their dependence on ATP to translocate substrates across membranes. This property has led to the generally accepted notion that the transport cycles of these proteins are unidirectional with the concomitant hydrolysis of ATP. As enzyme-mediated reactions are thermodynamically reversible, it can be argued that the unidirectional transport observed for ABC transporters reflects a kinetic irreversibility under physiological conditions rather than a mechanical irreversibility. In agreement with this idea, it has been demonstrated that mammalian Na/K P-type ATPases can mediate a reversed transport cycle under ATP-depleted conditions in the presence of inverted Na/K concentration gradients, resulting in the backward movement of Na^+ and K^+ with the synthesis of ATP. Here we report that in a similar way, the ABC multidrug export pump LmrA in *Lactococcus lactis* can be forced to run in a reversed transport reaction.

In the presence of an inwardly directed ethidium concentration gradient in ATP-depleted cells of L. lactis, LmrA mediated the reverse transport (or uptake) of ethidium with an apparent K_t of 2.0 μM. The uptake reaction was competitively inhibited by the known LmrA substrate vinblastine and was significantly reduced by the E314A mutation in the membrane domain of the transporter, which also inhibited its efflux activity. Similar to efflux, LmrA-mediated ethidium uptake was significantly reduced by the E512Q replacement in the Walker B region of the nucleotide-binding domain of the transporter. This mutation strongly inhibited the drug-stimulated ATPase activity of LmrA. Further studies in whole cells and proteoliposomes containing functionally reconstituted, purified LmrA showed that the uptake of ethidium by this ABC transporter was coupled to a reversed catalytic cycle and resulted in the net synthesis of ATP. Taken together, these results demonstrate that the conformational changes required for drug transport by LmrA are (i) not too far from equilibrium under ATP-depleted conditions to be reversed by appropriate changes in ligand concentrations, and (ii) not uniquely coupled to ATP hydrolysis but associated with a reversible catalytic cycle.

I-E c-5. Structural and functional characterization of Vma5p (subunit C) of the yeast vacuolar ATPase

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Vacuolar-type H^+-adenosine triphosphatase (V_1V_O-ATPase) is one of the most fundamental enzymes in nature. V-ATPases are responsible for the regulation of ion-concentration in the intracellular acidic compartments and have various physiological functions, including membrane and organelle protein sorting, neurotransmitter uptake, cellular degradative processes, and cytosolic pH-regulation. They consist of a membrane-embedded sector V_O, which contains the ion channel, a central connecting stalk, and an extrinsic complex V_1 (A3B3CDEFGyHz), in which ATP hydrolysis takes place (1). A phenomenon of V-ATPase is the reversible dissociation of V_1 from the V_O domain as an in vivo regulation, resulting in decrease of Mg-ATPase activity and proton pumping at the membrane, which is restored in the reassembled V_1V_O-holoenzyme. It has been hypothesized that subunit C plays a central role in the reversible reassembly of both domains by binding as an anchor protein to the actin-based cytoskeleton and controlling the linkage of the cytoplasmic V_1 complex with the actin filaments (2).

In the studies presented, the solution structure of Vma5p (subunit C) of the yeast V-ATPase has been restored. For the first time, the ability of Vma5p to bind ATP has been demonstrated by photoaffinity labeling and fluorescence correlation spectroscopy using the photosensitive ATP analogue 8-N3-3′ -biotinyl-ATP and the fluorescent ATP analogue BODIPY-FL-ATP, respectively.

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I-E c-6. Crystal structure of a central stalk subunit C and reversible association/dissociation of V-ATPase

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The vacuole-type ATPases (V-ATPases) exist in various intracellular compartments of eukaryotic cells to regulate physiological processes by controlling the acidic environment. V-ATPase and the F-type ATP synthase (F-ATPase) are evolutionary related and share the rotary mechanism coupling ATP synthesis/hydrolysis and proton translocation across the membrane. However, these two types of ATPase show significant differences. Reversible association/dissociation of the V_1 (soluble) and the V_O (membrane bound) domain is a unique activity regulation mechanism compared to F-ATPase. Subunit composition and structure in the stalk region of V-ATPase, which connects the V_O and V_1 domains, are suggested to be significantly different from those in F-ATPase. Thus, this region is possibly responsible for the association/dissociation of the complex.

The crystal structure of the subunit C of *Thermus thermophilus* V-ATPase, homologous to eukaryotic V-ATPases, has been determined at 1.95 Å resolution and located into the holo-enzyme complex structure obtained by single particle analysis as suggested by the results of subunit cross-linking experiments [Iwata, M., et. al., (2004) PNAS, 101, 59–64]. The result shows that V-ATPase is substantially longer than the related F-ATPase, due to the insertion of subunit C between the V_1 (soluble) and the V_O (membrane bound) domains. Subunit C, attached to the V_O domain, seems to have a socket like function in attaching the central-stalk subunits of the V_1 domain. This architecture seems essential for the reversible association/dissociation of the V_1 and the V_O domains, unique for V-ATPase activity regulation.

I-F: Quinol-Acceptor Oxidoreductases

I-F c-1. The quinone chemistry of BC complexes

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The quinone chemistry that gives rise to the rather unusual strict bifurcation of electron transfer at the Q_o site of the cytochrome bc complexes remains controversial. I review recent ideas on this reaction and propose a 'logic-gated' binding mechanism that combines classical quinone electrochemistry with specific hydrogen bonding requirements and results in a reversible reaction at the Q_o site that minimises unwanted side-reactions that could otherwise undermine the efficiency of the Q-cycle proton/electron coupling mechanism. The resultant model utilises individual steps of single electron and proton transfer to provide the overall 'concerted' reaction mechanism, and suggests new frameworks for considerations of Q_o-site occupancy and the nature of the antimycin-inhibited state that are also relevant to considerations of the possible mechanisms of superoxide anion generation at this site.

I-F c-2. Structure–function relationship in the cytochrome BC_1 complex

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The cytochrome bc_1 complex (ubiquinol:cytochrome *c* oxidoreductase, QCR) couples electron transfer to translocation of protons across the inner mitochondrial membrane, thereby contributing to the electrochemical proton gradient that conserves the free energy of the redox reaction for production of ATP. The multisubunit, membrane protein complex operates as a functional homodimer and contains three essential catalytic subunits: cytochrome *b*, cytochrome c_1, and the Rieske protein. The molecular mechanism of the enzyme, known as modified Q cycle, is not fully understood.

X-ray crystallographic analysis of yeast QCR at high resolution (Hunte C. et al., 2000, Structure 8, 669) and of the complex with cytochrome *c* bound (Lange C. and Hunte C., 2002, Proc. Natl. Acad. Sci. USA 99, 2800) reveals details of the catalytic sites of the complex, which are important for electron and proton transfer. Structural characterisation of the binding of a hydroxyquinone anion QP site inhibitor supports the model that His181 of the Rieske protein and Glu272 of cytochrome *b* are direct ligands of ubiquinol in the enzyme–substrate complex. Based on this structure the importance of Tyr279 for positioning of the substrate in the binding pocket is suggested. Furthermore, the structure provides experimental evidence for the rotational displacement of Glu272 and supports its role in the suggested proton transfer pathway (Palsdottir H. et al., 2003, J. Biol. Chem. 278, 31303).

The role of selected amino acid residues for substrate binding and proton transfer is studied by site-directed mutagenesis. Enzyme kinetics are characterised and variants analysed by EPR and FTIR.Tightly bound phospholipid molecules including cardiolipin appear to be important for the structural and functional integrity of the complex (Lange et al., 2001, EMBO J. 20, 6591; Palsdottir H. and Hunte C., Biochim. Biophys. Acta, in press). The number of retained phospholipid molecules in QCR preparations and crystals can be selectively controlled during purification, thereby influencing the enzyme activity. The importance of specific binding sites is probed by site-directed mutagenesis and the characterization of the variants will be presented.New information is obtained for the electron transfer complex between yeast QCR and cytochrome *c*. The impact of ionic strength and redox state on the binding interaction is discussed.

I-F c-3. The structure of the cytochrome b_6f complex from a green alga

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The photosystem I and II drive oxygenic photosynthesis, however, they can only do so by interacting with the multisubunit cytochrome b_6f complex. This complex couples the electron transfer between the two photosystems with the generation of a transmembrane electrochemical proton gradient using an analogous Q-cycle mechanism as the mitochondrial and bacterial cytochrome bc_1. The crystal structure of the green alga *Chlamydomonas reinhardtii* (Stroebel et al 2003, Nature 426:416) bears similarities with its mitochondrial counterpart but displays several unique features. The quinone reduction site Q_i harbours an unexpected haem, called haem ci, that is not present in the cytochrome bc_1. This haem has atypical properties, it is bound to the cytochrome b_6 by one thioether linkage in the transmembrane region near the stromal side, a quite unusual situation for a cytochrome from the c group. Furthermore, it has no proteic axial ligand, there is on one side a water molecule or hydroxyde ion interacting with the propionate of haem b_H, suggesting a strong coupling between the two haems. On the other side, a bulky phenylalanine side chain prevent strong interaction with the haem. It suggests that it is a high-spin haem. Phylogenetic comparison, corroborated by the structure of the complex from the cyanobacteria *Mastigocladus laminosus* (Kurisu et al., 2003 Science 302:5647) indicate that the haem ci is present not only in all chloroplastic and cyanobaterial cytochrome b_6f complexes, but also in the menaquinone oxidoreductase from the firmicutes. It results in a quite different Q_i site comparing with the one of cytochrome bc_1 and thus explaining the absence of inhibition by antimycin and the lack of semiquinone EPR signal. The general characteristics of the haem ci are in agreement with the one from the G cytochrome described in *Chlorella sorokiniana* by spectroscopy in vivo by Lavergne (1983, Biochem Biophys Acta 725:25). The position of this haem near the stromal side support the idea of a reinjection of electron to the cytochrome b_6f in the cyclic electron transfer around the photosystem I. In addition to the haem ci, the cytochrome b_6f hold two other cofactors not present in the cytochrome bc_1: a chlorophyll a and a beta-carotene, which are not interacting with each other, leaving us with a puzzling problem about their functions.

I-F c-4. Half-of-the-sites reactivity of the yeast cytochrome bc_1 complex

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We previously reported that stigmatellin and MAO-stilbene exhibit negative cooperativity in binding and inhibition of the yeast cytochrome bc_1 complex. These are competitive inhibitors of the enzyme that bind to the ubiquinol oxidation site at center P and are generally considered mimetics of intermediate states during ubiquinol oxidation. On the basis of this indirect evidence we suggested that the bc_1 complex oxidizes ubiquinol by an alternating, half-of-the-sites mechanism [Gutierrez-Cirlos, E. B. and Trumpower, B. L. (2002) J. Biol. Chem. 277, 1195–1202]. We have now obtained direct evidence for half-of-the sites reactivity during ubiquinol oxidation by analyzing the presteady state and steady state activities of the isolated bc_1 complex under conditions that allow the first turnover of ubiquinol oxidation to be observable in cytochrome c_1 reduction. In the presence of antimycin and at pH 8.8, where the redox potential of the iron–sulfur protein is approximately 200 mV, the amount of cytochrome c_1 reduced by several equivalents of ubiquinol in the presence of antimycin corresponded to only half of that present in the bc_1 complex. Similar results were obtained with a bc_1 complex with a mutated iron–sulfur protein of equally low redox potential. Experiments in which reduction of several equivalents of cytochrome *c* was monitored also showed only half of the bc_1 complex participating in quinol oxidation. The extent of cytochrome *b* reduced corresponded to two b_H hemes undergoing reduction through one center P per dimer, indicating electron transfer between the two cytochrome *b* subunits. Antimycin stimulated the ubiquinol–cytochrome *c* reductase activity of the bc_1 complex at low inhibitor/enzyme ratios. This stimulation could only be fitted to a model in which half of the bc_1 dimer is inactive when both center N sites are free, becoming active upon binding of one center N inhibitor molecule per dimer, and there is electron transfer between the cytochrome *b* subunits of the dimer. These results are consistent with an alternating half-of-the-sites mechanism of ubiquinol oxidation in the bc_1 dimer. We propose that this is a previously unrecognized mechanism for regulating respiration and protecting against superoxide anion formation by which bc_1 activity is adjusted in response to the redox poise of the ubiquinol/ubiquinone pool.

I-F c-5. Structure–function studies of the trypanosome alternative oxidase

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The parasite *Trypanosoma brucei* is the causative agent for sleeping sickness in humans and nagana in cattle. In the bloodstream form of the life-cycle, respiratory cytochromes are absent and the parasite is dependant on the trypanosome alternative oxidase (TAO) as the only respiratory oxidase. The parasite uses glycolysis for energy production. Therefore reoxidation of reducing equivalents is essential. This occurs through a glycerol-3-phosphate system consisting of glycerol-3-phosphate dehydrogenase, present in the glycosome, ubiquinone and TAO located in the mitochondrion. TAO, a ubiquinol; oxygen oxidoreductase, accepts electrons directly from ubiquinol but does not support oxidative phosphorylation.

TAO has been unequivocally confirmed as a member of the cyanide-insensitive alternative oxidase protein family found in plants, fungi, yeast and increasingly in protozoans. Three regions of TAO have significant homology to the C-terminal region of other alternative oxidases. Highly conserved residues include the Glu-X-X His motifs proposed to bind iron. Our research on the plant alternative oxidase has identified key residues, including those involved in ligating the di-iron centre [Albury et al (2002). J. Biol. Chem. 277, 1190–1194] and has led to our proposals of the catalytic mechanism [Affourtit et al (2002). FEBS Lett. 510, 121–126]. We have also identified residues potentially involved in quinone-binding.

The residues Tyr-275 and Trp-206, that we propose to be involved in the catalytic mechanism, together with the tyrosines Y253, Y266 and Y299 are all highly conserved in TAO. We are currently using our yeast expression system, together with site-directed mutagenesis to investigate the role of these residues in the catalytic cycle and in quinone-binding.

Work supported by the Wellcome Trust.

I-F c-6. Resolving protein–quinone interactions using pulsed EPR spectroscopy

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Quinones molecules are ubiquitous in living organisms. They are found either within the lipid phase of the biological membrane (quinone pool) or are bound in specific binding sites within membrane-bound protein complexes. The biological function of such bound quinones is determined by their ability to be reduced and/or oxidised in two successive one-electron steps. As a result, quinones are involved as one- or two-electron donors or acceptors in a large number of biological electron transfer steps occurring during respiratory or photosynthetic processes.

The intermediate formed by a one-electron reduction step is a semiquinone, which is paramagnetic and can be studied with EPR spectroscopy. Detailed studies of such states can provide important structural information of these intermediates in such electron-transfer processes.In this study, we focus on the redox-active ubiquinone from the cytochrome bc_1 complex (ubiquinol:cytochrome *c* oxidoreductase, QCR) from *S. cerevisiae* at the so-called Q_N site. Although the location of the Q_N binding pocket is quite well known from crystallographic studies, details about its exact binding are less clear. Currently three different X-ray crystallographic studies suggest three different binding geometries for Q_N.

Using the yeast system a range of EPR spectroscopic techniques are applied to characterise the Q_N binding site. High-field EPR spectroscopy is used to resolve the g-tensor, while pulsed Electron Nuclear DOuble Resonance (ENDOR) spectroscopy is used to map out the spin density distribution within Q_N- itself. Selective isotopes (in the environment) are applied to further characterise its binding within the protein using both deuterium-ENDOR and 2-dimensional proton-HYperfine Sublevel CORrElation (HYSCORE) spectroscopy. Nitrogen-Electron Spin Echo Envelope Modulation (ESEEM) spectroscopy together with an inversion recovery filter (REFINE)(1) is applied to resolve the question of whether the observed nitrogen modulations arise from interactions to Q_N- or to the Rieske iron–sulphur centre(2).

These results are compared with the corresponding information available on other protein binding sites e.g., in photosystem I and/or on model systems. They are discussed with regard to the location and potential function of Q_N in the enzyme.

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II-A: Mitochondrial Dynamics

II-A c-1. Molecular machinery of mitochondrial motility, fusion and fission

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Mitochondria are amazingly dynamic organelles. They continuously move along cytoskeletal tracks and frequently fuse and divide. These processes are important for maintenance of mitochondrial functions, for inheritance of the organelles during cell division, for cellular differentiation and for apoptosis (Westermann, 2002, EMBO Rep. 3:527–531). The understanding of the molecular mechanisms determining mitochondrial dynamics requires the identification of the proteins involved, combined with their functional characterization by genetic, biochemical and cytological approaches. As the machinery of mitochondrial behaviour has been highly conserved during evolution, it can be studied in yeasts and other fungi as model organisms.

To identify novel components essential for mitochondrial morphogenesis, we screened 4800 yeast deletion strains for mutants with aberrant mitochondrial distribution and morphology, MDM. The data obtained by the screen provide a comprehensive picture of the cellular processes and molecular components required for mitochondrial morphogenesis in a simple eukaryotic cell (Dimmer et al., 2002, Mol. Biol. Cell, 13:847–853). Functional characterization of mdm mutants revealed novel components involved in mitochondrial fusion, division, motility and mitochondrial DNA inheritance (Messerschmitt et al., 2003, J. Cell Biol. 160:553–564; Fritz et al., 2003, Mol. Biol. Cell 14:2303–2313).

Transport of mitochondria is actin-dependent in budding yeast, and microtubule-based in the filamentous fungus *Neurospora crassa* and in human cells. To identify components involved in microtubule-dependent mitochondrial motility, we developed biochemical and microscopic assays to monitor the interaction of mitochondria with microtubules in vitro (Fuchs et al., 2002, J. Cell Sci. 115:1931–1937). Using these assays, we have identified and functionally characterized a novel motor protein of the kinesin family, that is mediating mitochondrial motility in *Neurospora*.

II-A c-2. Class V myosin-dependent systems for mitochondrial distribution in the budding yeast

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In the budding yeast *Saccharomyces cerevisiae*, mitochondrial distribution to the daughter cell (bud) is dependent on actin and a class V myosin (Myo2p). Our genetic analysis revealed that the Myo2p-dependent process is composed of two independent pathways, the Mmr1p-dependent and Ypt11p-dependent pathways. Mmr1p resides preferentially on the bud-localizing tubules of mitochondria and properties of Mmr1p strongly suggest that Mmr1p is a component of the mitochondrial Myo2p-receptor that links Myo2p to mitochondria to polarize the mitochondrial movement toward the bud. The time-lapse images of the Mmr1p-dependent movement of mitochondria are matched with the model that the Mmr1p-Myo2p complex guides mitochondrial tubule along actin cytoskeleton, running through the mother cell to the bud. In contrast to Mmr1p, Ypt11p, a rab-type GTPase, localizes bud cortex, depending on the Ypt11p ability to associate with Myo2p. Ypt11p is essential for mitochondrial distribution into the bud when the Mmr1p pathway is abolished, showing synthetic lethal interaction between ypt11-deficiency and mmr1-deficiency. The time-lapse images revealed that the Ypt11p-dependent movement of mitochondrial tubules is highly dynamic and looks different from that driven by Mmr1p. To clarify the mechanism of the Ypt11p-dependent system, we surveyed factors, which are required for the Ypt11p system, by mutational analysis using the synthetic lethal phenotype between mmr1-deficiency and defect in the Ypt11p system. The phenotypes of the isolated mutants strongly suggest that membrane dynamics around bud periphery participates in the Ypt11p-depndent system. We propose that the mitochondrial dynamics through communication between mitochondria and other cellular membranes acts as an inheritance system of mitochondria. To speculate how eucaryote acquires systems for mitochondria inheritance, the yeast case may be suggestive. Actin is essential in mitochondrial distribution in the budding yeast, while microtubules play a main role in the distribution in higher eucaryote and the fission yeast. Although the Mmr1p scheme resembles to the microtubule-motor-driven system in higher eucaryote, systems for actin and tubulin were diverse in early timing of evolution. Therefore, it is conceivable that the Ypt11p-dependent system is a strong candidate for the evolutional derivative from the prototype system for mitochondrial inheritance, prevailing before developing the system using motor proteins.

II-A c-3. Organization and dynamics of human mitochondrial DNA

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Heteroplasmic mutations of mitochondrial DNA (mtDNA) are an important source of human diseases. The mechanisms governing transmission, segregation and complementation of heteroplasmic mtDNA-mutations are unknown but depend on the nature and dynamics of the mitochondrial compartment as well as on the intramitochondrial organization and mobility of mtDNA. We show that mtDNA of human primary and immortal cells is organized in several hundreds of nucleoids that contain a mean of 2–8 mtDNA-molecules each. Nucleoids are enriched in mitochondrial transcription factor A and distribute throughout the entire mitochondrial compartment. Using cell fusion experiments, we demonstrate that nucleoids and respiratory complexes are mobile and diffuse efficiently into mitochondria previously devoid of mtDNA. In contrast, nucleoid-mobility was lower within mitochondria of mtDNA-containing cells, as differently labeled mtDNA-molecules remained spatially segregated in a significant fraction (37%) of the polykaryons. These results show that fusion mediated-exchange and intramitochondrial mobility of endogenous mitochondrial components are not rate-limiting for intermitochondrial complementation but can contribute to the segregation of mtDNA molecules, and thus of mtDNA mutations, during cell growth and division.

II-A c-4. Energy substrate modulates mitochondrial structure and oxidative capacity in cancer cells

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Comparative analysis of cytoplasmic organelles in a variety of tumors relative to normal tissues generally reveals a strong diminution in mitochondrial content and in oxidative phosphorylation capacity. However, little is known about what triggers these modifications, and whether or not they are physiologically reversible. We hypothesized that energy substrate availability could play an important role in this phenomenon. The physiological effects of a change in substrate availability were examined on a human cancer cell line (HeLa), focusing specifically on its ability to use glycolysis vs. oxidative phosphorylation, and the effect that energy substrate type has on mitochondrial composition, structure and function. Changes in oxidative phosphorylation were measured in vivo by a variety of techniques, including the use of two novel ratiometric GFP biosensors, the expression level of OXPHOS and some glycolytic enzymes were determined by Western blot, mtDNA content was measured by real-time PCR and mitochondrial morphology was monitored by both confocal and electron microscopy.

Our data show that the defective mitochondrial system described in cancer cells can be dramatically improved by solely changing substrate availability, and that HeLa cells can adapt their mitochondrial network structurally and functionally to derive energy by glutaminolysis only. This could also provide an explanation for the enhancement of OXPHOS capacity observed after tumor regression or removal. Our work demonstrates that the pleomorphic, highly dynamic structure of the mitochondrion can be remodeled to accommodate a change in oxidative phosphorylation activity. We compared our finding on HeLa cells with those for nontransformed fibroblasts to help distinguish the regulatory pathways.

II-A c-5. OPA_1 requires mitofusin-1 to promote mitochondrial fusion

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The regulated equilibrium between fusion and fission of mitochondria is essential to maintain integrity of the organelle. Mechanisms that coordinate fusion of mitochondrial membranes are largely uncharacterized in mammalian cells. OPA_1 encodes a dynamin-related protein associated with inner mitochondrial membrane and mutated in autosomal dominant optic atrophy. It is unclear whether and how OPA_1 participates in mitochondrial fusion. Here we show that expression of OPA_1 promoted high levels of mitochondrial tubulation. Mutagenesis experiments showed that the GTPase and the C-terminal coiled coil domains of OPA_1 are both essential for the tubulation process. Real time imaging and fusion assays coupled to genetic analysis indicated that OPA_1 propels a process of mitochondrial fusion requiring mitofusin-1 but not mitofusin-2. In complex, OPA_1 and mitofusin-1 cooperate to fuse mitochondria, revealing a biological role for OPA_1 and a specific functional difference between outer membrane mitofusin 1 and 2.

II-A c-6. Mitochondrial fission: what for?

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In various eukaryotes, the morphology of mitochondria depends on the balance between fusion and fission processes. If mitochondrial fusion appears to be essential for the inheritance of mitochondrial DNA, less is known about the role of mitochondrial fission. Because this process is highly dynamic and occurs continuously in cells, it is conceivable that it may play an essential function in cell homeostasis. To understand the role of mitochondrial fission in HeLa cells, we have designed several siRNA targeting DRP-1. These RNAi were efficient in down regulating the levels of the DRP-1 protein. As a consequence, mitochondria became filamentous and highly interconnected. We observed that preventing mitochondrial fission had a profound effect on the cell cycle since a high proportion of cells were unable to enter into mitosis. These results were confirmed with primary cultures of fibroblasts. The relationship between mitochondrial morphology and the cell cycle is currently under investigation.

II-B: Channels in Coupling Membranes

II-B c-1. Modulation of the mitochondrial Ca^{2+} uniporter

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During cell activation, mitochondria play an important role in Ca^{2+} homeostasis thanks to the presence of a fast and specific Ca^{2+} channel in its inner membrane, the mitochondrial Ca^{2+} uniporter. This channel allows mitochondria to buffer local cytosolic [Ca^{2+}] changes and controls the intramitochondrial Ca^{2+} levels, thus modulating a variety of phenomena such as respiratory rate, Ca^{2+}-dependent secretion or apoptosis. The activity of the uniporter depends steeply on the cytosolic [Ca^{2+}]. However, little else is known on the physiological regulation of its activity. We have described recently that SB202190, an inhibitor of p38 mitogen activated protein kinase, strongly activates the uniporter in intact and permeabilized cells (Montero et al., FASEB J. 16, 1955–1957 (2002)). We have now found that a series of natural plant flavonoids, widely distributed in foods, produced also a strong stimulation of the mitochondrial Ca^{2+} uniporter. Their effect had the same magnitude than that induced by SB202190 (>10-fold increase in the Ca^{2+} uptake rate), developed without measurable delay and was rapidly reversible. In intact HeLa cells, the mitochondrial Ca^{2+} peak induced by histamine was also largely increased by the flavonoids, while the cytosolic one was little modified. Stimulation of the uniporter by either flavonoids or SB202190 did not require ATP, suggesting a direct effect on the uniporter not mediated by protein phosphorylation. The effects of the flavonoids on the mitochondrial Ca^{2+} uniporter occurred at low concentrations, close to those which can be reached in plasma after ingestion of flavonoid-rich food. Therefore, these compounds could modulate the activity of the uniporter under physiological conditions.

II-B c-2. Mitochondrial ATP-regulated potassium channel

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In 1991 the mitochondrial ATP regulated potassium channel (mitoK_{ATP} channel) was identified in the inner membrane of heart mitochondria. Similar to plasma membrane channels, mitochondrial channel is inhibited by antidiabetic sulfonylureas and activated by potassium channel openers (KCOs). What is important, in cardiac muscle mitoK_{ATP} channel was found to play an important role in protection of cardiomyocytes against ischemia/reperfusion injury. Activation of mitoK_{ATP} channel by KCO-diazoxide seems to mimic ischemic preconditioning in heart muscle. Cardioprotective action of mitochondrial KCOs involves probably multiple mechanisms: including effects on cellular apoptosis. In the present work we have analysed the effects of oxidative stress on neonatal rat cardiac ventricular myocytes. Moreover, reconstitution of mitoK_{ATP} into lipid bilayer was performed. Cytoprotective action of potassium channel openers such as diazoxide was also studied in skeletal musceol and neuronal cells.

The work in authors laboratory was supported in part by the State Committee for Scientific Research (grants 6P04A02321, 3P04A05025, 6P04A01019, PBZ-MIN-001/P05/11) and in part by NATO Collaborative Grant LST.CLG.979217 and by the grant of the Roche Organ Transplantation Research Foundation (ROTRF 860428181).

II-B c-3. A tetrameric voltage-dependent anion channel mediates cytochrome *c* release from mitochondria

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The voltage-dependent anion channel (VDAC) plays a central role in apoptosis, participating in the release of apoptogenic factors such as cytochrome *c*. The molecular mechanisms by which VDAC forms a protein-conducting channel for the passage of cytochrome *c* are unclear. The present work approaches this problem by addressing the oligomeric status of VDAC and its role in the release of cytochrome *c*. Chemical cross-linking of isolated mitochondria or purified VDAC fixed VDAC into dimers to tetramers. Fluorescence Resonance Energy Transfer between differentially labeled VDAC further supports VDAC oligomerization. Mitochondrial cross-linking prevented both PTP opening and release of cytochrome *c*, yet had no effect on electron transport or Ca^{2+} uptake. Cross-linking had no effect on the channel properties of bilayer-reconstituted purified VDAC, but inhibited release of encapsulated cytochrome *c* within VDAC-proteoliposomes or via bilayer-reconstituted VDAC. Moreover, encapsulated but not soluble cytochrome *c* induced VDAC oligomerization. These results suggest cytochrome *c*-induced VDAC oligomerization as a novel mechanism for cytochrome *c* crossing the outer mitochondria membrane, with transfer occurring via the large flexible pore formed between individual subunits of a VDAC oligomer.

II-B c-4. Evidence from patch-clamp experiments for cell-protective effect of permeability transition pore blockade

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It has been suggested that opening of the permeability transition pore (PTP) in the inner mitochondrial membrane is an important step in the signaling cascade leading to apoptosis. Consequently, blockade of the PTP should reduce apoptosis e.g., in neurodegenerative diseases in type-II neurons (mitochondria involved in cascade). We therefore characterized the PTP, proved its sensitivity to Fas/CD95-activation (Loupatatzis et al., 2002, Cell Physiol Biochem 12:269), and tested the blocking effect of agents known to exhibit neuroprotective effects (Andrabi et al., 2004, FASEB J 18:869). Single channel events of the PTP were recorded from mitoplasts (i.e., vesicles of inner mitochondrial membrane) of rat liver cells by means of the patch-clamp techniques. Pipettes were pulled from borosilicate glass with resistances of 8–17 MΩ. Basic solutions consisted out of 150 mM KCl, 10 mM K-HEPES, pH 7.2.

The PTP showed the typical single-channel behaviour with the largest amplitudes of >1 nS, numerous substates, and increased open probability (Po) at depolarizing potentials. Po could be dose-dependently increased by Ca^{2+} (EC50 = 10 μM) and blocked by Cyclosporin A. Lysate from Fas/CD95-activated T-lymphocytes with low Ca^{2+} increased the Po as compared with a lysate from nonactivated cells demonstrating an activating effect of the apoptosis cascade. Adding one of the common dopamine-D2-agonists to the PTP caused a dose-dependent decrease of the Po. This result could be confirmed by a swelling assay, where PTP-dependent swelling was considerably reduced by the agonist. It was shown elsewhere that Parkinson patients treated by the same dopamine agonists at tissue concentrations comparable to those used in our experiments showed improved motor scores after several years. In addition, the pineal hormone melatonin dose-dependently decreased the Po of the PTP (IC50 = 0.8 μM) and reduced several parameters of apoptosis thereby reducing brain damage in rats induced by a 2 h middle cerebral artery occlusion. We therefore conclude that blockade of the PTP may have a neuroprotective effect.

II-B c-5. Cyclophilin D, Cyclosporin A and the modulation of the mitochondrial permeability transition in Cyclophilin D-null mice

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The Permeability Transition Pore (PTP) is a high conductance mitochondrial channel that regulates the permeability of the inner mitochondrial membrane (IMM). The permeability transition due to opening of the PTP in vitro has dramatic consequences on mitochondrial function (collapse of the $\Delta\mu H$, depletion of pyridine nucleotides) and structure (rupture of the outer membrane and release of cytochrome *c*). This process has long been studied as a potential target for mitochondrial disfunction in vivo, for example as occurs during ischemia/reperfusion injury, and as a mediator of programmed cell death through the release of intermembrane proteins active on the apoptotic machinery. Despite detailed functional characterization of the PTP its molecular components have not yet been definitively established. A key feature of the PTP is its inhibition by Cyclosporin A (CsA), which has become a standard diagnostic tool for the characterization of the PTP. Much evidence clearly points to the mitochondrial cyclophilin isoform (CyP-D) as the target for the CsA inhibition of the PTP. To answer basic questions related to the role of CyP-D on PTP modulation, the partecipation of the PTP in the apoptotic program, and its role in specific pathological processes, we have created a mouse line in which the expression of the CyP-D has been eliminated by ''knock-out'' strategies that remove the first three protein coding exons. Mice homozygous for the disrupted cyp-D gene are viable and do not display obvious developmental defects. Our preliminary experiments indicate that (i) CyP-D-null mitochondria can undergo a permeability transition after the uptake of larger Ca^{2+} loads than wild type mitochondria; (ii) Mitochondria from CyP-D-null and wild type strains show a comparable sensitivity to other PTP inducers such as diamide and phenylarsine oxide; (iii) The PTP of mitochondria lacking CyP-D cannot be inhibited by CsA, while it is still sensitive to Ubiquinone 0. These findings indicate that CyP-D is not essential for PTP opening yet it contributes to sensitize the channel to Ca^{2+}; and that CyP-D mediates the desensitizing effects of CsA on the PTP.

II-B c-6. Role of a novel mitochondrial potassium channel in apoptosis

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Alterations of ion fluxes through plasma- and organellar membranes are a hallmark of apoptosis. Electron microscopy and immunological studies localized the Shaker-family member potassium channel Kv1.3—at present exclusively identified in the cell membrane—to the inner mitochondrial membrane. Patch clamp experiments revealed activity of a channel in the same membrane which displayed characteristics compatible with those of to Kv1.3. Blockade of mitochondrial Kv1.3 by the two specific inhibitors, Margatoxin and Shk sea anemone toxin, triggered crucial events for the induction of apoptosis, including generation of reactive oxygen species, Cyclosporin A-sensitive mitochondrial membrane depolarization as well as release of cytochrome *c*. Mitochondria from Kv1.3 deficient CTLL-2 cells never showed Kv1.3-like activity in electrophysiological experiments and failed to respond to Margatoxin and Shk. Likewise, cells lacking Kv1.3 were resistant to apoptosis mediated by tumor necrosis factor a, C6-ceramide or staurosporine, while retransfection of Kv1.3 restored apoptosis. The present data indicate an important role of mitochondrial Kv1.3 in apoptosis.

II-C: Secondary Active Transport Systems

II-C c-1. Functional genomics of plant membrane proteins

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Plant cells are highly compartmentalized and rely on an extensive intracellular and intercellular exchange of solutes to connect metabolic pathways of different compartments or plant tissues. Many membrane proteins will be involved in response and tolerance reactions of plants to environmental challenges (water-, salt-, temperature-stress, uptake of nutrients etc.).We aimed to identify and classify the complete set of proteins containing putative transmembrane domains of the model plants *Arabidopsis thaliana* and *Oryza sativa*. These numbers mount up to approximately 5800 and 7800 amino acid sequences, respectively. To collect the sequences and prediction data for these membrane proteins/genes, a novel web-accessible dynamic database, termed "ARAMEMNON" was created. Inter alia, this database is able to cluster all membrane proteins with similarities above a certain threshold to the query sequence into groups according to their similarities among each other.

To functionally characterize a subset of these proteins from *A. thaliana*, different approaches are followed including the identification of plant transporters by functional complementation of specific yeast mutant strains, the development and use of custom-made cDNA arrays that contain a set of *A. thaliana* membrane protein genes for RNA expressing profiling, and the isolation and characterization of *A. thaliana* loss-of-function mutants.

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II-C c-2. Structural basis for substrate translocation of the *Escherichia coli* glycerol-3-phosphate transporter, GlpT

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The glycerol-3-phosphate transporter (GlpT) from the inner membrane of *E. coli*, an antiporter that exchanges inorganic phosphate for glycerol-3-phosphate, plays an important role in lipid biosynthesis and carbon utilization. GlpT belongs to the major facilitator superfamily, which represents the largest group of secondary active membrane transporters in the cell. Using X-ray crystallography, the three dimensional structure of GlpT was recently solved (Huang, Lemieux, et al., Science 2003, 301:616–620). The 3.3 Å resolution structure of GlpT reveals two domains connected by a long central loop. The amino- and carboxyl-terminal domains of the protein, each containing a six-helix bundle, are related by a pseudo twofold symmetry. Between the two domains and open to the cytoplasm is a centrally located substrate-translocation pore. Two arginine residues at the closed end of the pore comprise the substrate-binding site. Biochemical experiments show that upon substrate binding the protein adopts a more compact conformation. The crystal structure suggests that GlpT operates in a single-binding-site, alternating-access mechanism via a rocker-switch type of movement of the N- and C-terminal domains. Furthermore, GlpT may serves as a structural and mechanistic paradigm for other members of the major facilitator superfamily.

II-C c-3. Yeast as a model system for studying glucose transport and sensing

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In the yeast *Saccharomyces cerevisiae* uptake of hexoses is mediated by a large family of transporter proteins. These transporters differ in their substrate specificities, their kinetic properties and their expression profiles. Their activities at the plasma membrane are controlled by a complex network of transcriptional regulation, regulation of intracellular trafficking as well as protein–protein interactions. Two of the transporter proteins have no detectable transport activity and serve, rather, as nutrient sensors. These proteins activate signalling pathways in response to detection of glucose in the external medium, and control transcription of genes encoding hexose transporters (Boles, E and André, B, 2004, In: Molecular Mechanisms Controlling Transmembrane Transport, Topics in Current Genetics; Eds: Boles E, Krämer R; pp. 121–153, Springer-Verlag).

Deletion of at least 20 genes was necessary to block uptake of hexoses in *S. cerevisiae* completely (Wieczorke et al., 1999, FEBS Lett 464, 123–128). The resulting hxt-null yeast strain provides a valuable tool for the cloning and characterization of hexose transporters from other organisms.

The production of properly folded and functional membrane proteins in heterologous expression systems is currently an enormous challenge. One important application is the production of large quantities of membrane proteins for structure determination. On the other hand, microbial expression systems are well suited for studying the biochemical and pharmacological properties of membrane proteins. We have expressed the human glucose transporters GLUT1 and GLUT4 in the yeast hxt-null strain (Wieczorke et al., 2003, Cell Physiol Biochem 13, 123–134). Molecular analyses revealed that a major problem in their correct delivery to the plasma membrane is the presence of ergosterol in yeast membranes in contrast to cholesterol in mammalian membranes. Moreover, a second bottleneck is the final fusion of secretory vesicles with the plasma membrane. With this knowledge we were able to construct a novel heterologous expression system for human GLUT glucose transporters.

II-C c-4. Novel inter- and intrasubunit transport-relevant contact sites between residues of the mitochondrial phosphate transport protein

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The three Cys of the yeast (*S. cerevisiae*) mitochondrial phosphate transport protein (PTP) subunit were replaced with Ser. The seven mutants (single, double, and complete Cys replacements) were expressed in yeast and the homodimeric mutant PTPs purified from the mitochondria and reconstituted. The pH gradient-dependent net phosphate (Pi) transport uptake rates catalyzed by these reconstituted mutants are similar to those of the wild type. Aerobic media inhibit only the Pi uptake rates catalyzed by PTPs with the conserved (yeast and bovine) Cys28. This inhibition in the proteoliposomes is 84% to 95% and can be completely reversed by dithiothreitol. Transport by the wild type as well as by all mutant proteins with Cys28 is more than 90% inhibited by mersalyl. When dithiothreitol is removed from purified single Cys mutant proteins, only the mutant protein with Cys28 appears as a homodimer in a nonreducing SDS polyacrylamide gel. Thus the function-relevant transmembrane helix A, with Cys 28 about equidistant from the two inner membrane surfaces, is in close contact with parts of transmembrane helix A of the other subunit in the functional homodimeric PTP. The results identify for the first time not only a transmembrane helix contact site between the two subunits of a homodimeric mitochondrial transport protein but also a contact site that if locked into position blocks transport.

Ser158 is located within the matrix loop connecting transmembrane helices C and D of PTP. The mutant Ser158Thr PTP is transport-inactive. His32 is located within helix A and Thr79 five residues outside the N-terminal of helix B. Replacing either one with Ala yields transport-inactive PTP. The structure of a subunit of the bovine ADP/ATP translocase complexed with carboxyatractyloside (Pebay-Peyroula et al. Nature 426, 39, 2003), sequence similarities between members of the mitochondrial transport protein family, and evidence that transmembrane A helices (Cys28) are part the subunit/subunit contact site, together suggest that the Ser158 site is at the subunit/subunit contact site that keeps transport by the two subunits 180 degrees out of phase. The data also suggest that His32 and Thr79 are sufficiently close to one another to act as coupling site between phosphate and proton cotransport. (Supported by NIH GM57563).

II-C c-5. The function of two novel families of metal-ion transporters chaperons

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We generated yeast null mutant lacking all three genes encoding metal ion transporters Smf1,2,3Δ. The phenotype of the triple null mutant could be complemented either by SMF1 or by SMF2 or by the mammalian transporter DCT1.We used the $ZnCl_2$ sensitivity of Smf1,2,3Δ to screen for genes that will suppress this phenotype and identified ZSP1 (Zinc Sensitive Phenotype 1). A null mutation in ZSP1 gene is sensitive to $ZnCl_2$ in the medium. Through treatment of Zsp1Δ with EMS, we found a mutant that grew in the presence of $ZnCl_2$ but was sensitive to EGTA. By transformation of this mutant with a yeast library we identified a novel gene named STC1 (Smf Transporter Chaperone 1) and its deletion results in EGTA sensitivity. Null mutations in a novel homologous yeast gene (STC2) also results in sensitivity to EGTA. Overexpression of Smf1p or Smf2p in the background of Stc1Δ or Stc2Δ does not recover the EGTA sensitivity. However overexpression of the short construct of Smf1p or DCT1 (the rat SMF1 homolog) complement this phenotype. Both STCs were expressed in the ER Golgi fractions when run on sucrose gradient, and were present in multiprotein complexes. We suggest a role for Stc1p and Stc2p in chaperoning Smf1p into the plasma membrane and therefore being crucial for its function.

II-C c-6. Characterization of L-methionine transport in *Corynebacterium glutamicum*: identification of the first bacterial methionine excretion system

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In the biotechnological important amino acid producing bacterium *Corynebacterium glutamicum* L-methionine transport was analysed in detail. Both import and export are energy dependent processes and their regulation is crucial for the cell.

Biochemical characterization of methionine uptake in *C. glutamicum* revealed two different systems: a primary transporter with a Km of ~ 0.5 μM and a secondary transporter with a Km of ~ 15 μM. Data base searches using the *E. coli* methionine uptake system MetD as query resulted in the identification of the primary active L-methionine uptake system in *C. glutamicum*. The expression of the corresponding gene cluster was shown to be regulated by the McbR repressor.To characterize methionine export, an L-methionine containing dipeptide loading system was established. The dipeptide is taken up by the cell and hydrolyzed in the cytoplasm. Upon methionine containing dipeptides addition, the internal methionine concentration increased drastically followed by a slower decrease. Induction of an export system was assumed to be responsible for this observation. DNA array experiments revealed two possible open reading frames involved in methionine export, encoding a putative multidrug resistance transporter (MDR) and a putative amino acid exporter. Whereas neither deletion nor overexpression of the MDR system had an influence on the methionine export, deletion of the putative amino acid exporter led to a strong increase of internal methionine. This system was identified as the main methionine export system. The regulation of the corresponding gene depends on the internal methionine concentration.

II-D: Physiology and Pathology of Energy Dissipation

II-D c-1. Central role of the respiratory chain complex 1 in the tuning of oxidative phosphorylation efficiency

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Uncoupling oxidative phosphorylation (ox-phos) affects reactive oxygen species (ROS) homeostasis and cell signaling. Among several mechanisms modulating ox-phos efficiency, the nature of reducing equivalent supply to the respiratory chain (RC) plays an important role. If acetylCoA oxidation in Krebs' cycle leads to three NADH and one FADH2, beta-oxidation produces an equal amount of each whereas glycolysis only leads to NADH. The transfer of reducing equivalents into the matrix is another regulatory step: the electrogenic malate/aspartate shuttle translocates NADH while the glycerol 3-phosphate/dihydroxyacetone phosphate shuttle, a non delta p-dependent pathway, transfers electrons from cytosolic NADH to the matricial quinone pool. Hence, any change in delta p affects the supply of electrons upstream or downstream of complex 1. In addition, the nature of cellular substrates also modulates uncoupling consequence on energy dissipation and ATP synthesis. We have shown that uncoupling intact liver cells with dinitrophenol (DNP) transiently increases oxygen consumption while delta psi and ATP/ADP ratio are collapsed when carbohydrates are exogenous substrate. However, with fatty acids, DNP uncoupling leads to a pronounced and sustained respiration increase while ATP and delta psi are only moderately affected. Hence, uncoupling with carbohydrate leads to a respiratory chain inhibition probably by exhaustion of RC electron supply (NADH), while uncoupling with fatty acid (FADH2) leads to maximal rates of respiration and energy dissipation. The reciprocal regulation of delta p and complex 1 activity is further strengthened by the near-equilibrium state of complex 1 allowing a reverse electron flux from FADH2 to NAD and NADH formation at the expense of delta p. This reverse electron flux affects ox-phos efficiency and ROS homeostasis. This ROS production related to complex 1 reverse electron flux is currently not believed to be physiologically relevant because it is evidenced with sucinate and abolished by ADP addition. However, we recently observed that such ROS production was not abolished by ADP addition when mitochondria were energized by glutamate-malate plus succinate, a more physiological situation. This finding led us to consider that this specific ROS production might represent the sensing system of the forward/reverse flux through complex 1.

II-D c-2. The search for mechanisms underlying thermogenesis in skeletal muscle: beyond uncoupling proteins

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Impairments in glucose and lipid metabolism in skeletal muscle, a hallmark of obesity and type 2 diabetes, have long been attributed to impaired thermogenesis in skeletal muscle. Yet, little is known about the cellular and molecular mechanisms that mediate skeletal muscle thermogenesis in response to key neurohormonal systems (leptin, catecholamines) implicated in the protection of skeletal muscle and other organs/tissues from excessive lipid storage and lipotoxicity. Against this background, this paper first addresses the current debate about whether skeletal muscle UCP3 (a homologue of the uncoupling protein that control mitochondrial proton leak and thermogenesis in brown adipose tissue) can be considered as a physiological mediator of thermogenesis or as a regulator of lipids as fuel sustrate. It then focuses upon emerging evidence from our laboratory that the direct effects of leptin in stimulating thermogenesis in skeletal muscle might be dependent upon substrate cycling between de novo lipogenesis and mitochondrial lipid oxidation. Impairment in signalling pathways that affect this substrate cycle, which links glucose and lipid metabolism to thermogenesis, would lead to impairments in glucose uptake and lipid homeostasis—with implications for skeletal muscle insulin resistance and lipotoxicity.

II-D c-3. Uncoupling protein activation decreases the superoxide anion production in muscle mitochondria submitted to anoxia/reoxygenation in vitro

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A small portion of the oxygen consumed by aerobic cells is converted to superoxide anion at the level of the mitochondrial respiratory chain. This harmful radical can impair cellular structures and functions. Mitochondria have been reported to be damaged in vivo (after ischemia–reperfusion) and in vitro (model of isolated mitochondria anoxia-reoxygenation used to investigate superoxide anion generation together with specific damage at the level of mitochondrial oxidative phosphorylation, Du et al, 1998, Free Radical Biology and Medicine, 25:1066–1074). Superoxide anion or its derivatives are proposed to activate uncoupling proteins (UCPs) and on the other hand activation of uncoupling proteins has been shown to decrease the production of superoxide. Here the in vitro anoxia-reoxygenation model is used with isolated mitochondria containing UCP_3 and 2. Superoxide anion is detected by electron paramagnetic resonance spin trapping with POBN-ethanol. Muscle mitochondria respiring in state 4 are driven to anoxia, then submitted to three and nine cycles of reoxygenation-anoxia in the presence or absence of palmitic acid which activates muscle UCPs. EPR spectra of samples collected after 1, 3 or 9 cycles of anoxia exhibit an increase of the signal without palmitic acid, the latter decreasing the signal in a concentration dependent way. These results suggest that the activation of UCPs by free fatty acids could protect cells from oxygen radical during oxygen deprivation in vivo.

This work was supported by grant of the Belgian Fonds de la Recherche Fondamentale Collective. R.N. was recipient of FRIA fellowship. P.D. is recipient of FRIA fellowship.

II-D c-4. Fatty acid uncoupling and mitochondrial carrier function

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The mitochondrial metabolite transporters form a protein superfamily. They display high substrate specificity and generally operate according to a simultaneous mechanism. Despite this specific carrier mechanism, several members of the family have been shown to switch to a channel/pore mode. This altered carrier function has probably pathophysiological significance like their involvement in the permeability transition pore (PTP).

High fatty acid levels are a common complication of diseases like obesity, diabetes, etc. The lipotoxicity disrupts cell function and several paths could lead to cell death. Lipoapoptosis is often related to a ceramide-dependent pathway but fatty acid oxidation can also increase ROS levels that would trigger apoptosis. Additionally, fatty acid uncoupling could also initiate the apoptotic cascade. Several mitochondrial carriers have been shown to participate in the uncoupling action of fatty acids and it has been proposed that they catalyse the translocation of the fatty acid anion. Fatty acids have recently been shown to induce the classical cyclosporin-sensitive permeability transition (Bernardi P et al. (2002) Vitam Horm 65:97–126). The assay conditions did not require the protonophoric action of fatty acids and thus it must be interpreted as due to the modification of carrier function. We hypothesized that when there exist abnormally high fatty acid levels, mitochondrial carriers would not be involved in the specific translocation of the fatty acid anion but rather the opening of the PTP. This effect would support the notion that mitochondrial carriers act as sensors for the presence of abnormally high levels of dangerous species (ROS, fatty acids, etc.) or critical energetic conditions (low membrane potential, ATP/ADP depletion, high Ca^{2+}, etc.).The uncoupling proteins (UCPs) are members the mitochondrial carrier family that mediate a regulated discharge of the proton gradient generated by the respiratory chain. This energy dissipatory mechanism would serve functions like thermogenesis, lower ROS production, etc. The uncoupling protein from brown fat (UCP1) is activated by fatty acids at nontoxic concentrations hence in agreement with their role as cytosolic second messengers of noradrenaline. This activation would have little in common with the fatty acid effects on other carriers and we have shown that fatty acid transport cannot be the underlying mechanism (Rial E et al. (2004) Biochim Biophys Acta 1608:122–130).

II-D c-5. Growth yield homeostasis in yeast under respiratory conditions: role of the Ras/cAMP pathway

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It is well documented that the growth yield of microorganisms depends on the fraction of ATP utilized for biomass synthesis per se compared to that used for cell maintenance. During aerobic growth, the growth yield may also be a function of the yield of ATP synthesis by oxidative phosphorylation (i.e., ATP/O ratio), a parameter which can vary according to the functional steady state of mitochondria. This steady state is controlled in situ by the adaptation of the energy producing system to energy consumption. In this respect, the enthalpic growth yield of yeast aerobic cultures has been assessed by direct microcalorimetry during the transition from exponential growth to stationary phase. Under these conditions, the ATP turnover largely decreased whereas, the growth yield remained quite constant. We have shown that this steady yield requires a strict dependency between mitochondrial content within a cell and energy demand throughout the transition period. This shows that as long as the cells grow, they are able to adapt their mitochondrial content in such a way that the growth yield is maintained. Under reciprocal conditions, i.e., when one is able to modulate the mitochondrial content, this strict dependency between mitochondrial content and energy demand (growth rate) also applies. This point out a tight regulation of energy demand and energy supply in such a way that the growth yield remains constant in a great variety of physiological situations. The Ras/cAMP/PKA signaling pathway is known to regulate adaptations of yeast cells upon environmental and metabolic transitions. We have shown that this pathway is involved in the regulation of this process. Overactivation of this pathway can lead to situations where the relationship between growth rate and mitochondrial amount is modified. This leads to a decrease in growth yield.

II-D c-6. The yeast mitochondrial unspecific channel as a possible regulator of oxidative phosphorylation

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Mitochondrial permeability transition (PT) is an ill understood phenomenon. In mammalians, PT does trigger cellular death, both as a program, (apoptosis) or as a consequence of ischemia–reperfusion. In yeast however, programmed death does not seem to operate. Even hypoxia is not a problem as the organism is facultatively aerobic. Thus, the yeast mitochondrial unspecific channel (YMUC) seems to be an ideal model to study the possible alternative function(s) of PT, other than death. The opening of the YMUC was regulated by Calcium or Magnessium or by two organic alkylguanidines (octyl-guanidine (OG) and hexyl-guanidine (HG)) (Pérez-Vázquez et al., (2003) J Bioenerg Biomemb, 35:231–240). The rate of oxygen consumption in state 3 and in state 4, the transmembrane potential and the rate of mitochondrial swelling were used to monitor the state of the YMUC. The presence of 0.4 mM phosphate was nos sufficient to close the YMUC, however, it did increase the sensitivity to calcium (The I50 decreased from 50 to 0.3 mM) and to magnessium (I50 decreased from 5 to 0.83 mM). The calcium concentration needed to close the YMUC was higher than the concentrations usually found in the cell. Nonetheless, the additive effects of calcium with magnesium and phosphate suggest that these may act in concert to control the opening of the YMUC in the cell. The possible physiological meaning of this control is to be determined. It does seem of interest that calcium is very high during the cellular cycle or whenever yeast mate, times at which a tighlty coupled mitochondrion is needed to produce large amounts of ATP. In regard to the reversibility of the Calcium promoted closing of the YMUC, the effect of the hydrophobic cations octyl-guanidine and hexyl-guanidine were explored. Low concentrations fo both OG (12.5 μM) and HG (25 μM) closed the YMUC. In addition, when the reversibility of closing was assayed by exposing swollen mitochondria to polyethylen-glycols of different MW, the effect of OG on the YMUC was still present inhibiting recontraction, while Ca did release the YMUC allowing its opening. OG seems to be an interesting tool for the study of both the PTP and the YMUC.

II-E: Reactive Nitrogen Species and Reactive Oxygen Species in Mitochondria

II-E c-1. Cellular metabolic responses to no-mediated mitochondrial inhibition

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Nitric oxide (NO) inhibits cytochrome *c* oxidase (1) in a reversible manner (2) by competing with oxygen (3,4). Despite the mechanism and potential pathological consequences of such effect has been fully documented (5), there is no experimental evidence showing the physiological role of such a bioenergetic interaction at the cellular level. Here, we present recent evidence (6) strongly suggesting that the reversible inhibition of mitochondrial respiration by endogenous or exogenous NO can result in a rapid activation of the glycolytic pathway in rat astrocytes in primary culture. The mechanism responsible for this effect was cyclic GMP independent and involved NO-dependent stimulation of AMP-activated protein kinase (AMPK), as judged by RNA interference approach. The activation of AMPK resulted in the increase in fructose-2,6-bisphosphate, i.e., the most potent positive effector of phosphofructokinase-1. In fact, allosteric activation of this regulatory enzyme was responsible for the increased glycolytic flux initially triggered by NO. From these results, we suggest that a physiological role of NO-mediated inhibition of mitochondrial respiration would be the modulation of glycolysis through a cell signalling pathway involving AMPK.

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Funded by MCYT (SAF2001-1961), JCYL (SA081/04; SA020/02) and FIS (03/1055).

II-E c-2. Mitochondria and reactive oxygen species in programmed death phenomena

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(1) Many kinds of apoptotic signals are amplified by mitochondria and mitochondria-generated reactive oxygen species (ROS). Moreover, such signals can be produced by mitochondria themselves, resulting in opening of the permeability transition pore (PTP) and in release of the ‘‘death proteins’’ hidden in the intermembrane space. PTP opening (i) can be a result of ROS production and (ii) can increase the ROS level due to exhaustion of mitochondrial antioxidants. (2) Long-lived PTP entail ‘‘a suicide’’ of mitochondrion (mitoptosis), dead mitochondria being eliminated by autophagosomes. Alternative mechanism of mitoptosis was recently discovered in our group. After treating cell culture by uncoupler and respiratory chain inhibitor, mitochondria (which under such conditions cannot form ATP but can hydrolyze it) were found to be concentrated in some regions of cytosol, surrounded by a membrane and then exiled from the cell. (3) To study collective apoptosis, our group has developed a new method of confronting cell cultures. It was found that staurosporine- or TNF-treated cells sent a death signal to the nontreated cells. The signal in question proved to be mitochondrially produced hydrogen peroxide since (i) the treated cells release hydrogen peroxide, maintaining its concentration in the medium at level of several microM, (ii) the cell-to-cell signal transmission was interrupted by added catalase or by mitochondria-targeted antioxidant MitoQ, which, according to M. Murphy and coworkers, blocks the hydrogen peroxide-induced apoptosis.

II-E c-3. The existence of a mitochondrial nitrite-reductase

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Nitric monoxide (NO) exerts a great variety of physiological functions. L-Arginine supplies amino groups which are transformed to NO in various NO-synthase-active isoenzyme complexes. NO-synthesis is stimulated under various conditions causing accumulation of stable NO-metabolites in the tissue. The major oxidation product found is nitrite. Elevated nitrite levels were reported to exist in a variety of diseases including HIV, reperfusion injury and hpovolemic shock.Denitrifying bacteria such as *Paracoccus denitrificans* have a set of periplasmatic cytochromes (cyt cd1, cyt bc) which were shown to be involved in nitrite reduction activities. Mammalian mitochondria have similar cytochromes which form part of the respiratory chain. Like in bacteria quinols are used as reductants of these types of cytochromes. The observation of one-electron divergence from this redox-couple to external dioxygen made us to study whether this site of the respiratory chain may also recycle nitrite back to its bioactive form NO. Thus, the aim of the present study was to confirm the existence of a reductive pathway which reestablishes the existence of the bioregulator NO from its main metabolite nitrite. Our resuslts show that respiring mitochodnria readily reduce added nitrite to NO which was made visible by nitrosylation of deoxyhemoglobin. The adduct gives characteristic triplet-ESR-signals. Using inhibitors of the respiratory chain for chemical sequestration of respiratory segments we were able to identify the site where nitrite is reduced. The results confirm the ubiquinone/cyt bc1 couple as the reductant site where nitrite is recycled. The high affinity of NO to the heme-iron of cytochrome oxidase causes an impairment of mitochondrial energy-linked respiration however, under physiological oxygen concentration the inhibition was neglectible [Kozlov, A.V.,Staniek, K. and Nohl, H. (1999) FEBS Lett. 454, 127–130]. The major significance of mitochondria for recycling NO from nitrite was recently shown by the release of NO from NO-donors such as glycerol trinitrate (GTN). GTN first releases nitrite which requires heart mitochondria to be tranformed to NO [Kozlov, A.V., Dietrich, B. and Nohl, H. (2003), British J. Pharmacol. 139, 989–997].

II-E c-4. Nitric oxide and cytochrome *c* oxidase

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Cell respiration is inhibited reversibly by nitric oxide (NO), and the reaction may be of pathophysiological relevance. Cytochrome *c* oxidase (CcOX) is the terminal acceptor of the mitochondrial respiratory chain. NO acts as potent, rapid and reversible inhibitor of CcOX by reacting, in competition with oxygen, with the heme a_3-Cu_B site of CcOX (1). NO reacts with the reduced cytochrome a_3^{2+} or the oxidised Cu_B forming, respectively, the nitrosyl-[a_3^{2+}-NO] or the nitrite-[a_3^{3+}-NO_2^-] bound derivative of the enzyme (2). Both inhibited states, although with different kinetics, recover full activity by dissociation of either NO or nitrite from the active site.We have shown in vitro as in coltured cells that both adducts can be detected, although to a different extent depending on conditions, and particularly on the concentration of reduced cytochrome *c* (2,3). Consistently, a theoretical model predicts that the nitrosyl-based mechanism prevails at high electron flux through the respiratory chain, whereas the nitrite-based one under low fluxes. Experiments carried out in air-saturated buffer with coupled mitochondria, indicates that both state III and state IV mitochondria follow predominantly the nitrite pathway, although state III mitochondria sometimes appeared to accumulate the nitrosyl adduct. Interestingly, it has been also suggested that at high electron flux, upon reversal of inhibition, NO is not released into the bulk as such, but as nitrite via transient formation of $ONOO^-$ in the active site of the enzyme (4).

Acknowledgements: The Ministero dell' Istruzione Università e Ricerca (MIUR) of Italy (PRIN ''Bioenergetica'' and FIRB, RBAU01F2BJ_001) and the Fondazione Italiana Sclerosi Multipla are gratefully acknowledged.

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II-E c-5. Induction of reactive oxygen species production in complex I of mitochodrial respiratory chain by photodynamic treatment of hela cells

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It is well known that the process of programmed cell death, or apoptosis take part in other human diseases including cancer and neurodegenerative disorders. Many such disorders can caused by formation of reactive oxygen species (ROS) in mitochondria, which appears first by leakage of electrons from complexes I and III of respiratory chain to molecular oxygen and formation of superoxide anion radical (O_2^-). With the recognition of the central role of mitochondria and ROS in apoptosis, there is a need to develop of novel tools to investigate selectively mitochondrial ROS. Some of human diseases, according with excessive cell division and formation of malignant cells can be treated by photoactivation of photosensitizers, which lead to local flash of reactive oxygen species. We examined ROS production in HeLa cells induced by photoactivation of Mitotracker Red, a photosensitiser, which selectively accumulates in mitochondria and lead to production of singlet oxygen $^1O_2$ (another reactive form). In our experiments we loaded HeLa cells by MitotrackerRed and DCFH-DA (2′ ,7′ -dihydrodiclorofluoresceindiacetate), a fluorescent probe, which accumulates in cells and fluoresce when it oxidized by ROS. For ROS detection we use a fluorescent microscopy and Scion Image (software for quantitative assessment of fluorescence). We detect the ROS accumulation by estimating intensity of DCF fluorescence after photoactivation of Mitotracker Red through a certain intervals. In control cells (loaded by only mitotracker and DCF) we observe a very modest increasing of DCF fluorescence, but in cells, loaded by rotenone or myxothiazol (inhibitors of complex I or III of respiratory chain, respectively) in addition to previous probes, DCF fluorescence grows more rapidly than in control cells. Thus we believe that we detect ROS make from complex I of respiratory chain. This higher ROS production was fully prevented by pretreatment of HeLa cells with antioxidant *N*-acetylcysteine (NAC) or DPI. Oligomycin, a inhibitor of FO-subunit of H^+-ATPase had no effect for this ROS production.

II-E c-6. P66SHC is a signal transduction red-ox enzyme

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P66Shc is the first gene identified in mammals whose mutation prolongs life span.P66Shc is a splice variant of p52Shc/p46Shc, a cytoplasmic signal transducer involved in the transmission of mitogenic signals from tyrosine kinases to Ras. P66Shc has the same modular structure of p52Shc/p46Shc and contains a unique N-terminal region; however, it is not involved in Ras regulation but rather functions in the intracellular pathway that converts stress signals into apoptosis. Indeed, p66Shc deletion in mice protects cells and tissues form stress-induced apoptosis. Moreover, p66Shc is crucial for the execution of p53-dependent apoptosis; as revealed by the finding that overexpression of p53 is not able to trigger apoptosis in the absence of p66Shc.A fraction of p66Shc localizes within mitochondria, where, following proapoptotic stimuli, it induces the collapse of the trans-membrane potential, triggers mitochondrial permeability transition and, consequently, the release of cytochrome *c*. Another functional property of p66shc is the ability to increase the intracellular concentration of reactive oxygen species, thus producing oxidative stress.

We have recently found that p66Shc executes electron transfer with the cytochrome *c* and generates hydrogen peroxide by the mitochondrial electron transfer chain. Through this mechanism, p66Shc regulates mitochondrial swelling and apoptosis.

The role of p66Shc-generated H_2O_2 as signaling molecule for apoptosis and other intracellular functions is discussed.

Keywords: P66Shc; Cytochrome *c*; Electron transfer; Reactive oxygen species; Apoptosis; Signaling

II-E c-7. Feedback regulation of oxidative stress: fatty acid hydroperoxides are cycling substrates of mitochondrial uncoupling protein UCP2

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Mitochondria are major source of the reactive oxygen species (ROS). Superoxide (O_2^-) as a primary product is formed at sites of Complexes I and III. It can be rapidly dismuted to H_2O_2. At pH 6.8, about 1% of O_2^- is hydrated to hydroperoxyl radical $HO_2^{\cdot}$ (pKa 4.8). H_2O_2 may be converted into hydroxyl radical OH; by Fenton reaction in the presence of Fe^{2+}. It is not superoxide, but HO_2 and $OH^{\cdot}$ (diffusing max. 2 nm, i.e., ~ half of a lipid bilayer) which attack mtDNA, proteins, and initiate lipoperoxidation. Fatty acid hydroperoxides (FAOOH) belong to major lipoperoxidation intermediates. If lipid side chains are cleaved off by the oxidative-stress induced mitochondrial phospholipase A2, released FAOOH may activate function of mitochondrial uncoupling protein-2 (UCP_2) (becoming UCP_2 substrates), which was proposed to decrease mitochondrial ROS production. Such mechanism would provide a feedback downregulation of the oxidative stress.Fatty acids (FA) were previously found to induce protonophoric function of UCP_2 by a cycling mechanism: FA^- anions are conducted by UCP_2, while protonated FA flips across the lipid bilayer and carries H^+. Assuming this mechanism for FAOOH, we studied the ability of UCP_2 to interact with synthesized linoleic acid hydroperoxides (LAOOH, mix of four isomers). We report that LAOOH causes rapid flip-flop-dependent acidification of liposomes, comparable to linoleic acid (LA)-dependent acidification, hence is able to carry H^+ across the membrane. Using *E. coli*-expressed UCP_2 reconstituted in liposomes we found that LAOOH also induced GDP-sensitive H^+ uniport with approximately threefold higher affinity than LA. LAOOH also initiated GDP-sensitive charge compensating K^+ influx, indicating the existence of $LA(OOH)COO^-$ uniport mediated by UCP_2. For this K^+ influx, similar differences in affinities were found when comparing $LA(OOH)COO^-$ and LA anion flux. These data clearly indicate that FA hydroperoxide anions, like other FA, are transported by a flipase mechanism mediated by UCP_2, leading to fatty acid cycling and uncoupling. The results point out that the proposed activation of UCP_2 by superoxide (O_2^-) (Murphy, M.P., et al. (2003) J. Biol. Chem. 278, 48534–48545) may be due to uncoupling by FAOOH. FAOOH thus promote feedback downregulation of ROS production in mitochondria. (Supported by grants A5011106 to PJ, 1K03002 and 204/04/0495 to MJ; NIH grants TW01487 to PJ and KDG; DK56273 to KDG).

III-A: Mitochondria in Aging and Degenerative Diseases

III-A c-1. Testing the mitochondrial theory of ageing

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Aging is often described as an extremely complex process affecting all of the vital parameters of an individual. Abundant evidence implicates mitochondria in aging and we focus on the three main components of the mitochondrial theory of aging: (i) increased reactive oxygen species (ROS) production, (ii) mitochondrial DNA (mtDNA) damage accumulation and (iii) progressive respiratory chain dysfunction. Experimental evidence shows a relationship between respiratory chain dysfunction, ROS damage and aging in most of the model organisms. Point mutations and deletions of mitochondrial DNA accumulate in a variety of tissues during ageing in humans. The possible causative effect of these mitochondrial DNA mutations has been intensely debated because of their low abundance and purely correlative connection with ageing. We have now addressed this question experimentally by creating homozygous knock-in mice expressing a proofreading-deficient version of the nucleus-encoded catalytic subunit of mitochondrial DNA polymerase. These mice develop a mitochondrial DNA mutator phenotype with a 3–5-fold increase in levels of point mutations as well as increased levels of aberrant (deleted) mitochondrial DNA. This increase in somatic mitochondrial DNA mutations leads to a reduced life span and induces premature onset of phenotypes associated with ageing.

III-A c-2. Bioenergetics shapes cellular death pathways in Leber's Hereditary Optic Neuropathy (LHON): a model of mitochondrial neurodegeneration

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Leber's hereditary optic neuropathy (LHON) was the first maternally inherited disease to be associated with point mutations in mitochondrial DNA and is now considered the most prevalent mitochondrial disorder. The pathology is characterized by selective loss of ganglion cells in the retina leading to central vision loss and optic atrophy, prevalently in young males. The pathogenic mtDNA point mutations for LHON affect complex I with the double effect of lowering the ATP synthesis driven by complex I substrates and increasing oxidative stress chronically.Our studies of LHON cybrids in glucose medium show that LHON mutations severely reduce ATP synthesis driven by complex I substrates, whereas complex II-dependent ATP synthesis is not significantly affected. However, the defective ATP synthesis observed with complex I substrates was reflected in a normal or only slightly reduced cellular content of total ATP indicating a compensatory mechanism, at least in cultured cells. In galactose medium, while the ATP content of control cybrids did not change, the LHON cybrids suffered a profound depletion with time and underwent a rapid increase of cell death, which was apoptotic by various criteria such as chromatin condensation, DNA laddering and cytochrome *c* release. However, caspase-3 was not activated and mitochondrial EndoG and AIF were released into the cytosol. Thus, a caspase-independent pathway of cellular death is suggested, which may depend on the rapid ATP depletion suffered by LHON cybrids incubated in galactose. The parallel marked changes in antioxidant enzymes found during the time-course of galactose experiments, also reveal a relevant role played by oxidative stress.The LHON cybrid model sheds light on the complex interplay amongst the different levels of biochemical consequences deriving from complex I mutations in determining neurodegeneration in LHON and suggests an unsuspected role of bioenergetics in shaping cell death pathways.

Acknowledgements. This work was supported by Telethon Fondazione Onlus Italy (grant n. GGP02323) to V.C.

III-A c-3. Calcium sensitivity of complex I-dependent respiration and of permeability transition in skeletal muscle mitochondria of huntington disease 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 huntingtin (htt) protein. Several lines of evidence suggest that energy metabolism is impaired in HD, although its role in pathogenesis remains uncertain. Alterations in energy metabolism were found in the brain and in muscle of HD patients. In addition, in purified mitochondria from HD patients and from brains of a transgenic mouse model expressing full-length htt showed that these mitochondria depolarize to unusual small amounts of Ca^{++} (Panov et al., Nat. Neurosci. 2002). We therefore investigated mitochondrial function in skeletal muscle of the widely used transgenic R6/2 mouse model for HD, expressing exon 1 of htt with an elongated polyglutamine stretch in comparison to the wild type animals at different Ca^{++} concentrations. The incubation medium for isolated mitochondria and for skinned muscle fibers contained 75 mM mannitol, 25 mM sucrose, 100 mM KCl, 10 mM KH_2PO_4, 0.5 mM EDTA, 5 mM $MgCl_2$, 20 mM Tris−HCl, pH = 7.4 and 0, 25 or 100 μM $CaCl_2$. Without Ca^{++} the respiratory rates of skeletal mitochondria in HD mices were under state 3 conditions with pyruvate/malate 67 ± 21 and with succinate 36 ± 12 nmol O_2/min/ mg fiber. Succinate respiration scaled pyruvate respiration (SRPR) was 190 ± 30%. The RCI for pyruvate as substrate was 5.8 ± 1.3 and the leak respiration was 8.8 ± 3.3 nmol O_2/min/ mg fiber. These data were not different from the corresponding data of WT animals. With increasing Ca^{++}, however, the pyruvate dependent state 3 respiration was remarkably inhibited in HD mices whereas the succinate dependent respiration remained more or less constant as indicated by the decreasing SRPR 182, 66, and 39 in the presence of 0, 25 and 100 μM Ca^{++}, respectively. In WT animals the corresponding SRPR were 195, 175 and 146 due to a significantly less pronounced inhibition of pyruvate respiration by Ca^{++}. With swelling measurements it was shown, that in isolated HD mitochondria the rate of permeability transition (PT) is much higher than in WT mitochondria for both substrates pyruvate/malate and for succinate.

It is concluded that in skeletal muscle of HD mices those mitochondrial properties are altered which have relations to Ca^{++} signalling an to PT. Data further support the point of view, that HD is not only a disease of CNS but that at least the skeletal muscle is affected too.

III-A c-4. The yeast NADH dehydrogenase as a therapeutic agent for complex I defects

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Mitochondria of *Saccharomyces cerevisiae* lack complex I but instead have the rotenone-insensitive NADH dehydrogenase (Ndi1). The Ndi1 enzyme is composed of a single polypeptide, does not pump protons, and contains noncovalently bound FAD and no iron–sulfur clusters. We have previously shown that the NDI1 gene encoding the Ndi1 protein can be functionally expressed in two complex I-deficient mutant cells and that the Ndi1 enzyme was capable of compensating respiratory deficiencies caused by defects in the host complex I. To extend the potential use of this enzyme to repair complex I deficiencies in vivo, we constructed a recombinant AAV vector carrying the NDI1 gene (rAAV-NDI1). With rAAV-NDI1 as the gene delivery method, we were able to achieve high transduction efficiencies (nearly 100%) even in nonproliferating cells.

After successful introduction of Ndi1 into cultured cells, we have started a project to deliver the NDI1 gene in vivo. We carried out injections of rAAV-NDI1 into muscles and brains of mice and rats. In the case of muscles, expression of the Ndi1 protein was observed around the injection sites as early as 2 weeks postinjection. The expression levels were apparently higher in the type I (mitochondria-rich) cells than in the type II cells. In brains, we stereotaxically injected rAAV-NDI1 in the substantia nigra and the striatum. All injections were unilateral. Again, the Ndi1 protein was clearly identified by anti-Ndi1 antibody after 2–3 weeks of injection only on the ipsilateral side. The expressed Ndi1 seemed to be localized to mitochondria in all tissues tested. It is, however, important to determine whether the expressed Ndi1 is functionally active. Therefore, NADH dehydrogenase activity was assessed in tissue sections by use of NADH and tetrazolium dye as the substrates. Dense, blue/purple spots were visible in the areas or cells in which Ndi1 was immunochemically identified. We conclude that the Ndi1 enzyme expressed in vivo is functionally active. We have also examined inflammatory response possibly caused by the injection of rAAV-NDI1. So far, hematoxyline/eosin staining of the tissues showed negative results, indicating no significant inflammatory incidents in the injected area.

All data suggest that the Ndi1 enzyme has a great potential as a molecular remedy for complex I defects including sporadic Parkinson's disease.

III-A c-5. Alterations of mitochondrial function in epilepsy and neurodegenerative diseases

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The impairment of mitochondrial function in brain has been reported for several neurogenerative diseases including amyotrophic lateral sclerosis, Alzheimers disease and Parkinson's disease. We have investigated putative alterations of neuronal energy metabolism in the hippocampus of patients with temporal lobe epilepsy and an animal model of temporal lobe epilepsy-in pilocarpine-treated chronic epileptic rats. In hippocampal samples of patients with temporal lobe epilepsy associated with Ammon's horn sclerosis we observed selectively in hippocampal CA3 subfield decreased citrate synthase normalised activities of the respiratory chain complexes I and IV and of the tricarboxylic acid cycle enzyme aconitase. Similar findings were obtained in the hippocampus of pilocarpine-treated chronic epileptic rats. These alterations of respiratory chain function in epilepsy can be attributed—as previously described for amyotrophic lateral sclerosis—to decreased copy numbers of mitochondrial DNA. This finding and the decreased activities of the highly oxygen radical-sensitive enzyme aconitase point to an important role of oxidative stress in the development of neurodegeneration-related dysfunction of mitochondrial energy metabolism. A vicious cycle of oxygen radical-induced damage of mitochondria which stimulates further oxygen radical production by mitochondria could be one of the factors promoting neuronal cell death. To test this hypothesis we performed detailed in vitro studies of superoxide and H_2O_2 production of isolated rat and human brain mitochondria. We observed that most of superoxide radicals are produced by complex I and that a high production of reactive oxygen species is a feature of respiratory chain-inhibited mitochondria or of reversed electron flow. Furthermore, our results indicate that the site of superoxide generation at complex I is the flavine mononucleotide moiety. Short-term incubation of rat brain mitochondria with H_2O_2 in the lower mM range induced at almost unchanged mitochondrial intactness increased superoxide production at this site. Therefore, we propose that in pathological states of the brain reactive oxygen species activate a self-accelerating vicious cycle leading to mitochondrial damage and neuronal cell death.

III-A c-6. Collagen VI muscular dystrophies from animal models to human therapy: (I) mitochondrial bioenergetics and mechanisms of apoptosis in myoblast cultures from Ullrich patients

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Collagen VI (CVI) is an ECM protein forming a microfilamentous network with a broad distribution in several organs, including skeletal muscle. Inherited mutations of COL6 genes in humans cause two forms of muscular dystrophies: Bethlem myopathy (BM) and Ullrich scleroatonic muscular dystrophy (UCMD). The pathogenic mechanisms involved in BM and UCMD are still unknown, and there is currently no effective treatment for patients affected by these disorders. To gain insight into the function of CVI in muscle, we generated knockout mice by targeted inactivation of the Col_6a_1 gene. $Col_6a_1 -/-$ mice lack CVI and they have an early onset myopathic phenotype strongly resembling human CVI disorders (1). A similar, although milder, myopathic phenotype is present in $Col_6a_1 +/-$ (heterozygous) mice, which have half the amount of CVI due to gene haploinsufficiency (1). By studying skeletal muscle fibers isolated from $Col_6a_1 -/-$ mice, we discovered a latent mitochondrial dysfunction due to increased opening of the permeability transition pore (PTP) (2). We have been able to show that lack of CVI is the cause of increased PTP opening, and that this mitochondrial event sets in motion the executioner mechanism of apoptosis in vitro and in vivo. The ability of cyclosporin A (an inhibitor of PTP opening) to rescue myopathic alterations of $Col_6a_1 -/-$ mice both in vitro and in vivo (2) suggests a pharmacological strategy to treat patients affected by congenital CVI deficiency. The key question is to what extent the pathogenesis of human CVI disorders coincides with that we have demonstrated in $Col_6a_1 -/-$ mice. Preliminary results indicate a) that the apoptotic rate of myoblast cultures established from two UCMD and one BM patient is higher than that of cultures from control donors; and b) that cyclosporin A is able to prevent the increase of nuclear apoptosis.

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III-B: Biogenesis of Energy Transducing Systems

III-B c-1. Structure, function and biogenesis of the cytochrome bc_1 complex in bacteria

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The cytochrome bc_1 complex is an important, membrane associated, energy transducing enzyme that is involved in major metabolic pathways such as respiration and photosynthesis. Currently, our understanding of its structure and function is well advanced, but that of its biogenesis is only now emerging. The bacterial enzyme is the simplest among the members of this family of protein complexes, and it has an intertwined dimeric structure, with each monomer constituted of three distinct subunits. These subunits are the cytochrome c_1 that is anchored to the membrane by a carboxyl-terminal helix and that carries a c-type heme covalently attached to the apocytochrome; the cytochrome *b* that is constituted of eight trans membrane helices axially coordinating two b-type hemes; and the iron–sulfur (Rieske) protein subunit that is anchored to the membrane with an amino-terminal helix and that bears a high redox midpoint potential [2Fe–2S] cluster. During the cytochrome bc_1 maturation, each subunit acquires its specific cofactor via a specific cellular pathway, and follows a distinct protein translocation route to integrate itself into the membrane. Then, an apparently ordered process occurs to stabilize these subunits and to assemble them into a functional dimeric enzyme. Here, the cellular processes involved in cytochrome bc_1 biogenesis will be reviewed, and better known steps and components of c-type cytochrome maturation required for cyt c1 subunit, and of periplasmic redox homeostasis required for the catalysis of critical disulfide bonds within the enzyme will be described using *Rhodobacter capsulatus* as a model organism.

III-B c-2. Role of oxa1p in the assembly of mitochondrial respiratory complexes

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The assembly of the respiratory complexes is an intricate process that requires the participation of nuclear encoded factors that are not intrinsic components of the complexes. One of these factors, Oxa1p, is an inner-membrane protein conserved from bacteria to eukaryotic organelles, which seems essential for the cotranslational membrane insertion of respiratory complex subunits. In yeast mitochondria, the absence of Oxa1p leads to a complete loss of respiration and is associated with a rapid degradation of several membrane subunits of the Cox, ATPase and bc1 complexes. Oxa1p can be divided into three domains conserved in all Oxa1p homologues suggesting that this structural organization is crucial for its function: a central hydrophobic domain, a hydrophilic intermembrane space domain, and a hydrophilic matrix. This matrix domain includes the C-terminal tail that is thought to interact with mitochondrial ribosomes.

In an effort to systematically examine how the Oxa1p structure relates to its function(s) and interaction(s) with its substrate(s), we have introduced point mutations and large deletions into the various domains of the protein. These mutants allowed us to show the importance of the first TM domain, as well as a synergetic interaction between the first loop and the C-terminal tail that both protrude into the matrix. We have also isolated genetic and multicopy suppressors that can compensate for the respiratory deficiency of various oxa1 mutants. We found that single mutations that introduce a positive charge into the TM domains of two subunits of the bc1 complex, cytochrome c_1 or Qcr9p, can compensate for the absence of Oxa1p. This bypass of Oxa1p by mutated TM domains suggests an important role for the transmembrane domain of Oxa1p in the process of respiratory subunit membrane insertion. We have also characterized a high copy number suppressor, OMS1, which encodes a member of the methyl-tranferase family. We show that Oms1p is a mitochondrial inner membrane protein inserted independently of Oxa1p. Oms1p presents one putative TM segment and its C-terminal domain carrying the methyl-transferase-like domain is located in the intermembrane space. Overexpression of OMS1 appears to stabilize both mutant and wild type Oxa1p. We will discuss the possible role of Oms1p on the intermembrane space domain of Oxa1p.

III-B c-3. Complex I assembly in human cells in health and disease

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The 46 subunit containing complex I (CI) is the largest complex of the oxidative phosphorylation (OXPHOS) system. The assembly of this complex is still poorly understood. Insight in the assembly will provide information about the nature of many unexplained CI deficiencies and might reveal the possible disease mechanisms. Therefore we studied the assembly of complex I in human cells in which complex I assembly was temporally blocked. After releasing this block, CI assembly resumes and assembly intermediates can be observed. We can distinguish three or possibly four distinct preassembled subcomplexes suggesting that complex I assembly is a semisequential process (subcomplexes are formed which are joined together to form a fully assembled complex). The complex I assembly factor CIA30 is not part of holo-complex I but it appears associated with at least the ND1 containing subcomplex. Moreover, this assembly factor is altered in expression and association in a patient with complex I deficiency.

We show an important decrease in the levels of intact complex I in 15 patients harboring mutations in nuclear-encoded complex I subunits, indicating that complex I assembly and/or stability is compromised. Different patterns of low molecular weight subcomplexes are present in these patients, suggesting that the formation of the peripheral arm is affected at an early assembly stage. Interestingly mutations in complex I genes can also affect the stability of other mitochondrial complexes, with a specific decrease of fully assembled complex III in patients with mutations in NDUFS2 and NDUFS4. This study improves our understanding of the molecular basis of complex I deficiency and aids to the selection of candidate genes for pathogenic mutations.

III-B c-4. Upregulation of mitochondrial dna synthesis and of oxidative phosphorylation complex biogenesis by the von Hippel Lindau factor

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The von Hippel Lindau factor is a tumor suppressor gene, the invalidation of which can cause cancers such as the clear cell renal carcinoma (CCRC). The protein pVHL is part of a complex that catalyses ubiquitination of hydroxylated alpha subunit of the hypoxia-inducible factor (HIF), which leads to its degradation by the proteasome. In cells lacking pVHL or in the absence of oxygen, HIF is stabilized and activates transcription of genes implied in oxygen homeostasis, glycolysis, regulation of iron metabolism and cell cycle [1]. Although no direct role of HIF has yet been demonstrated on the expression of genes encoding mitochondrial proteins, low rates of electron transfer and poorly coupled ATP synthesis are observed in CCRC [2], as in many other aggressive types of cancers [3]. Previous studies had shown that the transfection of the vhl gene in vhl-deficient cells originating from CCRC patients could suppress their ability to form tumors when they were injected to nude mice. In the present work, we show that pVHL increased contents of mtDNA and of respiratory chain complexes as well as their function and permitted the cells to rely on their mitochondrial ATP production to grow in the absence of glucose. The levels of mitochondrial transcription factor, Tfam, or of the redox-responsive nuclear transcription factors NRF1 or NRF2 mRNA known to be involved in OXPHOS complex biogenesis were not significantly modified by vhl transfection. Moreover, the lack of vhl-induced increase of COX IV, COX Vb or COX VIb mRNAs indicates that the nuclear-encoded COX subunit transcription program was not regulated by vhl. However, vhl-transfection increased the Tfam protein content in parallel to that of mtDNA, as well as those of OXPHOS subunits. These data suggest a new direct or indirect pVHL-dependent regulation of mitochondrial biogenesis, likely involving posttranscriptional induced mechanisms.

III-B c-5. Cytochrome oxidase subunit 2 mutations and their effect on copper transfer

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The respiratory chain enzyme cytochrome oxidase is composed of 13 subunits. The three core catalytic subunits (1, 2 and 3) are encoded by the mitochondrial genome while the remainder are nuclear-encoded. The subunits assemble in a complex process to form a functional dimer in the inner mitochondrial membrane. Cytochrome oxidase also contains several prosthetic groups, which are thought to be inserted at intermediate stages of assembly.

We are studying the effect of mutations in subunit 2 on assembly of cytochrome oxidase. Subunit 2 coordinates Cu ions to form the initial acceptor of electrons from cytochrome *c*. The mutations R159K and G156E, in the C-terminal extramembrane region of subunit 2, impair assembly and reduce catalytic activity. Suppressor mutations in the subunit 2 Cu-binding region restore assembly and activity of the enzyme, although R159K and G156E are not spatially close to this site.

Characterisation of the R159K mutant reveals a behaviour similar to that of mutants of Sco1p and Cox17p, required for transfer of Cu to cytochrome oxidase. We hypothesise that R159K impairs interaction of Sco1 with the enzyme, preventing efficient Cu transfer, and that the suppressor mutations increase efficiency of Cu binding.

III-B c-6. Yeast mitochondrial biogenesis is regulated via the camp protein kinase TPK3

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In most organisms, the cellular mitochondrial content is modulated by environmental stimuli and/or in response to energy demand changes. Several cAMP targets and transcription factors seem to be involved in the upmodulation of mitochondrial biogenesis when energy demand increases. In the yeast *Saccharomyces cerevisiae*, the Ras/cAMP/protein kinase pathway is involved in many physiological adaptations of cells upon environmental changes. Among the adaptation processes occurring during the transition between exponential growth to stationary phase, the downmodulation of the cytochrome content and of the respiratory activity of yeast cells plays a role in the maintenance of high growth yield. The question is therefore raised as to the role of the Ras/cAMP-protein kinase A pathway in this adaptative process. We have previously shown that mutant strains exhibiting an increased or decreased activity of this pathway have a coordinated change in mitochondrial enzyme equipment. The question thus arose as to whether a specific kinase might be involved in this process. We show that out of the three cAMP protein kinases in yeast, TPK3 is the one involved in mitochondrial biogenesis. Indeed, when grown on nonfermentescible carbon source TPK3- cells have a significantly decreased amount of mitochondria. Respiratory rates, enzymatic equipment as well as mitochondrial DNA amount are decreased in this strain. Moreover, isolated mitochondria from the TPK3- strain display distinct energetic features when compared to the wild type, pointing out a role of Tpk3p in the biogenesis of the mitochondrial respiratory chain.

Posters

I-A: F-Type ATP Synthases

I-A p-1. Amino acid substitutions at contact sites of alpha and beta subunits close to catalytic sites in the TF_1-ATPase: one set affects stability and another affects cooperativity between catalytic sites

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In crystal structures of MF_1, the guanidinium of betaR191 is within 3 Å of the carboxylate of alphaD347 in all alpha/beta pairs. The alpha3beta3gamma (a3b3g) subcomplexes of TF_1 containing the aD347N, aD347E and bR191K single substitutions and the aD347E/bR191K, aD347R/bR191D and aD347R/bR191E double substitutions have been characterized (MF_1 residue numbers are used throughout). Whereas the aD347E mutant has 60% of wild-type ATPase activity and partly dissociates during native PAGE, the aD347N mutant is inactive and completely dissociates during native PAGE. In contrast, the bR191K mutant has the same activity as wild-type and stays intact during native PAGE. The aD347E/bR191K mutant has 60% of wild-type activity and partly dissociates during native PAGE. However, the aD347R/bR191D and aD347R/bR191E mutants are inactive and completely dissociate during native PAGE. Thus, interaction of the guanidinium of bR191 with the carboxylate of aD347 is required for stability and optimal activity of the TF_1 subcomplex.

It is well-known that the aS370F mutant of EF_1 hydrolyzes substoichiometric ATP, but lacks multisite ATPase activity, indicating that the substitution affects cooperativity between catalytic sites. In the crystal structure of MF_1 containing MgADP-fluoroaluminates bound in two closed catalytic sites, the guanidinium of bR337 is 2.9 Å from the carbonyl oxygen of aS370 and 3.7 Å from a methyl group of aV371 in the half-closed catalytic site containing MgADP and sulfate. To test the possibility that the bR337/aS370 contact might be transient during multisite ATP hydrolysis, the aV371C, bR337C and aV371C/bR337C mutants of TF_1 have been characterized. Both single mutants have 50% of wild-type ATPase activity. The reduced double mutant has 30% of wild-type ATPase activity. CuC_{l2} inactivates the nucleotide-free double mutant by cross-linking two a/b pairs. Treatment of the reduced double mutant with MgADP bound to either one or two catalytic sites with AlCl3 and NaF causes slow formation of the MgADP–fluoroaluminate complex in a single catalytic site and rapid formation of MgADP–fluoroaluminate complexes in two catalytic sites, respectively. In contrast, MgADP–fluoroaluminate complexes do not form in catalytic sites upon treatment of the cross-linked double mutant under identical conditions. These results support the premise that the guanidinium of bR337 transiently interacts with the carbonyl oxygen of aS370 during cooperative, multisite ATP hydrolysis.

I-A p-2. A single-point mutation in the ATP synthase of *Rb. capsulatus* impairing the stability of the protonmotive force-activated state

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The single-point mutation gammaM23-K introduced in the ATP synthase of *E. coli* has been reported to perturb the coupling efficiency between ATP hydrolysis and proton pumping as measured with the ACMA assay (1). Supporting this conclusion, the ATP synthesis rate was more affected compared to wild-type than the ATP hydrolysis rate, by about threefold (2). In addition, a study of interaction between subunits indicated that, in the mutated complex, the epsilon subunit inhibition of ATPase activity was not relieved upon binding of F_1 to the membrane as observed in the wild-type (2).

With the aim of further investigating the uncoupling process in a photosynthetic system in which analysis of the kinetics of the phosphorylating proton fluxes is possible (3), we have introduced this same mutation in the ATP synthase of *Rb. capsulatus*.In this organism, ATP synthesis and hydrolysis rates were impaired to a similar extent, both to approximately 1/3 of wild-type. Analysis of phosphorylating proton fluxes and associated ATP synthesis in the mutated and wild-type enzymes has not revealed uncoupling.However, the protonmotive force-activated state (measured as the transient increase of the ATP hydrolysis rate upon addition of uncouplers to energised vesicles), decayed extremely fast compared to wild-type. In agreement with this finding, the coupled proton flux through F_oF_1 induced by a single flash, which is usually observed in the wild-type enzyme in the presence of ADP and Pi, was completely absent. We conclude that the gammaM23-K mutated ATP synthase of *Rb. capsulatus* is an excellent system for studying the mechanism of ATP synthase activation by the protonmotive force.

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I-A p-3. Inhibition and reactivation of single F_1-ATPase molecules by tentoxin

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F_1-ATPase, the hydrophilic part of FoF_1 ATP synthase, hydrolysis ATP into ADP and phosphate. It acts as a molecular motor: during catalysis one part of the enzyme, the γ subunit, rotates within a ring of three α and three β subunits [1]. Tentoxin is a cyclic tetra-peptide produced by fungi, which acts as a phytophathogenic toxin, inhibiting F_1-ATPase of sensitive plant species [2]. Whereas low concentrations ($\sim 10^{-8}$ M) of the toxin block the ATP hydrolysis activity, higher concentrations($\sim 10^{-4}$ M) surprisingly reactivate the enzyme [3].

Here we investigate individual molecules of engineered, tentoxin-sensitive F_1-ATPase from the thermophilic Bacillus PS3 in the presence of tentoxin using the polystyrene bead detection method [4]. We show that while low tentoxin concentrations suppress rotation, under reactivating conditions the rotary movement of the γ subunit commences again, but with new characteristics compared to the enzyme in the absence of tentoxin [5]. The implications of these findings for the mechanism of inhibition and reactivation will be discussed.

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I-A p-4. Bovine heart atpsynthase dimerization: involvement of F_0 and F_1 subunits

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ATP synthase exists in both monomeric and dimeric forms in bovine heart (1) and yeast (2) mitochondria. In yeast, ATP synthase dimerization is mediated by subunits e, g, f and b of F_0 sector (2). Although bovine IF_1 can bind two F_1 domains simultaneously in solution (3), in neither yeast nor bovine heart does dimerisation require the binding of the inhibitor protein IF_1, The goal of this study was to provide information about the subunits involved in ATPsynthase dimerization in bovine heart mitochondria using limited proteolysis.

Submitochondrial particles were treated with trypsin or chymotrypsin, extracted by Triton X-100 and analysed by 1DBN-PAGE followed by 2DSDS-PAGE coupled to MALDI-TOF/MS. 1DBN-PAGE revealed that neither proteolytic enzyme altered the monomer/dimer ratio. 2DSDS-PAGE coupled to MALDI-TOF/MS of both monomer and dimer showed a similar progressive limited cleavage of the F_1 major subunits and no degradation of F_0. These results indicate that the same F_1 domains are exposed and accessible to proteolysis in monomer and dimer, demonstrating that dimerization does not result in shielding of such F_1 domains. This suggests that F_1 domains do not mediate ATP synthase dimerization (2).

Similar digestions were carried out using mitoplasts rather than submitochondrial particles. In this case, 1DBN-PAGE revealed that trypsin promoted monomerization of the dimer. 2DSDS-PAGE coupled to MALDI-TOF/MS showed that in both monomer and dimer the e subunit, whose carboxyl terminus is exposed on the membrane cytosolic side (4), was selectively cleaved by trypsin. Conversely, when mitoplasts were treated with chemotrypsin, no alteration of monomer/dimer ratio was observed, nor degradation of e subunit, as expected on the basis of the carboxyl terminus sequence (4). These results indicate that even in mammals, dimerization of e subunit has an essential role in the interface cohesion between ATP synthases.

Acknowledgements: MURST Cofin.

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I-A p-5. Simulation of subunit rotation in F_0F_1-ATP synthase

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Cellular ATP production is catalysed by membrane-bound F_0F_1-ATP synthase. An internal rotation of subunits couples the chemical reaction at three binding sites in the F_1 part to proton translocation through the membrane-integrated F_0 part. We apply single-molecule fluorescence resonance energy transfer (FRET) to examine the rotary subunit movements. Rotation is divided into three major steps with constant FRET level corresponding to three relative orientations (M. Diez et al. 2004 Nat Struct Mol Biol 11, 135). For epsilon-subunit rotation we have found distinct dwell times for the three different orientations, indicating heterogeneous catalytic rates at the three binding sites (M. Börsch et al. 2004 Biophys J 86, 181A, Part 2 Suppl). To proof our single-molecule FRET analysis and to evaluate the statistical significance we develop computer simulations of the signals, that help to unravel critical parameters. These simulations strongly support nonequal catalytic rates.

I-A p-6. Subunit g of the yeast ATP synthase forms homodimers and is involved in the oligomerization of ATP synthase complex

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The unique membrane-spanning segment of the ATP synthase subunit g, that contains a conserved putative dimerization GxxxG motif, was altered in yeast by site-direct mutagenesis. These alterations led to the loss of subunit g and the loss of dimeric and oligomeric forms of the yeast ATP synthase. In these strains, mitochondria displayed anomalous morphologies with onion-like structures. A specific mutant, with a unique cys in the intermembrane C-terminus, allows to determine that two subunits g are in close proximity. The disulfide bond formation between subunits g in intact mitochondria increased the stability of an oligomeric structure of the ATP synthase in digitonin extracts. The data show the involvement of the membrane-spanning segment of subunit g in the formation of supramolecular structures of mitochondrial ATP synthases and are in favour of the existence in the inner mitochondrial membrane of associations of ATP synthases whose masses are higher than those of ATP synthase dimers.

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I-A p-7. N-terminal fusions to the epsilon subunit of ATP synthase

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Recently we have provided in vivo evidence consistent with rotation in the *Escherichia coli* ATP synthase (Cipriano, D.J., Bi, Y., and Dunn, S.D. (2002) J. Biol. Chem. 277, 16782–16790). By fusing proteins to the C-terminus of epsilon, rotation of the gamma-epsilon rotor was stopped, blocking ATP synthesis. The fusions also resulted in the partial uncoupling of ATP hydrolysis, and loss of ability of epsilon to inhibit ATPase activity of soluble F_1. The latter activity is specific to the C-terminus of the subunit. Adding protein mass to the C-terminus of epsilon likely interferes with the proper interactions of epsilon with the remainder of F_1. Thus interactions of the C-terminus of epsilon with F_1 are essential for proper energy coupling during ATP hydrolysis. We have proposed a model for epsilon subunit function: that epsilon maintains the efficiency of ATP synthase by preventing rotation in the wrong direction depending on whether ATP or ADP is bound to the enzyme.

This work is continued by creating a number of N-terminal fusion proteins to the epsilon subunit in the presence and absence of a C-terminal deletion. Results from ATP hydrolysis assays, proton pumping assays, growth rates and yields, as well as western blot analysis, will reveal key results that directly test our model for epsilon subunit function.

I-A p-8. Diazoxide effect on F_1 catalysis of beef heart F_0F_1 ATPsynthase: enhancement of IF_1 inhibitor protein binding

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Diazoxide, a selective opener of the mitochondrial ATP-sensitive K^+ channel (mitoKATP), is a cardioprotective drug which mimics ischemic preconditioning (1). It has been reported that diazoxide enhances F_0F_1ATPsynthase inhibition during ischemia, but the mechanistic link between opening of mitoKATP and inhibition of ATPsynthase remains obscure (2, 3).

Our data demonstrate that diazoxide directly interacts with the F_1 sector of beef heart F_0F_1ATPsynthase markedly promoting the binding of IF_1 to beta subunit. More specifically, the treatment of soluble F_1 with one equivalent of diazoxide was sufficient to decrease the Kd of IF_1–F_1 complex at low pH. Such effect was revealed only on the cycling enzyme, while no effect was observed in the absence of Mg-ATP. However, diazoxide binding occurred independently from the catalysis, as shown by the structural changes induced by the drug in not cycling F_1 and revealed by CD spectra. In addition, kinetic analysis of ATP hydrolysis demonstrated that diazoxide slows the release of the inhibitory Mg-ADP suggesting that the drug exerts a stabilising role on Mg-ADP bound in the catalytic site of the beta subunit adopting the tight conformation (betaDP). In accordance with these data, a stabilising effect of Mg-ADP at the nucleotide binding domain (NBD) has been reported also for KATP channel (4). These results suggest that diazoxide binds to beta subunit at NBD, which is highly conserved in the ATP-binding cassette protein family (5), therefore inducing nucleotide stabilisation and favouring F_1 conformation suitable for IF_1 binding. Finally, diazoxide also increased IF_1 binding to membrane bound F_1, while it did not influence the energization-dependent IF_1 release.

We suggest that the mechanism described may contribute to cardioprotection favouring IF_1 binding and consequent F_0F_1ATPsynthase inhibition during ischemia, when the enzyme works in reverse and hydrolyses ATP (6, 7).

Acknowledgements: MURST Cofin 2002.

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I-A p-9. The 3D-reconstruction of the A1-ATPase from *Methanosarcina mazei* Gö1 facilitates a structural comparison of the evolutionary linked A-/F- and V-type ATPases

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The archaeal A1AO ATPsynthase/ase belongs to the fundamental proteins involved in cell energy cycle. It is composed of a water-soluble A1 ATPase and an integral membrane subcomplex, AO. ATP is synthesized and hydrolyzed in the A1 headpiece, consisting of an A3B3 domain, and the energy provided for or released during that process is transmitted to the membrane-bound AO domain. The energy coupling between the two active domains occurs via the so-called central stalk, an assembly proposed to be composed by the subunits C, D and F [1,2], where subunit D has been shown to be the structural and functional homologue of the F_1-g and V_1-E subunit, respectively [1].

Here, we present the first three dimensional reconstruction of the A1 ATPase from *M. mazei* Gö1 at 3.2 nm resolution [3]. The A1 consists of a headpiece of 10.2 nm in diameter and 10.8 nm in hight, formed by the six elongated subunits A_3 and B_3. At the bottom of the A_3B_3 complex a stalk of 3.0 nm in length can be seen. The A3B3 domain surrounds a large cavity that extends throughout the length of the A_3B_3 barrel. A part of the stalk penetrates inside this cavity and is displaced at an angle of 20 toward an A-B-A triplet. This three dimensional model allows comparison with the 18 Å structure of the V1 ATPase from M. sexta [4] and the crystal structure of the bovine heart F_1 ATPase [5]. In addition to the structural data, chemical crosslinking experiments have been carried out to elucidate the structural alterations of the subunits during ATP cleavage, showing nucleotide depended alterations of the A_1 ATPase stalk subunits C, D and F relatively to the A_3B_3 hexamer [1,3]. These data provide structural und functional information toward a fuller understanding of the mechanistical events occurring in this and the evolutive related F_1 and V_1 ATPases, particularly regarding the central, rotating, coupling element of these enzyme complexes.

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I-A p-10. The high affinity metal binding site in beef heart mitochondrial F_1 ATPase: an EPR spectroscopy study

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The high affinity metal binding site of isolated F_1-ATPase from beef heart mitochondria was studied by High Field CW-EPR and Pulsed EPR spectroscopy, using Mn(II) as a paramagnetic probe. The protein F_1 was fully depleted of endogenous Mg(II) and nucleotides (stripped F_1 or $MF_1(0,0)$) and loaded with stoichiometric Mn(II) and stoichiometric or excess amounts of ADP or AMPPNP. Mn(II) and nucleotides were added to $MF_1(0,0)$ both subsequentially and together as preformed complexes. Metal-ADP inhibition kinetics analysis was performed showing that Mn(II) enters one catalytic site on a b subunit. From the HF-EPR spectra the ZFS parameters of the various samples were obtained, showing that different metal-protein coordination symmetry is induced depending on the metal nucleotide addition order and on the protein/metal/nucleotide molar ratios. ESEEM technique was used to obtain information on the interaction between Mn(II) and the 31P nuclei of the metal coordinating nucleotide. In the case of the samples containing ADP, the measured 31P hyperfine couplings clearly indicated coordination changes related to the metal nucleotide addition order and the protein/metal/nucleotide ratios. On the contrary, the samples with AMPPNP showed very similar ESEEM patterns, despite of the remarkably differences present among their HF-EPR spectra. This fact has been attributed to changes in the metal site coordination symmetry due to ligands not involving phosphate groups. In this investigation we showed that the divalent metal triggers the high affinity conformation of the catalytic site and ADP binds the preformed Mn(II)–$MF_1(0,0)$ complex to give the Mg(II)-ADP inhibited form upon tri-site catalytic activity. By studying protein samples prepared in different ways, we were able to prove for MF_1 what was previously demonstrated for *E. Coli* F_1 and CF_1: the active role of the divalent cation in creating a competent catalytic site upon binding.

I-A p-12. The distance between the gamma- and the a-subunit of the ATP synthase determined by single-molecule fluorescence resonance energy transfer

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F_oF_1-ATP synthases are membrane-bound multisubunit enzymes catalysing ATP synthesis driven by a proton motive force across the membrane. Mechanically the coupling is achieved by intramolecular rotation of subunits. In *E. coli* the 'rotor' consists of the gamma-epsilon-c10 subunits. The 'stator' is formed by the subunits alpha3, beta3, delta, b2 and a. We investigated the changes in distance between the a-subunit and the gamma-subunit by fluorescence resonance energy transfer (FRET) with single holoenzymes incorporated in liposomes. An enhanced green fluorescent protein was fused to the c-terminus of the a-subunit and the gamma-subunit was labelled with Alexa 568. During catalysis a repeating sequences of three FRET states, i.e., three different distances, was observed, indicating the stepwise rotation of the gamma-subunit relative to the a-subunit. This set of three distances was used to calculate the position of the FRET donor at the a-subunit with respect to the FRET acceptor at the gamma-subunit in a three-dimensional model. We compared these results with previous FRET measurements of the gamma-subunit relative to the b-subunit. From the differences in the sequence of FRET states during ATP hydrolysis the relative position of the a-subunit in the holoenzyme can be allocated.

I-A p-13. Charge displacements during ATP-hydrolysis and synthesis of the Na^+-transporting F_OF_1-ATPase of *Ilyobacter tartaricus*

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Transient electrical currents generated by the Na^+-transporting F_oF_1-ATPase of *Ilyobacter tartaricus* were observed in the hydrolytic and synthetic mode of the enzyme. Two techniques were applied: A photochemical ATP concentration jump on a planar lipid membrane and a rapid solution exchange on a solid supported membrane. We have identified an electrogenic reaction in the reaction cycle of the F_oF_1-ATPase which is related to the translocation of the cation through the membrane bound Fo subcomplex of the ATPase. In addition, we have determined rate constants for the process: For ATP hydrolysis this reaction has a rate constant of $15-30\ s^{-1}$ if H^+ is transported and $30-60\ s^{-1}$ if Na^+ is transported. For ATP synthesis the rate constant is $50-70\ s^{-1}$.

I-A p-14. Induction and properties of proton slip in the ATP synthase from *Rhodobacter capsulatus*

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We investigated the F_OF_1-ATP synthase in chromatophores (subbacterial vesicles) of the purple phototrophic bacterium *Rhodobacter capsulatus*. Steps of the protonmotive force (*pmf*) were induced by flashing light, the transmembrane voltage was monitored by electrochromic carotenoid band-shifts proton uptake and release were followed by added pH-indicators, and the concomitant ATP yield was measured by the luciferin-luciferase system.

We found that in the absence of nucleotides *pmf* induced uncoupled proton flow (slip) through the ATP synthase (B. Feniouk, et al. (2001). *Biophys. et Biochim. Acta*, 1506,189–203). Here we characterise in detail the induction and the properties of proton slip.

(i) Induction: Slip was induced only in the absence of nucleotides. Transmembrane voltage ($\Delta\psi$) alone was sufficient for that; no transmembrane pH difference was required. The $\Delta\psi$ threshold for slip induction was 150 mV. Once induced, the enzyme conformation producing a slipping behaviour persisted for at least 2 h, even after addition of ADP or ATP. Slip induction was prevented in the presence of ADP or ATP at concentrations greater than 100 nM.

(ii) Proton conduction: The proton conductance for slip was twofold lower than that found for the proton transport through bare F_O which was stripped of F_1. There was no detectable voltage threshold for proton slip, as well as for proton transport through bare F_O. An important difference between these two modes of proton transport was that slip was inhibited at pH below 7.

I-A p-15. Contribution of intersubunit hydrogen bonds and salt bridges to the F_1F_O ATP synthase mechanism

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Single molecule experiments of the F_1-ATPase gamma subunit rotation were measured with dark field microscopy using a gold nanorod as a probe of rotation. The ability to form specific hydrogen bonds or salt bridges between the gamma subunit and the alpha or beta subunits of *E. coli* F_1-ATPase was eliminated by the creation of 43 site-directed mutants. Several of the mutant strains grew very slowly or not at all on succinate suggesting that these intersubunit interactions were important for ATP synthase activity. With rare exception, the yield and subunit composition of purified F_1-ATPase purified from these strains was not affected by the mutations. Several of the mutations decreased F_1-ATPase Mg-ATPase activity significantly and a few increased the activity. In some cases, the mutation caused a large differential effect on ATP synthase and ATPase activities. Several mutants altered the propensity of the enzyme to entrap inhibitory Mg-ADP as measured by the magnitude of the acceleration of ATPase activity induced by LDAO. These studies identify specific hydrogen bonds and salt bridges between the gamma subunit and the alpha and beta subunits that contribute significantly to the ability of F_1 to change subunit conformations during its catalytic cycle.

I-A p-16. The subunit h of the F_1F_O ATP synthase of *Saccharomyces cerevisiae*

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Subunit h, a 92-residue-long, hydrophilic, acidic protein, is a component of the yeast mitochondrial F_1F_o ATP synthase. This subunit, homologous of the mammalian factor F_6, is essential for the correct assembly and/or functioning of this enzyme since yeast cells lacking it are not able to grow on nonfermentable carbon sources. The construction of cysteine-containing subunit h mutants and the use of bismaleimide reagents has provided insights into its environment. Cross-links were obtained between subunit h and subunits alpha, f, d and 4. These results and secondary structure predictions allowed us to build a structural model and to propose that this subunit occupies a central place in the peripheral stalk between the F_1 sector and the membrane. In addition, the subunit h was found to have a stoichiometry of one in the F_1Fo ATP synthase complex and to be in close proximity to another subunit h belonging to another F_1Fo ATP synthase in the inner mitochondrial membrane. This subunit could be highly implicated in one of the two dimerisation interfaces of the F_1Fo ATP synthase. Taking into account the disruption of the subunit h coding gene (ATP14) leads to a very high proportion of Rho-/Rhoo cells, a doxycycline-regulatable expression system was used to study the impact of the absence of the subunit h on the assembly and the activity of the enzyme.

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I-A p-17. F_1F_O-ATP synthase operon of *Nonomuraea* SP. ATCC 39727

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Nonomuraea sp. ATCC 39727 is a filamentous actinomycete, producer of a glycopeptide antibiotic, which is used as a precursor of dalbavancin. This microorganism is a poorly characterised species: one aspect of the physiology of the bacterium is the absolute oxygen requirement for growth.In some prokaryotes such as *E. coli* the genes for ATP synthase are clustered in a single operon in the order atpIBEFHAGDC (1), while other species, have split atp operons (2,3).

The atp operon of *Nonomuraea* sp. ATCC 39727 was sequenced using conventional polymerase chain reaction technique. It consisted of nine open reading frames arranged in the order atpI(i), atpB(a), atpE(c), atpF(b), atpH(d), atpA(a), atpG(g), atpD(b) and atpC(e), which was identical to that found in *E. coli* (1) and many other bacteria.

The deduced aminoacid sequences of the nine atp genes were aligned with the ATP synthase subunits from other species. The greatest homology was observed in the F_1-b subunit whose primary structure showed up to 79% identities with that of some phylogenetically close bacterial species (*Th. fusca*, *S. coelicolor*, *S. lividans*). Moreover, a 64% identity was also shown with the bovine mitochondrial subunit so that *Nonomuraea* sp. b subunits could be detected in Western blotting experiments with the antibody raised against the bovine mitochondrial b subunit.

The presence of a functional membrane-bound F_1F_o-ATP synthase has been shown by the measure of the ATP-driven proton translocation in *Nonomuraea* sp. everted membrane vesicles, using ACMA as transmembrane DpH probe. The ATPase activity was strictly dependent upon the presence of Mg^{2+} ion and was inhibited by the addition of inhibitor specific for F-type ATPase like oligomycin and venturicidin. This activity was low in the exponential phase of growth, showing an increase at the transition to the stationary phase (4).

Acknowledgements: This work was performed as a part of M.I.U.R. industrial research project " Nuove Tecnologie per lo studio di microrganismi altoproduttori di antibiotici" coordinated by Aventis Bulk S.p.a., Italy.

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I-A p-18. Structural studies of the yeast F_1-F_O ATP-synthase

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The F_1F_o-ATP synthase produces ATP from ADP and Pi by using the proton motive force generated across the inner mitochondrial membrane by the respiratory chain. The yeast enzyme is a 600 kDa complex that contains 14 distinct subunits. The X-ray structure of the bovine F_1-ATPase (alpha3, beta3, gamma, delta, epsilon) has been solved at 2.4 Å resolution (a) and an unrefined model of a subcomplex (alpha3, beta3, gamma, delta, c10) has been obtained from a yeast ATP-synthase crystal diffracting up to 3.9 Å (b). A 32 Å model of the intact bovine ATP-synthase has recently been determined by cryo electron microscopy (c). Structural data and direct observation of the rotation of the gamma subunit(d) inside the catalytic subunits (alpha,beta) have led to a rotary motor mechanism model with a rotor part (gamma,delta,epsilon,c10) and a stator part (alpha3,beta3,OSCP,4,d,h,f,8,6,i).

Despite all the structural work that has been performed on this enzyme, little is known on the stator part of this complex and on interactions between membrane subunits. In order to get a higher resolution model and to obtain data on the whole complex we are developing several structural complementary approaches on the yeast ATP-synthase. Here, we present a new purification protocol of a modified enzyme and our preliminary work on 2D and 3D crystals as well as atomic force microscopy images.

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I-A p-19. Inhibition sites on F_1-ATPase from bovine heart mitochondria

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High-resolution crystallographic studies of a number of inhibited forms of bovine F_1-ATPase have identified four distinct inhibitory sites: the catalytic site (Abrahams et al., 1994 Nature 370, 621–628; Orriss et al., 1998 Structure 6, 831–837; Gibbons et al., 2000 Nat. Struct. Biol. 7, 1055–1061; Menz et al., 2001 Cell 106, 331–341), the aurovertin B binding site (van Raaij et al., 1996 Proc. Natl. Acad. Sci. U. S. A. 93, 6913–6917), the efrapeptin binding site (Abrahams et al., 1996 Proc. Natl. Acad. Sci. U. S. A. 93, 9420–9424) and the site to which the natural inhibitor protein IF_1 binds (Cabezón et al., 2003 Nat. Struct. Biol. 10, 744–750). However, the binding sites for other inhibitors, such as polyphenolic phytochemicals (Zheng and Ramirez, 2000 Br. J. Pharmacol. 130, 1115–1123), cationic dyes and amphiphilic peptides (Bullough et al., 1989 Biochim. Biophys. Acta 975, 377–383), have remained undefined. By employing structural and kinetic analyses, we have identified the binding sites for a number of these compounds. Several of these novel inhibitors bind to the known regulatory sites. An understanding of these inhibitors may enable rational development of therapeutic agents for the treatment of several disorders linked to the ATP synthase, including ischaemia–reperfusion injury and cancer.

I-A p-20. The transmembrane domain of subunit b of the *Escherichia coli* F_1F_O ATP synthase is sufficient for H^+-translocating activity together with subunits a and c

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Subunit b is indispensable for the formation of a functional H^+-translocating F_O complex both in vivo and in vitro. Whereas the very C terminus of subunit b interacts with F_1 and plays a crucial role in enzyme assembly, the C-terminal region is also considered to be necessary for proper reconstitution of F_O into liposomes. Here, we show that a synthetic petide (residues 1–34 of subunit b (b1–34) [Dmitriev, O., Jones, P. C., Jiang, W., Fillingame, R. H. (1999) J. Biol. Chem. 274, 15598–15604)] corresponding to the membrane domain of subunit b was sufficient in forming an active F_O complex when coreconstituted with purified ac subcomplex. H^+ translocation was shown to be sensitive to the specific inhibitor N,N′ -dicyclohexylcarbodiimide, and the resulting F_O complexes were deficient in binding of isolated F_1. This demonstrates that only the membrane part of subunit b is sufficient as well as necessary for H^+ translocation across the membrane, whereas the binding of F_1 to F_O is mainly triggered by C-terminal residues beyond Glu-34 in subunit b. Comparison of the data with former reconstitution experiments additionally indicated that parts of the hydrophilic portion of the subunit b dimer are not involved in the process of ion translocation itself, but might educate subunits a and c in F_O assembly. Furthermore, the data obtained functionally support the monomeric NMR structure of the synthetic b1–34.

I-A p-21. EXAFS analysis of the structure of Ca and Ca-nucleotides bound to chloroplasts ATP synthase

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ATP synthase from chloroplasts has three active sites each located on one of the three beta subunits. We have measured the binding of Ca to enzyme in the absence and the presence of added nucleotides in order to identify the sites and to relate them to the active sites of the enzyme. It was found that both binding constants and the cooperative interactions between the binding sites are changed in the presence of nucleotides. There was positive cooperative binding of Ca in the presence of ADP and in the inhibition of ATP hydrolysis and similarities between the Kd and Ki for binding and inhibition, respectively. Correlation between binding and kinetic parameters and evaluation of the Km/ Kcat ratio revealed that the lake of proton pumping in the presence of CaATP was partially due to the fact that the stroke produced by CaATP was not sufficiently large to rotate the turbine against the electrochemical potential formed across the membrane. However, these data were not sufficient by themselves to explain the reason why Ca ion that catalyze hydrolysis could not catalyze synthesis of ATP.

We therefore, determined the structure of enzyme bound Ca by measurement of Ca XAFS in various forms of the enzyme in order to be able to determine structure–function relations. We were able to measure for the first time a dilute solution of Ca compounds at cryogenic temperature. We found that the striking differences between the structures of the Ca- and Mg-nucleotide bound to the active site of CF_1 can give a clue to the differences in catalysis in the presence of these two metal ions. The longer Ca–O bond distance (average of 2.4 Å) than the Mg–O (average 2.1 Å) explains the observe differences in binding of the metal-nucleotide that is indeed expected to become weaker in the presence of Ca ions.

There are six oxygen atoms about the Mg ion compared to more than six about Ca ion. Two of the ligands are attached to the phosphate oxygen in the MgAMPPNP and one the thrionine hydroxyl while the rest are bound to water molecules as seen in the crystallographic structure of ATP synthase. A larger number of water molecules about the Ca are expected to induce a more hydrophilic environment in the vicinity of the gama phosphate that favors the hydrolysis of this bond. The distorted binding of CaATP at the active site might also cause a smaller change in the conformational interaction between the beta and the gamma subunit and prevent proton pumping in the presence of CaATP.

I-A p-22. The role of the F subunit of thermus thermophilus V-ATPase on ATP hydrolysis and rotation

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The pH within many intracellular compartments of eukaryotes, such as Golgi apparatus, endosomes, and lysosomes, is regulated by vacuolar H^+-pumping ATPases (V-ATPases). A family of V-ATPases also exists in the plasma membrane of some bacteria including the thermophilic bacterium, Thermus thermophilus. V-ATPases are multi-subunit enzymes arranged as a peripheral V_1 portion responsible for MgATP hydrolysis attached to a membrane-embedded V_o portion containing proton pore. The V_1 portion of T. thermophilus is itself ATPase-active, hence called V1-ATPase. The V_1-ATPase is composed of four subunits with a presumed subunit composition of A3B3DF and is a molecular rotary motor, in which central rotor subunits, D and F, rotate within a hexaemric ring of A3B3.

To understand the role of the F subunit, we prepared A3B3D subcomplex and compared with A3B3DF (V_1-ATPase). ATPase activity of A3B3D was significantly lower than that of A3B3DF: Both catalytic turnover and affinity to ATP were decreased in A3B3D (Vmax = 20/s and Km = 0.49 mM) as compared to A3B3DF (Vmax = 31/s and Km = 0.26 mM). In addition, A3B3D was more susceptible to the MgADP inhibition during ATP hydrolysis. Binding of the isolated F subunit to A3B3D increase the ATPase activity to the extent close to that of A3B3DF. Above results indicate that the F subunit promotes the ATPase activity of V_1. This function is opposite from the inhibitory role of the e subunit of bacterial FoF_1-ATPase, of which the F subunit has been thought to be a functional homolog. The C-terminally trancated F(1–84) subunit was also bound to A3B3D but F(1–68) subunit was not, indicating the importance of the 69–84 stretch of the F subunit for the association with A3B3D. ATPase activity of A3B3DF(1–84) (Vmax = 24/s) was an intermediate between that of A3B3D and A3B3DF, suggesting a participation of C-terminal 22 residues of the F subunit in V_1 catalysis. In a single molecule observation, the beads attached to the D subunit rotated not only in A3B3DF but also in A3B3D. This implies that the F subunit is a dispensable component for rotation itself. Thus, the F subunit binds peripherally to the D subunit but promotes V_1-ATPase catalysis.

I-A p-23. Location of the C-terminus of subunit F6 in the peripheral stalk of ATP synthase

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The F_6 subunit of mitochondrial F_1F_o ATP Synthase is a 76 amino acid protein component of the peripheral stalk, which is comprised of single copies of the oligomycin sensitivity conferral protein (OSCP), and subunits b, d and F_6. The F_6 solution NMR structure revealed that it is a hairpin of two antiparallel α-helices. The aim of this study was to determine the orientation and location of F_6 in F_1F_o. A biotinylation signal sequence was fused to the C-terminus of the F_6 subunit of *Saccharomyces cerevisiae* to which avidin molecules were bound for localisation by electron microscopy. The avidin molecules and therefore the C-terminus of the labeled subunit were identified in a series of class averages generated from negatively stained single particles. We have succeeded in localising the C-terminus of F6 and can now better interpret its role in the peripheral stalk.

I-A p-24. Purification and crystallization of the V-ATPase subunits from thermus thermophilus

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The vacuole-type ATPases (V-ATPases) are commonly found in many organisms involved in a variety of physiological processes. The V-ATPases from Thermus thermophilus is solely responsible for aerobic ATP synthesis in this bacteria which lacks F-ATPase. The Thermus V-ATPase is composed of nine different subunits, which are arranged within the atp operon in the order of G–I–L–E–C–F–A–B–D, which encodes proteins with molecular sizes of 13, 71, 8, 20, 35, 12, 64, 54 and 25 kDa, respectively. This ATPase-active V1 domain, is composed of four subunits with a stoichiometry of $A_3B_3D_1F_1$, whereas the Vo domain, involved in proton translocation across the membrane, is an assembly of G, I, L, E and C subunits. Each Thermus V-ATPase subunit shows a sequence similarity to its eukaryotic counterpart.

Our ultimate goal of is to determine the holo V-ATPase structure, however, it is extremely difficult to obtain well-ordered crystals of this multisubunit complex. We are, therefore, trying to express and crystallise each subunit of this enzyme. So far, the subunits A, B, C, E, and F and a part of the subunit I have been over expressed in *E. coli*. Among them, the structure of the C subunit has been already solved and reported (1). We will present our recent progress on the crystallisation trials on these subunits.

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I-A p-25. Conformations of the beta subunit of F_OF_1-ATP synthase during ATP synthesis

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Conformational changes of the beta subunit in F_oF_1-ATP synthase are assumed to play key roles in rotation of the central shaft consisting of gamma, epsilon and c subunits in the process of ATP synthesis and hydrolysis reactions. A subcomplex of the F_1 portion, alpha3beta3gamma, was previously shown to undergo conformational changes in which two of the three beta subunits have the ‘‘closed’’ conformation at certain steps of ATP hydrolysis. This conformation was observed also in the case the enzyme was lapsed in the ADP inhibited form.

In the present study, conformations of the beta subunit in the complete F_1 (alpha3beta3gamma-delta-epsilon) and the holoenzyme F_oF_1 were investigated by intersubunit disulfide cross-linking which represents simultaneous closing of two beta subunits. In the case of F_1, cross-linking did not occur in the ADP-bound state, but turnover of ATP hydrolysis accelerated cross-linking. These results indicated that the conformations of the beta subunits changed in response to ‘‘up’’-and-‘‘down’’ conformational changes of the epsilon subunit. In the case of F_oF_1, the beta–beta cross-linking was not efficient during ATP synthesis, whereas exposure to ATP in the absence of proton motive force induced almost complete cross-linking. The results revealed that, in contrast to the case of ATP hydrolysis, simultaneous closing of two beta subunits is not essential or the dwelling time of the conformation is not long enough to form cross-linking in the process of ATP synthesis. In addition, the absence of cross-linking is consistent with the absence of ADP inhibition during ATP synthesis.

In summary, one of the nucleotide-bound beta subunits is expected to be in the half-closed conformation during ATP synthesis thus allowing the C terminal helix of the epsilon subunit to stay in the alpha3beta3 cavity without steric hindrance.

I-A p-26. The structure of the yeast F_1-ATPase at 2.8Å resolution

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The crystal structure of the yeast F_1-ATPase has been obtained at 2.8Å resolution. The enzyme was crystallized in the presence of AMP-PNP, ADP, and Mg^{2+}, which gave crystals in space group $P2_1$ ($a = 111.8$ Å, $b = 294.4$ Å, $c = 190.8$ Å and $\beta = 101.7^o$). There are three F_1 molecules (>9000 residues) in the asymmetric unit and six molecules in the unit cell. Thus, the crystal packing in these crystals is quite different from that of bovine F_1. The structure was solved by molecular replacement using the structure of the bovine F_1 without the nucleotides (and missing much of the γ- and all of the δ- and ε-subunits) and the model has been refined to an R_{free} of 29.4% and R_{factor} of 23.3%. The 2Fo−Fc density map clearly shows the presence of five bound nucleotides per F_1, with a nucleotide bound to three noncatalytic and to two catalytic sites with the remaining catalytic site (β_E) devoid of nucleotide. The conformations of the active sites are nearly identical to those of the bovine enzyme including the conformation of β_E. In addition to the nucleotides, the 2Fo−Fc map shows density for most of the γ-, δ-, and ε-subunits. It is clear that the structure of the yeast enzyme supports all of the major conclusions made from the structure of the bovine F_1-ATPase. This is the first high-resolution structure of a nonbovine F_1-ATPase, which shows the asymmetric features first observed in the bovine enzyme. (This work was supported by grants from the NIH (R01-GM067091, R01-GM066223, 1F06TW002379) to D.M.M. and MRC support to J.E.W. and A.G.W.L.).

I-A p-27. Crystal structure at 2.1 Å resolution of the membrane rotor domain of the V-type Na^+-ATPase from *Enterococcus hirae*

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In V-ATPases, ATP hydrolysis is coupled to translocation of protons through membranes by rotation of a ring of 16-kDa proteolipids in the membrane which has binding sites for protons. The proton translocation pathway probably lies between the ring and another hydrophobic protein as in the F-ATPases. A unique variant among the V-ATPases is the enzyme from Enterococcus hirae, which transports Na^+ ions rather than protons under physiological conditions. NtpK has a binding site for Na^+ ions. It is the homologue of the eukaryotic V-type 16-kDa proteolipid. Here, we have solved the crystal structure of the NtpK ring at 2.1 Å resolution. The structure shows that oligomers of Ntpk form a ring with 10-fold symmetry, 83 Å in diameter. Ten sodium ions are bound to the specific binding pockets and they are located on the external surface of the ring. More than 20 bound phospholipids are attached to the inside of the ring, suggesting that the inside of the ring is occupied by lipids. On the basis of the structure, we propose a model of ion translocation mechanism by the V-ATPase.

Keywords: Crystal structure; Na^+-dependent ATPase; V-ATPase; Na^+-binding site

I-A p-28. A novel mechanical model of ATP synthesis and hydrolysis in F_OF_1-ATP synthase

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The great achievement in the investigations of both ATPsynthase structure and its catalytic mechanism has brought to a new view on the regulation of ATP synthesis and hydrolysis in the cell. However, some problems remain unsolved. One of them is how one can reconstruct a whole process of complete enzyme cycle using known structural data. In order to partially clear this question a dynamic model of the enzyme has been created. The main purpose of this model was to explain quantitatively the mechanism of energy transformation from transmembrane electrochemical proton gradient into the energy of a chemical bond in ATP. The main and the most important features of the model is that the kinetic parameters of ATP synthesis such as the rate of ADP phosphorylation and the time of enzyme turnover are estimated on the base of the structural data only. This approach makes one possible to extend the list of ATP synthase properties which can be analyzed independently. In particular it was shown that the time of protons movement through F_O proteins essentially depends on membrane viscosity and elastic tension in gamma subunit. Moreover, the time of protonation/deprotonation of F_O protonophoric groups determines the peculiarity of the hydrophobic protein subunits rotation. Another merit of the model is an explicit analytical type of the parameters used. It means that the model can be simply and fast modified for corrected experimental data. Appropriate software has been developing.

I-A p-29. The C-ring of the F_1F_0 ATP synthase in native membranes is filled with lipids

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F_1F_0 ATP synthases are the smallest rotary motors known in nature. The rotor of these machines consist of the subunits gamma and epsilon and the membrane embedded c-ring. The number of c subunits in the ring varies between species and is shown to be 10 in yeast mitochondria, 11 in *Ilyobacter tartaricus* and 14 in spinach chloroplasts. The c-ring has a central cavity which in case of *I. tartaricus* has a diameter of 1.7 nm. Here we show that this cavity is filled with phospholipids.Site-directed mutagenesis was used to introduce cysteine residues within the inner helix of the *Escherichia coli* subunit c at positions 4, 8 and 11. Cells containing a single-cysteine mutant ATP synthase were labeled with sulfhydryl-specific photoactivatable cross-linking reagents. Two novel crosslinkers with a photoreacive aryldiazirine group and a commercially available benzophenone were used. It was shown that the cysteine residue of subunit c was specifically labeled. After light exposure subunit c cross-linked with lipids could be identified with MALDI-MS and phospholipase C digestion. Using Western blot analysis multimers of subunit c could be detected, but no cross-link formation between the subunits a and c was observed.

I-A p-30. Real-time pH-microscopy down to the molecular level by combined scanning electrochemical microscopy and single-molecule fluorescence spectroscopy

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A new technique combining scanning electrochemical microscopy (SECM) and single-molecule fluorescence spectroscopy (SMFS) was developed to accomplish locally and temporally defined pH adjustments in buffer solutions and on surfaces monitored by fluorescence alteration of pH-sensitive fluorophores in real-time. Local pH gradients were created by electrochemical generation of H^+ or OH^- during redox reactions at ultramicro- or nanoelectrodes with radii from 5 μm down to 35 nm. Ratiometric fluorescence measurements were performed with a confocal laser microscope using two detectors for different spectral regions. Time-resolved pH measurements were carried out with freely diffusing SNARF-1-dextran. For pH measurements on surfaces, total internal reflection fluorescence microscopy (TIRFM) was used in combination with a CCD camera. The fluorophore SNAFL-succinimidylester was bound to amino-terminated octadecylsilane-coated cover slips. Local pH determinations could be accomplished with an accuracy of 0.1 units. The measured pH profiles showed a strong dependence on the tip diameter, the buffer/mediator concentration ratio, and the tip-surface distance. As an application for bionanotechnology using SECM induced pH changes on the molecular level, the proton-driven ATP synthesis by single membrane-bound F_0F_1-ATP synthases was investigated. ATP synthesis resulted in stepwise subunit rotation within the enzyme, that was monitored by single-molecule fluorescence resonance energy transfer (FRET).

I-A p-31. Photoaffinity labeling of ATP synthase by biotinylated adenine nucleotides

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In order to characterize nucleotide binding sites of ATP synthases we have synthesized various monofunctional and bifunctional photoactivatable ATP analogs. The six nucleotide binding sites—three catalytic and three noncatalytic—of ATP synthases are located on the F_1 complex of the enzyme alternately at the interfaces between the major subunits alpha and beta as demonstrated by photoaffinity labeling and photoaffinity cross-linking.

The introduction of an additional biotin residue, yielding 3′-biotinyl-8-azido-ATP, is advantageous for an easy detection of labeled proteins. Irradiation of the F1-ATPase from the thermophilic bacterium PS3 (TF_1) in the presence of 3'-biotinyl-8-azido-ATP resulted in the nucleotide-specific inactivation of the enzyme as well as in the nucleotide-dependent labeling of alpha and/or beta subunits.

Dimerization of 3′-biotinyl-8-azido-ADP resulted in the formation of the bifunctional diadenine dinucleotide 3′-dibiotinyl-8-diazido-AP4A. Irradiation of TF_1 in the presence of 3′-dibiotinyl-8-diazido-AP4A yielded the nucleotide-specific inactivation of TF_1 and the nucleotide-dependent formation of alpha-beta cross-links.

In addition the biotinylated mono- and bifunctional photoaffinity labels could be used successfully to label the V_1-ATPase from Manduca sexta.

All these results demonstrate the suitability of the biotinylated azidonucleotides for photoaffinity labeling and photoaffinity cross-linking of ATP synthase complexes and V-type ATPases.

I-A p-32. Distances between the b-subunits in the tether domain of F_0F_1-ATP synthase from *E. coli*

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F_0F_1-ATP synthases couple proton translocation with the synthesis of ATP from ADP and phosphate. These enzymes consist of a hydrophilic F_1-part and a hydrophobic membrane-integrated F_0-part which are connected by a central and a peripheral stalk. In the *Escherichia coli* enzyme, the peripheral stalk consists of two b-subunits.

To obtain information about the orientation of the two b-subunits in the so called tether domain of F_0F_1, cysteine mutations were introduced and labeled with a nitroxide spin-label at positions b-40, b-51, b-53, b-62 and b-64. Conventional (9 GHz), high-field (95 GHz) and pulsed EPR spectroscopy revealed that all residues are in a relatively polar environment, with mobilities consistent with helix sites. Spin labeled mutants b-62 and b-64 are in a slightly more protic surrounding than the others.

Distances between the spin labels are determined and a model of the arrangement of the b-subunits is suggested. Binding of the nonhydrolysable nucleotide AMPPNP to the spin-labeled enzyme had no significant influence on the distances. We discuss our results to improve the knowledge about the peripheral stalk of F_0F_1-ATP synthases.

I-A p-33. The ion channel of F-ATP synthase is the target of toxic organotin compounds

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ATP is the universal energy currency of living cells and the majority of it is synthesized by the F_1F_0 ATP synthase. Inhibitors of this enzyme are therefore potentially detrimental for all life forms. Tributyltin chloride (TBT-C_l) inhibits ATP hydrolysis by the Na^+-translocating ATP synthase of *Ilyobacter tartaricus* or the H^+-translocating counterpart of *Escherichia coli* with apparent Ki of 200 nM. To target the site of this inhibition, we synthesized a tritium labeled derivative of TBT-Cl in which one of the butyl groups was replaced by a photoactivatable aryldiazirine residue. Upon illumination, subunit a of the ATP synthase becomes specifically labeled and this labeling is suppressed in the presence of the non labeled inhibitor. In case of the Na^+ ATP synthase labeling is also suppressed in the presence of Na^+ ions, suggesting an interference in Na^+ or TBT-C_l binding to subunit a. TBT-C_l strongly inhibits Na^+ exchange by the reconstituted *Ilyobacter tartaricus* ATP synthase. Taken together these results indicate that the subunit a ion channel is the target site for ATPase inhibition by toxic organotin compounds. TBT-Cl is the first known inhibitor interacting specifically with this site.

I-A p-34. V type ATPase/synthase from *Thermus thermophilus*

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VoV1-ATPases (V-ATPases) are the ATPase/ATP synthase superfamily which catalyzes the exchange of the energy between proton translocation across membranes and the energy of ATP hydrolysis/synthesis. They are widely distributed in different types of eukaryotic cells and some bacteria.

The VoV1-ATPase of Thermus thermophilus consists of an ATPase-active V1 part (ABDF), and a proton channel V_o part (CLEGI), but the arrangement of each subunit is still largely unknown. Exposure of the isolated Vo to acid or 8 M urea produced two subcomplexes, EGI and CL. Thus, C subunit (homologue of d subunit, yeast Vma6p) associates with L subunit ring tightly, and I (homologue of 100-kDa subunit, yeast Vph1p), E, and G subunits constitute a stable complex. Based on these observations and our recent demonstration that D, F, and L subunits rotate relative to A3B3, the results indicate that C, D, F, and L subunits constitute the central rotor shaft and A, B, E, G and I subunits comprise the surrounding stator apparatus in the V_oV_1-ATPase.Then, the crystal structure of the subunit C has been determined at 1.95 Å resolution and located into the holo-enzyme complex structure obtained by single particle analysis as suggested by the results of subunit cross-linking experiments. The result shows that V-ATPase is substantially longer than the related F-ATPase, due to the insertion of subunit C between the V_1 and the V_o domains. Subunit C, attached to the Vo domain, seems to have a socket like function in attaching the central-stalk subunits of the V_1 domain. This architecture seems essential for the reversible association/dissociation of the V_1 and the V_o domains, unique for V-ATPase activity regulation.

I-A p-35. Functional and structural domains of the ATPase inhibitor protein (IF_1) from bovine heart mitochondria

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The FoF_1-ATP synthase of coupling membrane catalyzes ATP synthesis driven by the protonmotive force generated by respiration. In mitochondria, ATP hydrolysis by the FoF_1 complex is specifically inhibited by the IF_1. IF_1 associates reversibly in a 1:1 stoichiometry with FoF_1, from which it is displaced by the protonmotive force, thus IF_1 has no significant effect on ATP synthesis. IF_1-(42−58) has been identified as the shortest segment of IF_1 which exerts the most potent, pH and temperature dependent activity on the F_oF_1 complex. Due to its flexible structure, determined by circular dichroism and [1H]-NMR spectroscopy, IF_1-(42−58) synthetic peptide can fold in helical and/or b-spiral arrangements that favour the binding to the F_oF_1 complex, where the native IF_1 binds. The native IF_1-(1−84) protein and the IF_1-(42−58) synthetic peptide inhibit more effectively the ATPase activity of the F_oF_1 complex compared to soluble F_1. Results of cross-linking and limited proteolysis experiments are presented showing that in the intact F_oF_1 complex "in situ", in the inner mitochondrial membrane of the bovine heart, the synthetic central segment of IF_1 (residues Leu_{42}−Lys_{58}) binds to the a and b subunits of F_1 in a pH-dependent process which results in inhibition of the ATPase activity. The C-terminal region of IF_1 binds, simultaneously, to the OSCP subunit in a pH-independent process. This binding keeps IF_1 anchored to the complex, both under inhibitory conditions, at acidic pH, and noninhibitory conditions at alkaline pH. Simultaneous binding of IF_1 to a and b subunits and OSCP which are component of the stator, and to F_1-g and e subunits which are component of the rotor, can provide a firm interaction of IF_1 with the F_oF_1 complex, thus blocking the rotary mechanism of catalysis. The pH-independent binding of IF_1 to OSCP might also prevent IF_1 loss from the complex at alkaline pH, set up in the matrix by respiration, conditions under which the binding at the inhibitory site in the b and a subunits is unlaced. In conclusion, we propose that each single IF_1 molecule binds to one F_oF_1 complex, with the central segment Leu_{42}−Lys_{58} bound at the inhibitory site at the a/b interface and the C-terminal region bound to F_o-OSCP.

I-A p-36. The requirement of ADP and Pi for the steady-state operation of proton-translocating ATPase in *Paracoccus denitrificans* membranes

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The ATP synthase in tightly coupled vesicles derived from *Paracoccus denitrificans* is not capable of ATP hydrolysis whereas the enzyme catalyzes rapid respiration-supported ATP synthesis. The proton-translocating ATPase activity is induced by brief exposure of the enzyme to proton-motive force (pmf) and declines if pmf falls down to a certain level [Zharova T.V., Vinogradov A.D. (2004) J.Biol.Chem. 279, 12319–12324]. The activation and deactivation of ATPase are due to pmf-dependent dissociation and uncoupling-dependent reassociation, respectively, of bound ADP from (to) its specific high affinity inhibitory site. Inorganic phosphate (Pi) is not essential for the energy-dependent activation of ATPase whereas the presence of Pi or arsenate is required for the steady-state pmf-generating self-sustained ATP hydrolysis. Half-maximal concentration of Pi needed for the steady-state ATP hydrolysis (20 μM) is close to that required for the respiration-supported oxidative phosphorylation. The initial rates of the energy-activated, pmf-generating ATP hydrolysis in the presence of Pi are the same whether the ATPase activity is assayed with or without ATP regenerating system (phosphoenolpyruvate plus pyruvate kinase). Only initial burst followed by rapid decline of ATP hydrolysis is seen at high pyruvate kinase/ATPase activity ratio, whereas the ATP hydrolysis proceeds at almost constant rate in the absence of ATP regenerating system. The higher pyruvate kinase is present in the assay (the lower steady-state ADP level is reached), the shorter initial burst of ATP hydrolysis is seen. Taken together these results show that certain level of the substrates for ATP synthase (products of ATPase) in the reaction medium is required for the steady-state operation of the pmf-generating, pmf-dependent ATP hydrolysis. If ATP hydrolysis and ATP synthesis catalyzed by the enzyme proceed via different mechanisms as it has been discussed earlier [Vinogradov, A.D. (2000) J.Exp.Biol. 203, 41–49] the data suggest that slow futile cycles occur when the enzyme operates either as pmf-supported ATP synthase or pmf-generating ATPase thus making the net production and/or utilization of ATP finely tuned to physiological demands.

Supported by Russian Foundation for the Fundamental Studies (grant 02-04-48679) and by the program Leading Schools in Science (grant 596.2003.4).

I-A p-37. Movements of epsilon-subunit in EF_0F_1-ATP synthase revealed by single-molecule fluorescence resonance energy transfer

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ATP synthesis is catalyzed by membrane-bound H^+-ATP synthases. The chemical reaction at the three binding sites in the F_1 part is assumed to be coupled to proton translocation through the membrane-integrated F_0 part by an internal rotation of the gamma-epsilon-cn-subunits. We investigated movements of the epsilon-subunit relative to the b-subunits by single-molecule fluorescence resonance energy transfer (FRET) using liposome-embedded F_0F_1. Three different FRET states corresponding to three distances between the epsilon-subunit and the b-subunits are observed and the sequence of FRET states during ATP synthesis and ATP hydrolysis are reversed. From these results we conclude an opposite rotation of epsilon in 120 steps during catalytic turnover. Moreover, the dwell times of the three FRET states are different, indicating individual catalytic rates for the three binding sites. In addition to the three orientations of epsilon during catalysis we observed different orientations of the inactive enzyme. A statistical analysis of FRET states shows asymmetric distributions of epsilon orientations for active and inactive states.

I-B: Ion Motive Redox Systems

I-B p-1. Tetrahydro derivatives mimic NAD(P)H at the hydride transfer site of transhydrogenase

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Transhydrogenase, from bacterial and mitochondrial membranes couples the redox reaction between NAD(H) and NADP(H) to proton translocation. It comprises three components: dI, which binds NAD(H), and dIII, which binds NADP(H), protrude from the membrane, whereas dII spans the membrane. 1,4,5,6-tetrahydro analogues of NAD and NADP were synthesised by reduction of NADH and NADPH, respectively, with dihydrogen over a palladium catalyst and then purified by ion-exchange chromatography. Both analogues inhibited ''reverse'' transhydrogenation by the intact enzyme and ''cyclic transhydrogenation'' by complexes made from recombinant dI and dIII. The two nucleotides were completely redox-inactive. The binding affinity of tNAD to isolated dI (measured by microcalorimetry) was similar to that of NADH (Kd ~ 20 μM). Experiments on the fluorescence emission of an engineered Trp residue showed that, like NADPH, the tNADP analogue bound very tightly to isolated dIII. Crystal structures of dI–dIII complexes were previously solved for proteins with either both nucleotides oxidised or both reduced (to block hydride transfer). Crystallisation of complexes with a combination of either tHNAD and $NADP^+$ or NAD^+ and tHNADP may yield structures whose hydride-transfer site more closely resembles that during physiological turnover. Preliminary X-ray data support this view.

I-B p-2. Two genes encoding the terminal oxidases from *Starkeya novella*

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The *Starkeya novella* (formerly *Thiobacillus novellus*, a sulfur oxidizing bacterium) cytochrome *c* oxidase has cytochrome *c* oxidase which shows clear proton pump activity in addition to electron transfer across the membrane. This purified enzyme is composed of two subunits which homologous to the two largest subunits of mitochondrial cytochrome *c* oxidase and shows an abnormal behavior. The effects of several nucleotides on the activity and molecular aspects of the oxidase were investigated. The oxidase which occurred as a dimer of the minimal structural unit (cytochrome aa_3) in the presence of 0.5% Tween-20 dissociated to monomers of the unit (cytochrome a_3) on addition of ATP, and a sigmoidal [S]-v curve in the oxidation of ferrocytochrome *c* catalysed by the dimeric oxidase became hyperbolic on addition of ATP. However, neither change of the molecular mass nor of the [S]-v curve was observed on addition of ADP, AMP, GTP, CTP or UTP in place of ATP. In order to analyze the mechanism of this enzyme, two operons coding for the genes of cytochrome *c* oxidases from *S. novella* were isolated from the genomic libraries and sequenced. The amino acid sequences deduced on the basis of their base sequences showed two kinds of the terminal oxidases were present in the bacterium: Alignment analysis among other species shows both *S. novella* oxidases have the highest homology with *Bradyrhizobium japonicum* (nitrogen fixable bacterium with soybeans) cytochrome *c* oxidases. The order of aa_3-type reading frames was coxB (encodes subunit II)-coxA (subunit I)-coxE (heme O synthase)-coxF (unknown)-coxG (assembly protein)-coxC (subunit III), and bb_3-type, in the case of *B. japonicum*, reading frames was coxP (subunit II)-coxO (subunit I)-coxN (subunit IIIA)-coxM (subunit IIIB). Although their amino acid sequences were respectively homologous to those of the oxidases from other organisms, the two sequences did not show high homology to each other. CoxB contained a partial sequence which was in perfect agreement with the N-terminal amino acid sequence of the subunit II of the purified oxidase.

I-B p-3. The binding of nucleotides to the intact proton-translocating transhydrogenase studied by calorimetry and FTIR spectroscopy

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Transhydrogenase, from bacterial and mitochondrial membranes couples the redox reaction between NAD(H) and NADP(H) to proton translocation [Jackson, J.B. FEBS Lett. 2003, 545, 18]. Using isothermal scanning calorimetry, we have measured heat changes accompanying the binding of NAD^+, NADH, $NADP^+$ and NADPH to detergent-dispersed, intact *E. coli* transhydrogenase. Kd values were 50, 400, 14 and 0.9 μM respectively. Nucleotide binding affinities were greatly lowered in ''dead-end'' complexes. IR spectral changes in thin films of intact transhydrogenase were measured by ATR-FTIR spectroscopy in nucleotide-perfusion experiments. Nucleotide binding to the protein was indicated by the appearance of characteristic absorbance bands. The nucleotide IR bands were sharpened relative to those in aqueous solution, reflecting a restriction to motion and a change in environment upon binding; some were subjected to a considerable shift indicating specific protein–ligand interactions. Importantly, concentration dependences approximately correlated with the Kd values for the different nucleotides listed above. This shows that nucleotide-binding affinity was not greatly affected by incorporation of transhydrogenase into the film. Nucleotide binding also caused changes in the amide I and amide II regions of the spectrum and led to a preliminary indication of changes in IR absorbance due to amino acid side chains in the protein. The reaction mechanism of hydride transfer will be discussed based on the present results with available X-ray crystal structures [Cotton, N.P.J. et al, Structure 2001, 9, 165].

I-B p-4. Crystal structure analysis of spinach plastocyanin mutants explain reactivity changes towards cytochrome *f* and photosystem 1

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Plastocyanin (Pc) transfers electrons from cytochrome *f* to photosystem 1 (PS1) in most oxygenic photosynthetic organisms. The electron transfer (ET) is mediated by His87 situated in the hydrophobic surface in the north region of Pc. We have studied the small but significant structural changes in this region for two different mutants using X-ray crystallography. The structure of the spinach G8D Pc mutant in the oxidized and reduced forms has been determined at pH 4.5–1.35 and 1.25 Å resolution, respectively. The overall fold of the Pc molecule remains the same during the oxidation–reduction process. There are not any large differences in the backbone conformation between the two forms of the protein except in the vicinity of Gln88 and Glu89 where the backbone in addition shows two conformations. The other protein studied, G8D/L12E [Jansson et al, (2003) Biochim. Biophys. Acta, 1607, 203], is a double mutant of Pc where residue 12, close to His87, is changed to Glu in addition to the G8D mutation introduced previously to enable crystallization [Xue et al. (1998) Protein Sci, 7, 2099]. The L12E mutation severely disturbs the ET from cytochrome *f* and to PS1. The crystal structure of G8D/L12E has been determined to 2.0 Å resolution. A comparison with the G8D structure reveals that structural differences are limited to the site of the L12E mutation. In particular, there is a small but significant change in the hydrophobic surface close to His87. This change is similar in magnitude to the one induced by a change in the redox state (see above). Our conclusion is that the affinities between Pc and its redox partners is highly dependant on subtle changes in the protein surface probably because of a disturbance of the complementarity between the proteins.

I-B p-5. Coupling through the "hinge" region: roles of individual amino acids in helix 13 and 14 of domain II in *E. coli* transhydrogenase

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The membrane domain, domain II, of proton-translocating *E. coli* transhydrogenase has not been structurally determined but a topology model of the 13 transmembrane helices has been established (1). Four of the helices, i.e., helices 3, 9, 13 and 14 consist of conserved amino acids to a high extent, 50–80%, and are therefore suggested to be involved in the formation of the proton channel in which a proton is translocated linked to the reversible redox reaction between NAD(H) in domain I and NADP(H) in domain III (2). Residue bD213 in the loop preceding helix 13 and the region following helix 14, including bR265, form a strongly conserved so called "hinge" region. The bD213–bR265 salt bridge is important in the reaction mechanism and mediates a conformational communication between domain II and domain III (3). Residue bN222 in helix 13 has an essential role in the proton translocation with an inhibitory affect on transhydrogenation and proton pumping activities to 5–20% if mutated (4). To elucidate the roles of individual amino acids in helix 13 and 14, which are the two most conserved a–helices in domain II, all residues between bP215–bS260 were replaced with a cysteine in a cysteine-free transhydrogenase. Activity measurements and the accessibility of cysteine residues introduced in dII to cysteine specific probes in the absence and presence of NADPH support the proposal (3) that a conformational communication occur between dII and dIII.

Keywords: Transhydrogenase; Cysteine scanning; Membrane protein

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I-B p-6. Permeability of the inner membrane of rat liver mitochondria to univalent cations was increased by thallium (I)

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Effects of $TlNO_3$ on swelling and respiration of isolated rat liver mitochondria were studied in the media containing 125 mM nitrate (KNO_3 or $NaNO_3$ or NH_4NO_3) or 250 mM sucrose. Swelling of nonenergized mitochondria rose with increase of TlNO3 concentration from 25 to 75 mM. The swelling increased in the series of sucrose $< KNO_3 < NaNO_3 < NH_4NO_3$ at the same thallium concentration. In all media used, T^{l+} has stimulated the state 4 respiration. With increase of $TlNO_3$ concentration, the 2,4-dinitrophenol (DNP)-uncoupled mitochondrial respiration rose in the sucrose medium, but, on the contrary, it was reduced in the nitrate media. 75 mM T^{l+} did not affects the state 3 respiration of mitochondria incubated in sucrose medium but the respiration was markedly reduced in experiments with the nitrate media. It is concluded that similarly to divalent heavy metals and regardless of mitochondrial energization, T^{l+} enhances permeability of the inner membrane to monovalent cations. However, T^{l+}, unlike divalent heavy metals, does not affect mitochondrial respiratory enzymes. It is suggested that the T^{l+}-induced decrease in the state 3 and DNP-stimulated respiration in experiments with the nitrate media may be due to a swelling-induced modification of spatial structure of the inner mitochondrial membrane (Doklady Biochem. Biophys. (2001) 378: 145–149).

I-B p-7. Preliminary characterization of a Na^+-dependent NADH:quinone oxido-reductase from *Marinobacter hydrocarbonoclasticus*

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Introduction: The marine bacterium Marinobacter hydrocarbonoclasticus ATCC 50418 (MH) was isolated from sediments collected in the gulf of Fos (French mediterranean coast) at the mouth of a petroleum refinery outlet polluted by hydrocarbons. This Gram-negative bacterium is a facultative aerobe hydrocarbon-degrading organism with a strict Na^+ growth requirement, but little is known about its bioenergetics under aerobic conditions.

Materials and methods: Cells were aerobically grown at 37 C in artificial sea water containing 0.6% yeast extract. NADH:O_2 activity was carried out on 50–100 nm diameter vesicles obtained by sonication of the purified membrane fraction while the NADH:menadione and NADH:Q1 oxido-reductase activities were assayed on an enzyme-enriched fraction obtained by extraction of native membranes with lauryl maltoside. Activities were assayed either polarographically or spectrophotometrically at 340 nm and at 25 C.

Results: The NADH:O_2 and NADH:quinone oxido-reductase activities were stimulated by monovalent cations including Li^+, Na^+ and K^+, with a maximal specific activity in the presence of 0.6 M NaCl. NADH oxidase activity of membrane vesicles was insensitive to CCCP (>0.08 mM) and amiloride, but stimulated by valinomycin indicating electrochemical transmembrane coupling arising from NADH-linked vectorial transport of ions different from protons. The steady-state initial rates displayed an hyperbolic behaviour at alkaline pH, with Vmax of ca. 20 μmol/mg/min, and Km values of 80–135 μM for the NADH:O_2, NADH:menadione and NADH:Q1 oxidoreductase activities. NADH:O_2 activity was sensitive to classical respiratory inhibitors but was, however, only slightly inhibited by very high concentrations of rotenone and capsaicin (100 μM), yet very sensitive to HQNO (a mixed-type inhibitor with a *K*I of 3.2 μM), Ag^+ (a noncompetitive inhibitor with a *K*I of 0.95 μM) and korormycin which are known to be strong specific inhibitors of Na^+-translocating NADH:quinone oxidoreductases. The results strongly suggest the presence in MH of a Na^+-NQR type NADH:quinone oxidoreductase that functions at alkaline pH.

Acknowledgements: Work supported by PRIN "Bioenergetica: Aspetti Genetici, Biochimici e Fisiopatologici" from MIUR of Italy. We thank Dr. Kyoko Adachi (Japan) for providing a koromycin sample.

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I-B p-8. Distance and orientation studies of the Cu_A fragment of cytochrome *c* oxidase with different cytochrome *c* by pulsed epr spectroscopy

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Whereas many bacteria have more than one terminal oxidase, up to six, in the respiratory chain, they and eukaryotes have only one cytochrome *bc* complex [1]. Among the subunit components of the *bc* complex, both cytochrome *b* and Rieske Fe/S protein are widely conserved in the organisms, while *c*-type cytochrome subunit is highly diverged into several classes, some of which show no sequence similarity to the class of cytochrome c_1 [2]. This finding is compatible to the view that the functional and evolutionary core of the *bc* complex is cytochrome *b* plus the peripheral domain of the Rieske Fe/S protein, and that different *c*-type cytochromes have been recruited several times independently during the molecular evolution.

In archaea, *c*-type cytochrome in general has been reported only for limited number of species, such as halophiles and a thermoacidophile, in contract to *a*/*o*-type and *b*-type cytochromes, which seem ubiquitous in the respiratory chains of archaea, Focusing on the homologues of cytochrome *bc* components, cytochrome *b* and Rieske Fe/S proteins are present in some archaea such as Sulfolobus species and compose supercomplexes with oxidase subunits, whereas cytochrome *c*-type components are missing even in those organisms. *Aeropyrum pernix* is a hyperthermophilic aerobic crenarchaeon isolated from a sea in Japan and the whole genome sequence has been reported. Here, we isolated a *c*-type cytochrome in the membrane fractions of the cell and identified it as the cytochrome *c*-type subunit of the *bc* complex. The structural gene contains CXXCHXnM motif but does not show high sequence similarity to cytochrome c_1 or the other classes of bacterial or eukaryotic *c*-type components.

Keywords: Cytochrome *bc* complex; *c*-type cytochrome; Extremophile; Archaea; *Aeropyrum pernix*

I-B p-9. Stability and folding of DIII from *E. coli* transhydrogenase in the presence of NADPH or $NADP^+$

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A rapidly growing research field of today deals with the question how proteins fold into a functional, three dimensional structure. In the present investigation the unfolding and refolding properties of the soluble domain dIII of nicotinamide nucleotide transhydrogenase (EC 1.6.1.1) from *E. coli* were determined. The processes were investigated in the presence of two different substrates, NADPH and NADP.

Nicotinamide nucleotide transhydrogenase (TH) is a membrane-bound enzyme located in the cytoplasmic membrane of bacteria and in the inner membrane of the mitchondria.

The enzyme catalyses the reversible reduction of $NADP^+$ by NADH coupled to a proton translocation across the membrane. TH consists of three domains, domain I and III that binds NAD(H) and NADP(H), respectively, and the proton translocating, membrane spanning domain II. For a review see [1]. The NADP(H) binding domain of *E. coli* TH, ecIII, contains 177 amino acids essentially adopting an nucleotide-binding fold. The structure is composed of an open twisted six-stranded parallel β-sheet which is flanked by helices on both sides of the sheet [2].

The folding properties of ecIII + NADPH or NADP have not been studied previously and, furthermore, the protein also represents a generally interesting case for investigating the correlation between ligand binding affinity and protein stability. We demonstrate that the higher affinity for NADPH is reflected as a slightly higher stability for the ecIII + NADPH complex compared to the ecIII + NADP complex, as indicated by the free energy of folding and midpoint of transition. However, the exposed surface area upon transition is not changed whether NADPH or NADP is bound.

We also conclude that the parameters describing the folding/refolding properties of ecIII are independent of the methods of use, i.e., far UV- circular dichroism (CD) or intrinsic tryptophan fluorescence.

Supported by the Swedish Research Council.

Keywords: Transhydrogenase; Folding; NADPH; NADP

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I-B p-10. Towards a test of the alternating-site mechanism for transhydrogenase

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Transhydrogenase, from bacterial and mitochondrial membranes couples the redox reaction between NAD(H) and NADP(H) to proton translocation. The coupling is achieved through conformational changes. Transhydrogenase comprises three components: dI, which binds NAD(H), and dIII, which binds NADP(H), protrude from the membrane, whereas dII spans the membrane. The protein is effectively a ''dimer'' of two dI–dII–dIII ''monomers'', although the subunit composition varies amongst species. Based on asymmetries in crystal structures of a complex formed from recombinant dI and dIII, we proposed an alternating-site mechanism for transhydrogenase: the conformational changes responsible for coupling run 180o out of phase in each monomer [Biochemistry 41, 4173–4185 (2002)]. We are attempting to test the hypothesis by creating dimers in which one monomer is wild-type and the other is inactivated by mutation. If the monomers are cooperative, as in an alternating-site mechanism, the ''heterodimer'' would be inactive but, if the monomers operate independently, then it would be 50% active. We are exploiting the *R. rubrum* transhydrogenase where, unlike in the *E. coli* and mammalian enzymes, the dI component exists as a separate polypeptide (it forms a dimer) that can be reversibly dissociated from dII–dIII. We have found that dI reaches binding equilibrium with dII–dIII in bacterial membrane vesicles in <30s; that is, the system is eminently suited to our experiements. Wild-type dI can be denatured and refolded to give a protein which can fully reconstitute dI-depleted membranes. Denatured and refolded mixtures of wild-type dI and the Q132N mutant of dI are only partially active in reconstitution. However, uncertainties over the relative binding affinities of wild-type dI homodimers, mutant dI homodimers and mutant/wild-type dI heterodimers prevent us from discriminating between the alternating- and independent-site models from these data. We are now constructing tagged proteins in which pure mutant/wild-type dI heterodimers can be isolated and these should provide an unequivocal test.

I-C: Mitochondrial NADH–Ubiquinone Oxidoreductase

I-C p-1. NADPH binding to the accessory 39 kDA subunit of complex I from *Yarrowia lipolytica*

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NADH: ubiquinone oxidoreductase (complex I) is the first and most complicated enzyme in the mitochondrial respiratory chain. The purified enzyme from the aerobic yeast *Yarrowia lipolytica* is composed of about 40 polypeptides: in addition to the 14 central subunits, 23 accessory subunits were identified [1]. We analysed one of these extra subunits which is homologous to the 39 kDa subunit of bovine heart complex I and is related to the SDR-enzyme family. The members of this family function in different redox and isomerization reactions and contain a conserved NAD(P)H-binding site[2]. It was proposed that the 39 kDa subunit may be involved in a biosynthetic pathway [3].

To test this hypothesis, we have mutated residues in the putative nucleotide binding site of the 39 kDa subunit. The mutation of a basic residue at the end of the second b-strand of the nucleotide binding fold that is responsible to the selective interaction with the 2′-phospate group of NADPH leads to impaired binding of the nucleotide, while 60% of the ubiquinone reductase activity in the mitochondrial membranes is retained.

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I-C p-2. AMP kinase is involved in the regulation of cardiac mitochondrial function

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AMP-activated protein kinase (AMPK) is involved in the homeostasis of energy metabolism by phosphorylating key effectors of glucose and fatty acid oxidation. As a metabolic sensor, it has been associated with mitochondrial biogenesis in skeletal muscle in response to exercise training. AMPK is phosphorylated and activated in pressure-overload cardiac hypertrophy and following exercise. Newly engineered mice, inactivated for the $AMPKa_2$ subunit ($AMPKa_2 -/-$) exhibit impaired insulin secretion and insulin sensitivity in vivo (Viollet et al 2003 JCI 111, 91–98), but nothing is known concerning cardiac mitochondrial function. Nine month-old deficient mice and their littermate control were used to investigate whether the $AMPKa_2$ deletion alters mitochondrial capacity, regulation and substrate utilization in the heart. There was no sign of cardiac hypertrophy between control and $AMPKa_2 -/-$ mice. Oxidative capacity and mitochondrial regulation were assessed on saponin permeabilized cardiac fibers. When the physiological substrates pyruvate or octanoate were used, significant ($\gg 30\%$) decrease in respiration rates were observed in $AMPKa_2 -/-$ mice. Respiration rate was significantly lower in $AMPKa_2 -/-$ with glutamate/malate (through complex I of the respiratory chain) as substrate (12.6 ± 1.2 vs. 16.8 ± 1.5 μmol O_2/min/g dry weight in control mice, $P < 0.05$), but was significantly higher with succinate (complex II) as substrate (23.2 ± 1.7 vs. 16.7 ± 1.3 μmol O_2/min/g dw in control, $P < 0.05$), suggesting a compensatory mechanism to the decreased complex I activity. Mitochondrial regulation by ADP and creatine was not affected in $AMPK -/-$ mice. Changes in the functional state of complex I and II of the respiratory chain were not accompanied by changes in the maximal activity of these complexes or of citrate synthase or cytochrome oxidase, suggesting that the regulation of these complexes is altered rather than their actvity. These results suggest that $AMPKa_2$ subunit is involved in regulating oxidative capacity and mitochondrial substrate utilization in cardiac muscle.

I-C p-3. Projection structure of the membrane domain of *E. coli* complex I

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The hydrophobic membrane domain was isolated from intact *E. coli* complex I by a novel procedure. Well-ordered two-dimensional crystals were obtained after reconstitution with a mixture of synthetic and *E. coli* lipids. Crystals belong to the space group p22121 and consist of crystallographic dimers with alternating orientations in the lipid bilayer. Projection maps of negatively stained crystals were calculated at 20 Å resolution. Comparison of these maps with previously published structures of *Neurospora crassa* complex I (Hofhaus, Weiss and Leonard, (1991) J. Mol. Biol. 221, 1027–1043) allowed us to suggest the likely position of the peripheral arm in the intact complex. Our projection structure is consistent with the distal location of the two large antiporter-like subunits NuoL and NuoM, as proposed from fragmentation studies (Holt, Morgan and Sazanov, (2003) J. Biol. Chem. 278, 43114–43120).

I-C p-4. Localization of the 39 KDa subunit from complex I from *Yarrowia lipolytica* and *Bos taurus*

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A characteristic feature of NADH:ubiquinone oxidoreductase (complex I) is its large number of subunits. The simplest form known so far exists in bacteria and consists of 14 different subunits. Homologues of these 14 ‘core’ subunits are found in all complex I species and contain all the redox cofactors of the enzyme and its substrate binding sites. In eukaryotes, a large number of subunits, with a less well understood role, add up to more than 35 subunits and a mass of about 1 MDa. From these ‘accessory subunits’, the 39-kDa subunit was shown to harbor a NAD(P)H binding site.

We studied the enzyme using electron microscopy and single particle image analysis. The structure shows the overall well known L-shape formed by a membrane associated arm and a matrix arm having roughly the same length. Using monoclonal antibodies we located the 39-kDa subunit within the *Yarrowia lipolytica* and *Bos taurus* complex I. For both studied species the epitope of the 49-kDa subunit was located on the cytosolic side in the junction between the two arms.

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I-C p-5. New insights on complex I subunit conservation among eukaryotes by investigating the *Chlamydomonas* complex I composition

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The mitochondrial complex I is the most intricate membrane-bound enzyme of the respiratory chain: 45 subunits were previously identified in the bovine enzyme. By combined genomic and proteomic approaches, we have attempted here to characterize the subunit content of the complex I from the unicellular green alga *Chlamydomonas reinhardtii*. Analysis by BN-PAGE coupled to tandem mass spectrometry (MS) has allowed the identification of 30 proteins in the *Chlamydomonas* enzyme. Comparison of the known complex I components from higher plants, mammals, worms and fungi with the MS data set and the translated sequences from the algal genomes revealed that the complex I in *Chlamydomonas* might be composed of 42 proteins for a total molecular mass of about 1000 kDa.

By compiling recent data, it appeared that a set of 27 proteins are widely conserved in eukaryotic complex I. We propose here five new subunit families (ESSS, PFFD, B12, B14.7, B16.6), extending to 32 the number of widely conserved complex I components. In parallel, many subunits seem to be specific to each eukaryotic kingdom (animals, fungi or plants). Whether some of these proteins represent less-conserved homologues, structural counterparts or are real lineage-specific components that would have been acquired or lost during the evolution in specific groups will be discussed.

I-C p-6. The phosphorylation of subunits of complex I from bovine heart mitochondria

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In bovine heart mitochondria and in submitochondrial particles, membrane associated proteins with apparent molecular weights of 18 and 10 kDa become strongly radiolabelled by [32P]-ATP in a cAMP dependent manner. In the presence of cAMP-dependent protein kinase, subunits of purified bovine complex I with the same apparent molecular weights are also phosphorylated. The phosphorylation sites of these subunits of complex I have been analysed by both solid-phase Edman degradation and mass spectrometric methods. By analysis of the radioactive 18 kDa band from both mitochondria and complex I, it was shown that serine-20 of subunit ESSS was phosphorylated uniquely, and that, contrary to previous proposals, subunit AQDQ was not phosphorylated. In the radioactive 10 kDa band, subunit MWFE was phosphorylated on serine-55. The possible effects of phosphorylation of these subunits on the activity and/or the assembly of complex I remain to be explored.

I-C p-7. Effects of complex I inhibitors on ROS production in beef heart submitochondrial particles

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The molecular mechanism of catalysis of Complex I is not completely understood principally due to lack of detailed structural information: it is the only enzyme of the respiratory chain to remain a "L shaped black box". The FMN is the entry point for electrons from NADH, while N_2 iron–sulfur cluster is considered to be the direct electron donor to ubiquinone. It is most likely located in the connection between the peripheral and the membrane arm suggesting that the ubiquinone headgroup could somehow reach up into the peripheral arm (Brandt et al. FEBS Lett. 2003, 545, 9–17) Complex I produces reactive oxygen species (ROS): N_2 was suggested to be the site of electron leak(Genova et al. FEBS Lett.2001,505,364–368). We have studied the effect of different Complex I inhibitors on ROS production to elucidate the mechanism by which N2 transfer electrons to coenzyme Q. The working hypothesis it is that during normal redox cycle the electron leak from Complex I is very low: it can be increased by the presence of inhibitors and enhanced by partial reduction of Coenzyme Q. We have tested ROS production from Complex I in presence of Rotenone and /or Coenzyme Q1 in beef heart submitochondrial particles following the fluorescence increase due to the oxidation of H2DCF. Rotenone blocks the electron flow from N_2 to CoQ pool so that with high NADH and in presence of rotenone N_2 is fully reduced allowing electron escape to oxygen as shown by the increase in the DCF fluorescence. There is a common agreement on the capability of CoQ_1 to interact with the physiological CoQ site: if N_2, in presence of rotenone, can reduce by one electron CoQ_1, its prooxidant activity should be enhanced. When inhibitors are absent control SMP and those treated with CoQ_1 show a similar pattern, while the effect of CoQ_1 is completely different if the inhibitor used is Antimycin A or Rotenone. In presence of Antimycin A CoQ_1 shows a protective effect, while in presence of Rotenone it induces a strong increase in ROS production. pHMB, that prevents the reductions of the iron–sulphur clusters, prevents ROS production confirming that the electrons escape site is related to N_2. We have checked the effect of different inhibitors on the ability of Complex I to produce ROS: Piericidine A and rolliniastatin-1 and -2 behave like Rotenone, whereas Stigmatellin, capsaicin, Coenzyme Q2 prevent the oxidation of DCF suggesting different sites of interaction.

I-C p-8. The redox-Bohr group associated with iron–sulphur cluster N2 in complex I

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The mitochondrial NADH:ubiquinone oxidoreductase or complex I (EC 1.6.99.3) translocates four protons per two electrons transferred from NADH to membrane ubiquinone [1]. The mechanism of this process and the number of redox centers participating in proton transfer still remain unclear. One of the most considered candidates is the iron–sulphur cluster N_2. Due to the pH dependence of its redox midpoint potential, N_2 was predicted to function in combination with a redox-Bohr group potentially accomplishing redox-dependent H^+ translocation.

Mutation of the histidine 226 of the 49 kDa subunit of complex I from Yarrowia lipolitica to methionine resulted in pronounced decrease of the redox midpoint potential of cluster N_2 and absence of its pH-dependence [2]. However, these change had only minor effects on electron transfer rates. We thoroughly investigated the proton translocating properties of wild type and mutant complex I. Both enzymes reconstituted into proteoliposomes exhibited no difference in their capacity to translocate protons across the membrane. The proton to electron stoichiometry of complex I measured in intact mitochondria was 4 H^+/2e- for both wild type and mutant strain.

We conclude that histidine 226 of the 49 kDa subunit is the redox-Bohr group associated with cluster N_2, but that this group is not directly involved in proton pumping.

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I-C p-9. Kinetic evidence for supramolecular assembly of the respiratory chain complexes I–III in bovine heart mitochondria

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The model of the respiratory chain where the enzyme complexes are independently embedded in the lipid bilayer of the inner mitochondrial membrane and are connected by randomly diffusing molecules, Coenzyme Q and cytochrome *c*, is mostly favored. However, multicomplex units can be isolated from mammalian mitochondria suggesting a model (the respirasome) based on direct electron channeling between complexes [Schägger H. et al. (2001) J. Biol. Chem. 276, 37861]. Kinetic testing using flux control analysis can discriminate between the two models [Lenaz G. (2001) FEBS Lett. 509, 151]: the former model implies that each enzyme may be rate-controlling to a different extent and that the sum of all flux control coefficients for the different enzymes should be 1 [Kacser A. et al. (1979) Biochem. Soc. Trans. 7, 1149] while in the latter the whole metabolic pathway would behave as a single supercomplex and inhibition of anyone of its components would elicit the same flux control. In particular, in the absence of other components of the oxidative phosphorylation apparatus (i.e., ATP synthase, membrane potential, carriers), such as in open nonphosphorylating submitochondrial particles (SMP), the existence of a supercomplex would elicit a flux control coefficient near unity for each respiratory complex and the sum of all coefficients would be well above unity [Kholodenko N.B. et al. (1993) FEBS Lett. 320, 71].

We investigated uncoupled bovine heart mitochondria (BHM) and SMP, devoid of substrate permeability barriers, for flux control analysis of Complexes involved in aerobic NADH oxidation (I, III, IV) and in succinate oxidation (II, III, IV). The flux control coefficients (Ci) were determined measuring the fractional change of either NADH or succinate oxidase activity induced by the infinitesimal change in the specific activity of each respiratory complex by titration with rotenone, carboxin, mucidin and cyanide as inhibitors of Complexes I, II, III and IV, respectively.

Flux control analysis over aerobic NADH oxidation in SMP indicates that Complex I is largely rate controlling ($Ci = 0.91$) while the level of control exerted by Complex III is intermediate ($Ci = 0.61$) and that of Complex IV is extremely low ($Ci = 0.06$). Threshold plots of residual NADH oxidase activity as a function of percent inhibition of each complex are almost linear for Complex I and III, but have a threshold at about 60% inhibition for cytochrome *c* oxidase.

We report kinetic evidence strongly suggesting the existence of functionally relevant association only between Complex I and III while Complex IV appears randomly distributed. It is possible that if any stable interaction with Complex IV exists, it escaped detection in our experimental samples most likely due to a pronounced abundance of not assembled molecules. Moreover, Complex II is rate limiting over succinate oxidation and clearly there is no channeling towards Complex III and IV.

I-C p-10. Monitoring the redox coupled conformational change in the NADH:ubiquinone oxidoreductase (complex I) from *E. coli* by means of FTIR difference spectroscopy and site directed mutagenesis

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The NADH:ubiquinone oxidoreductase, also known as respiratory complex I, couples the transfer of electrons from NADH to ubiquinone with the translocation of protons across the membrane. The working hypothesis is that redox-driven proton-translocation is at least partly accomplished by conformational changes. We used redox induced FTIR difference spectroscopy [1, 2] to describe the perturbation induced by mutations of a number of conserved amino acids on subunit NuoB. The method monitors the reorganization of the cofactors and their binding site upon electron transfer, including conformational changes in the protein and protonation of side chain groups.

The mutation E67N did not only prevent electron transfer, but also the conformational rearrangement of the complex during redox reaction. Electron transfer and the conformational rearrangement were restored in the E67D mutant enzyme. Replacement of C_{64}, a putative ligand of Fe/S cluster N_2, by either serine or alanine led to a complete loss of N_2, of the enzymatic activity, and of the conformational rearrangement. The coherence between proton translocation, electron transport, and conformational changes was further confirmed by the pH-dependence of the electrochemically induced reaction [3].

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I-C p-11. Reconstruction of human pathogenic mutations in complex I from the yeast *Yarrowia lipolytica*

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We have used the obligate aerobic yeast *Yarrowia lipolytica* to reconstruct and analyse human pathogenic mutations in nuclear coded subunits of mitochondrial complex I. An exon 2 skipping mutation in the human NDUFV2 gene which removes part of the mitochondrial targeting sequence and the processing site from the 24 kDa subunit precursor had been found to cause hypertrophic cardiomyopathy and encephalomyopathy [1]. By construction of a similar presequence deletion in the orthologous NUHM gene of *Y. lipolytica* we could show that neither mitochondrial import nor assembly into functional enzyme are impaired when a presequence remnant is retained on the mature 24 kDa subunit. Using site directed mutagenesis we had identified residues within the homologues of bovine subunits 49 kDa and PSST that interact with ubiquinone and quinone-like inhibitors. Based on these observations, we had proposed that a significant part of the ubiquinone binding pocket of complex I has evolved from the catalytic site of evolutionary related [NiFe] hydrogenases and that the structural fold has been conserved between the two enzymes. [2]. Significantly, several human pathogenic mutations have been discovered in recent years [3–5] which according to this "hydrogenase model" map close to the catalytic core of complex I. After reconstruction in *Y. lipolytica*, altered Michaelis–Menten parameters or reduced stability were observed in many cases, providing useful clues on the biochemical basis of the observed disease symptoms.

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I-C p-12. Disruption of conserved "accessory" subunits from neurospora crassa complex I

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The respiratory chain of the mitochondrial inner membrane includes a proton-pumping enzyme, complex I, which catalyses electron transfer from NADH to ubiquinone. This electron pathway occurs through a series of protein-bound prosthetic groups, FMN and around eight iron–sulphur clusters. *N. crassa* has been a useful genetic model to characterise complex I, a multisubunit enzyme compose of around 35 polypeptides of dual genetic origin [1]. The characterisation of mutants in specific proteins helped to understand the elaborate processes of the biogenesis, structure and function of this oligomeric enzyme. Ongoing with this work, we present the characterization of two new complex I mutants. We concentrated in subunits that are present in mitochondrial complex I, but absent from the bacterial "minimal" form of the enzyme, therefore known as "accessory" subunits.

The *N. crassa* 11.5 kDa polypeptide is homologous to the IP15 subunit of bovine complex I and contains four conserved cysteine residues that might be relevant for its function. The *N. crassa* 14 kDa polypeptide, homologue of bovine B16.6, is similar to the cell-death regulator GRIM-19, whose expression is induced by interferon-β and retinoic acid [2]. We have disrupted the fungal 11.5 and 14 kDa proteins and show that both subunits are required for the assembly/stability of complex I.

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I-C p-13. The rotenone-sensitive NADH:menaquinone oxidoreductase from the respiratory chain of *Rhodothermus marinus*

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The rotenone sensitive NADH:quinone oxidoreductase (Nqo) (complex I) is the first segment of most aerobic respiratory chains, catalysing the NADH:quinone oxidoreductase reaction, with the concomitant proton, or in some cases sodium, translocation across membranes. This is the largest enzyme of aerobic electron transport chains, and this far, the least understood. The bacterial protein is generally composed of 14 subunits, the so-called minimal functional unit, comprising all the prosthetic groups present in the eukaryotic counterpart, namely the iron–sulphur clusters and the dinucleotide binding motives.

The thermohalophilic bacterium *Rhodothermus marinus* possesses a rotenone-sensitive NADH:menaquinone oxidoreductase in its aerobic respiratory chain. EPR spectroscopy from the purified complex revealed the presence of at least five iron–sulphur centers: two $[2Fe–2S]^{2+/1+}$ and three $[4Fe–4S]^{2+/1+}$ centres [1].

The genomics of the *R. marinus* complex I are discussed and further insights in the biochemistry of the enzyme are presented.

I-C p-14. Synthesis and inhibitory action of novel acetogenin mimics with bovine mitochondrial complex I

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Studies on the inhibition mechanism of acetogenins, the most potent inhibitors of bovine heart mitochondrial complex I, are useful to elucidate the structural and functional features of the terminal electron transfer step of this enzyme. We synthesized acetogenin mimics which possess two alkyl tails without a gamma-lactone ring, named delta.lac-acetogenin, and examined their inhibitory action on bovine heart mitochondrial complex I. Unexpectedly, the delta.lac-acetogenin carrying two *n*-undecanyl groups elicited very potent inhibition comparable to that of bullatacin. The inhibitory potency of delta.lac-acetogenin markedly decreased with shortening the length of either or both alkyl tails, indicating that symmetric as well as hydrophobic properties of the inhibitor are important for the inhibition. Both acetylation and deoxygenation of either or both of two OH groups adjacent to the THF rings resulted in a significant decrease in inhibitory potency. In addition, the number of THF ring(s) and the stereochemistry around the hydroxylated THF ring moiety are crucial for the potent inhibition. These structural dependencies of the inhibitory action of delta.lac-acetogenins are in marked contrast to those of ordinary acetogenins. Double-inhibitor titration of steady state complex I activity showed that inhibition of delta.lac-acetogenin and bullatacin are not additive, though the inhibition site of both inhibitors is downstream of iron–sulfur cluster N_2. Our results indicate that the mode of inhibitory action of delta.lac-acetogenins differs from that of ordinary acetogenins. Therefore, delta.lac-acetogenins can be regarded as a novel type of inhibitors acting on the terminal electron transfer step of complex I.1).

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I-C p-15. Properties of NAD(P)H dehydrogenases isolated from bell pepper chromoplasts

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NADH–ubiquinone oxidoreductase complex (NDH-I) of the aerobic respiratory chain in mitochondria and bacteria consists of at least 14 different subunits (NuoA ~ N in *E. coli*) including a peripheral diaphorase subcomplex (NuoEFG), and functions as a redox-driven proton pump for oxidative phosphorylation. Recent genetic, immunological and biochemical studies on the photosynthetic electron transfer chain of plastids and cyanobacteria indicated that a plastid-type NAD(P)H-plastoquinone (PQ) oxidoreductase (pNDH-I) is involved in cyclic electron transfer around PS I in the light and chlororespiration in the dark. Isolated pea pNDH-I (Mr, 550 kDa) has been shown to be specific to NADH (Sazanov et al. (1998) Proc. Natl. Acad. Sci. USA 95, 1319–1324), but neither NuoEFG of the respiratory NDH-I nor FNR (ferredoxin-$NADP^+$ oxidoreductase) serves as a catalytic subunit(s) of pNDH-I.

For understanding the molecular basis of membrane bound NAD(P)H dehydrogenases in plastids, we prepared chromoplasts from nonphotosynthetic tissues (i.e., red fruits) of bell pepper Capsicum anuum, and isolated two major NAD(P)H-ferricyanide oxidoreductases by anione-exchange and size-exclusion HPLC. Peak 1 enzyme (Mr, ~ 130 kDa) eluted at ~ 60 mM NaCl from a Tosoh SuperQ-5PW column showed a pH optimum below 5.0 and was more specific to NADPH (Km of 0.1 vs. 0.3 mM for NADH). Peak 2 enzyme (Mr, 300–500 kDa) eluted at ~ 120 mM NaCl showed a pH optimum at 6.0 and was specific to NADH (Km of 0.05 vs. 0.4 mM for NADPH). Both fractions catalyzed electron transfer from NAD(P)H to ubiquinone-1, and were insensitive to dichmarol, flavone, DPI, SHAM, and TTA. Peak 1 was unable to reduce artificial electron acceptors like NBT or MTT, whereas the peak 2 activity was highly sensitive to SH reagents (NEM and pCMB). These observations indicate the presence of unique NAD(P)H dehydrogenases in plastids. Identification of their subunits is currently underway.

I-C p-16. The role of detergents in two-dimensional crystallisation and maintaining the stability of intact *E. coli* complex I

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The stability of intact complex I from *E. coli* was investigated in a number of different detergents.

The intact form observed in dodecyl-maltoside (DDM) was preserved after exchange into undecyl-maltoside (UDM), CHAPS, C12E9 and C12E8. Many other detergents disrupted the complex. Exchange into diheptanoyl-phosphatidylcholine (DHPC) resulted in the removal of subunits NuoL and NuoM from the otherwise intact complex, indicating a distal location of these large antiporter-like subunits in the membrane domain (Holt, Morgan and Sazanov, (2003) J. Biol. Chem., 278, 43114–43120). Two-dimensional (2D) crystals could be produced using only DDM or UDM as solubilising detergents for the enzyme and lipids. Using DDM, well-ordered crystals were obtained in several different crystal forms, depending on lipid composition. Our results indicate that dodecyl-maltoside is the optimal detergent for 2D crystallisation of *E. coli* complex I, probably because it provides the most stable environment for this large multisubunit membrane protein.

I-C p-17. Studying the effects of LHON-associated human mitochondrial ND6 subunit mutations on complex I activity and superoxide production using the bacterium *Escherichia coli*

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Respiratory chain complex I is a multisubunit enzyme in the inner mitochondrial membrane and it catalyzes the transfer of electrons from NADH to ubiquinone with a coupled translocation of protons across the membrane. Due to its size (molecular weight about 1000 kDa), complexity (number of subunits 42–45, and up to nine iron–sulfur clusters) and the lack of high resolution structural data, little is known about its catalytic mechanism. Besides, association of many mitochondriopathies with complex I makes it a challenging object of research.

Leber's Hereditary Optic Neuropathy (LHON) is a common complex I-linked disease typically caused by a mutation in some of the mitochondrially encoded ND subunits of complex I. ND_6 subunit has been shown to be a hot spot for mutations associated with LHON (Chinnery et al. Brain 2001 Jan; 124: 209–218). Biochemical analyses of the consequences of some of the LHON-associated mutations in ND_6 have pointed out that it might be involved in complex I mechanism by contributing to the ubiquinone binding site(s). Unfortunately, the limited number of naturally occurring mutations known and difficulties in site directed mutagenesis of mitochondrial DNA make it impracticable to study the role of ND6 in complex I function in more detail. The bacterial counterparts (the NDH-1's) show structural similarity and homology to complex I, and therefore are believed to comprise the catalytic core of this complex multisubunit enzyme. Being more amenable to genetic manipulation, the NDH-1's provide a useful model system for studying mitochondrial complex I.

By means of site-directed mutagenesis in *Escherichia coli* we have produced nuoJ mutants corresponding to LHON associated sequence changes in ND6 to study their effect on enzyme function. The effect of these mutations on growth rate in malate-YE medium necessitating efficient NDH-1 linked energy production was analyzed. In addition, enzyme activities and substrate affinities were measured, and the effects of the mutations on superoxide production will be presented. Our results suggest that some of these relatively highly conserved residues in ND_6 are essential for NDH-1 linked energy production in *Escherichia coli* since growth on malate as a main carbon source was severely hampered, and that mutagenesis of them reduce respiratory activity.

I-C p-18. Neurotoxicty of dehydroepiandrosterone is associated with inhibition of NADH:ubiquinone oxidoreductase

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Although several works demonstrate the neuroprotective activities of dehydroepiandrosterone (DHEA), some reports suggest also that it may act as an inhibitor of mitochondrial respiration. This study was therefore designed to find out whether DHEA is able to inhibit the respiratory activity also in neuronal mitochondria and, if yes, then to reveal the functional significance of that inhibition. Using different mitochondrial substrates we show here that DHEA suppress the mitochondrial respiration in permeabilized cerebellar granule cell culture by inhibiting mitochondrial NADH:ubiquinone oxidoreductase (complex I of the electron transport chain; IC50 about 30 μM). This effect was specific, e.g., not related to high lipophility of steroid structure, since cholesterol and some other steroids had no effect in this concentration range. Surprisingly, only progesterone had the same inhibitory effect on mitochondrial respiratory activity. DHEA inhibited NADH:ubiquinone oxidoreductase activity at similar IC50 also in neuronal cell culture lysates. Described effect of DHEA was present also in intact neurons: treatment with DHEA was associated with increased consumption of glucose and accumulation of lactate in culture medium. Moreover, DHEA induced neuronal death in concentrations above 10 μM that was most pronounced in conditions of glucose deprivation. In conclusion, this study provides direct evidence that DHEA inhibits mitochondrial NADH:ubiquinone oxidoreductase both in cell lysates and in situ in permeabilized neurons, and that this leads to impaired mitochondrial bioenergetic capacity associated with neuronal death.

I-C p-19. Effects of disruption of NUO-29.9 complex I gene on the catalytic activities of the *Neurospora crassa* enzyme

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The eukaryotic proton-pumping NADH:ubiquinone-oxidoreductase (complex I) is a part of mitochondrial respiratory chain. Complex I catalyzes the oxidation of NADH by endogenous ubiquinone, coupled to vectorial proton translocation across the mitochondrial membrane. The structure of NADH:ubiquinone-oxidoreductase is very complex. The enzyme from *Neurospora crassa* consists of more than 30 subunits, seven of which are mitochondrially encoded, and contains FMN and several iron–sulfur clusters as prosthetic groups (1). The catalytic properties of complex I are also very complicated. It was shown that in all preparations mammalian complex I exists in two slowly equilibrating forms—active and deactivated (2). Active and deactivated forms differ in their catalytic and structural properties. Only active form can catalyze rapid rotenone-sensitive NADH–ubiquinone reductase reaction. Deactivated form is not able to catalyze this reaction, but it can catalyze oxidation of NADH by artifitial electron acceptors. Only deactivated form is sensitive to the action of SH-inhibitors. It was discovered that complex I from *N. crassa* also exhibits active/deactive transitions though with different characteristics (3). The molecular mechanism of active/deactive transitions and subunits involved in this process remain unclear. Using of *N. crassa* complex I mutants can help to clarify this mechanism.

We have studied the nuo29.9 *N. crassa* mutant lacking the nuclear encoded 29.9 kDa subunit homologous to B_{13} subunit of bovine complex I (4). We have characterized the catalytic properties and active/deactive transition process of the mutant complex I. Our results have shown that in nuo29.9 the characteristics of active/deactive transitions differ from the analogues characteristics of the wild type enzyme.

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I-C p-20. Changes in Fe–S clusters of complex I from *Escherichia coli* upon purification studied by the analysis of EPR spectra of membranes and purified complex I

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Bacterial NADH: Ubiquinone Oxidoreductases type I consist of 13–14 subunits that are homologues to core subunits of mitochondrial Complex I; thus they can be considered as a ''minimal'' model of the mitochondrial enzyme. So far *E. coli* NDH-1 is the only bacterial Complex I, which can be isolated without falling apart into subcomplexes. The amino acid sequence of subunits that contain Fe–S clusters in complexes I from *E. coli* and mitochondria show strong similarity. However, EPR spectra of purified Complex I from these sources differ significantly [Ohnishi, BBA 1364 (1998) 186–206]. A unique 2Fe–2S cluster, N1c, with an unusual gz feature close to $g=2$ was found in purified *E. coli* Complex I [Leif et al. Eur. J. Biochem. 230 (1995), and our data]. We have analyzed EPR spectra of *E. coli* membranes and preparations enriched with Complex I, where the enzyme was intact and retained its natural quinone reductase activity. Spectra of 2Fe–2S clusters in these preparations were similar to those in mitochondria and the line at $g=2$ was not found. We conclude that the NADH dehydrogenase module of *E. coli* Complex I undergoes conformational changes upon further purification, which result in a decreased stability of one of the binuclear Fe–S clusters, and in its modification. Hence, N1c is not a native property of the *E. coli* enzyme but rather the result of degradation of other 2Fe–2S clusters.

I-C p-21. Functional roles of four conserved charged residues in the membrane domain subunit NuoA of the proton-translocating NADH-quinone oxidoreductase from *Escherichia coli*

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The bacterial H^+ (Na^+)-translocating NADH-quinone (Q) oxidoreductase (NDH-1) is composed of 14 different subunits (Nqo1-14/NuoA-N). Subunit NuoA (ND_3, Nqo_7) is one of the seven membrane domain subunits which are considered to be involved in H^+(Na^+) translocation. We determined the topology of the Paracoccus Nqo7 (NuoA) subunit. The Paracoccus Nqo7 subunit is composed of three transmembrane segments (designated TM_1-3 from the N- to the C-terminus) and its N- and C-terminal regions are directed toward the cytoplasmic and periplasmic phases of the membrane, respectively. The predicted topology places two highly conserved carboxyl residues (D79 and E81, *E. coli* numbering) in the middle of the TM_2. In addition, there are two conserved charged residues (K46 and E51) in the loop 1 (between TM_1 and TM_2) that faces the periplasmic phase and is involved in human mitochondrial diseases. More recently, our cross-linking study revealed direct interactions between subunits Nqo_7 (NuoA) and Nqo6 (NuoB) and between subunits Nqo7 and Nqo_4 (NuoC) [Kao, M.-C., Matsuno-Yagi, A., and Yagi, T. (2004) Biochemistry 43, 3750–3755]. The Nqo_6 (NuoB) subunit is considered to bear center N_2 which shows the highest Em values of all known cofactors in the NDH-1. Therefore, it was of interest to clarify structural and functional roles of these conserved charged residues in the Nqo_7 (NuoA) subunit. We constructed a nuoA knock-out mutant and site-specific mutants K46A, E51A, D79N, D79A, E81Q, E81A, and D79N/E81Q in the *E. coli* NDH-1 by utilizing chromosomal DNA manipulation techniques. In terms of immunochemical and NADH dehydrogenase activity-staining analyses, all site-specific mutants are similar to the wild-type, suggesting that NuoA site-specific mutations barely affect assembly of peripheral subunits in situ. In addition, site-specific mutants showed similar deamino-NADH-K3Fe(CN)6 reductase activity to the wild type. The K46A mutation scarcely inhibited deamino-NADH-Q reductase activity. In contrast, E51A, D79A, D79N, E81A, and E81Q mutation partially suppressed deamino-NADH-Q reductase activity to 30%, 90%, 40%, 40%, and 50%, respectively. The double mutant D79N/E81Q almost completely lost the energy-transducing NDH-1 activities, but did not display any loss of deamino-NADH-K3Fe(CN)6 reductase activity. It is hypothesized that D79 and E81 are both involved in the mechanism of the coupling site 1, but they may work in a compensatory manner.

I-C p-22. The position of central subunits within complex I from *Yarrowia lipolytica*

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Complex I is the largest and least understood enzyme of the respiratory chain. There is no structural information at high resolution available to date. Electron microscopy has proven to be a valuable technique to study the overall structure of complex I from various organisms. Complex I from *Yarrowia lipolytica* shows an L-shaped structure. Improving the resolution of the electron microscopic structure may lead to the discovery of new structural features.

With the help of monoclonal antibodies it was possible to identify the position of several subunits within the complex. The 49 and 30 kDa subunits were found in the peripheral arm of complex I at a considerable distance to the membrane. This imposes major restraints onto possible proton translocation mechanisms of the enzyme [1]. Immunolabeling of the NUWM subunit that is associated with the hydrophobic subunits of complex I from *Y. lipolytica* complex I allows the unambiguous assignment of the hydrophobic and peripheral arm in the electron microscopic structure of complex I [2].

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I-C p-23. The characterisation of factors which determine the catalytic activity of complex I from bovine heart mitochondria

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Complex I catalyses the transfer of electrons from NADH to ubiquinone, with the concomitant transfer of protons across the inner mitochondrial membrane. A pure, catalytically active, and monodisperse preparation of complex I from bovine mitochondria has long been sought, and has recently been developed in this laboratory. The poster presents a comprehensive investigation of the factors that determine the presence of catalytic activity in pure preparations of the enzyme: the quinone, flavin and phospholipids which are required for complex I activity were determined. Examination of the role of ionic strength in determining the magnitude of the catalytic activity led to the discovery that Zn2+ inhibits complex I reversibly at micromolar concentrations. The pH dependence of the inhibition and the point at which Zn2+ acts in the catalytic mechanism were determined.

I-C p-24. The flavoprotein (Fp) subcomplex of NADH:ubiquinone oxidoreductase (complex I) from bovine heart mitochondria

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Complex I (NADH:ubiquinone oxidoreductase) is the first membrane-bound enzyme complex of the electron transport chain in mitochondria. Complex I from bovine heart contains 46 subunits and at least 9 cofactors. Therefore it is very complicated, and so smaller, simpler fragments of the enzyme may present excellent alternative subjects for study. The smallest fragment of complex I which can carry out NADH oxidation is the Fp subcomplex. It contains only 2 subunits (the 51 and 24 kDa subunits) and 3 cofactors (a flavin mononucleotide (FMN) and a [4Fe-4s] cluster in the 51 kDa subunit, and a [2Fe-2S] cluster in the 24 kDa subunit). This poster describes the preparation of subcomplex Fp from complex I by chaotropic resolution follwed by ion-exchange and gel filtration chromatography. Western blotting, N-terminal sequencing and mass spectrometry confirmed the presence and stoichiometry of the 51 and 24 kDa subunits. Iron and FMN analyses, UV-visible spectroscopy and electron paramagnetic resonance (EPR) confirmed the cofactor composition, and activity assays (NADH: ferrycyanide and NADH:menadione) showed that the purified enzyme is catalytically competent. So far, all available data justifies the use of an isolated subcomplex as a model for the intact enzyme. Studies of Fp by EPR snd protein-film voltammetry, and attempts to crytallise the enzyme, have now been initiated.

I-C p-25. Modulation of complex I activity by thiol derivatisation, and identification of labelled thiol residues

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NADH:ubiquinone oxidoreductase (complex I) is the largest and least understood enzyme of the mitochondrial electron transport chain. It couples the transfer of electrons to the pumping of protons across the inner mitochondrial membrane. Decreases in complex I activity have been identified in a number of medical conditions which are associated with oxidative stress, such as Parkinson's disease. The work presented here aims to correlate changes in then oxidation state of free thiols, via their derivatisation, with decrease in complex I activity, and to identify exposed thiols on the surface of the enzyme. Derivatisation with iodoacetamide results in a drastic loss of rotenone-sensitive NADH:decylubiquinone exidoreductase activity. The critical site was determined to be after the sites at which hexacyanoferrate (III), hexaammineruthenium (III), and coenzyme Q_1 (rotenone insensitive) accept electrons, but before or at the (rotenone sensitive) Q-binding site. This conclusion will be confirmed by electron paramagnetic resonance (EPR), to show whether reduction of any of the [Fe-S] clusters is affected by thiol derivatisation. The subunits containing thiols which were derivatised, as the reaction progressed, were identified by labelling with ^{14}C-iodoacetamide. Initial results suggest that labelling of the membrane-bound subunits B14.7 and ND3 corresponds with the decrease in complex I activity; other subunits such as the 51 and 75 kDa subunits are derivatised on longer timescales but exert no further effect on the activity. Confirmation of the labelled subunits and identification of labelled residues will be carried out by 2D-gel electrophoresis and by MALDI-TOF mass spectrometry.

I-D: Heme-copper Oxygen Reductases

I-D p-1. Electrometric measurements on the N131D mutant of cytochrome *c* oxidase from *Paracoccus denitrificans*

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The aa3-cytochrome *c* oxidase from *Paracoccus denitrificans* belongs to the familiy of heme-copper oxidases. These proton pumps use the free energy of oxygen reduction to water to establish a transmembrane proton gradient. Both vectorial and substrate protons are supplied by either the D- or K-channel. The mutation of the asparigine N131 to an aspartate residue, located in the D-channel, leads to a very intriguing phenotype of the enzyme: The N131D mutant retains its wildtype electron transfer and oxygen reduction activity, but its function as a proton pump is abolished (Pfitzner et al. (2000) Biochemistry 39, 6756–6762; Pawate et al. (2002) Biochemistry 41, 13417–13423). It was suggested that the introduction of the aspartate does not impede proton transfer through the D-channel, but rather decreases the protonation efficiency of an acceptor site for pumped protons at the intraprotein exit of the channel (Namslauer et al. (2003) PNAS 100, 15543–15547).

Here we report the result of electrometric measurements with the BLM (black lipid membrane) technique. This method allows the time-resolved observation of charge displacement over the membrane in the cytochrome *c* oxidase reconstituted in proteoliposomes. The reaction is started by photoactivation via the artificial electron donor tris(2,2′-bipyridyl)ruthenium-(II)-dichloride. We have investigated several steps of the catalytic cycle for the N131D mutant. For the reductive phase of the cycle, i.e., the O->E and the E->R transition, the uptake of two electrons is compensated by the uptake of two protons through the K-channel. However, no proton is pumped in the second reduction step in contrast to the wildtype (Ruitenberg et al. (2002) Nature 417, 99–102). Using an excess of hydrogen peroxide the enzyme is coverted to the F-state. Upon electron injection, only the electronic phase is observed in the electrometric measurement. The results are supported by spectroscopic data and compared to the wildtype and another D-channel mutant, D124N. It seems that both mutants show the same behaviour in the kinetic measurements of single electron injections. On the other hand the N131D mutant shows wildtype activity in term of oxygen reduction, while the D124N does not. The implications for the uncoupling mechanism are discussed.

I-D p-2. Electrogenic reactions induced by flash-activated electron withdrawal from cytochrome *c* oxidase

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Oxidation of *P. denitrificans* cytochrome *c* oxidase (COX) was initiated by laser-flash triggered, Ru(bpy)3(+)-mediated electron withdrawal from the fully reduced enzyme. Methyl viologen (MV) served as a final electron acceptor. Ru(bpy)3(+) excitation initiates electron backflow from heme a to CuA, followed by electron redistribution between the two hemes. The kinetics of the reaction was monitored by electrometric measurements of electric potential (Verkhovsky et al. (1997) BBA 1318, 6–10) generated in COX-proteoliposomes. Each pulse-generated electric transient consisted mainly of two kinetic phases: a small, positive (20–40 us, 0.5–0.8 mV) and a large, negative (2–4 ms, ~ 5 mV). The amplitude of both phases reached apparent saturation at approx. 5 mM MV, but the ratio between the two amplitudes remains nearly constant. The slow but not the fast phase was inhibited by KCN and was absent in both KM354 and DN124 proton channel mutant enzymes. The fast phase therefore was identified as the electron transfer from heme a to CuA, and the slow phase, as proton transfer that follows the electron redistribution between two hemes. The sign of the slow phase indicates movement of protons in the "right" direction and we propose that it is coupled to rereduction of the enzyme. The switching mechanism (Wikström et al. (2003) BBA 1604, 61–65) between the "pumped" and "chemical" proton transfer pathways regulated by the redox state of the hemes might explain this unusual proton transfer behaviour.

I-D p-3. Theoretical studies of cytochrome *c* oxidase:mechanisms for O−O bond cleavage and proton pumping

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Due to recent fast methodological developments, quantum chemistry can be used to study enzymatic reaction mechanisms. One major advantage with the quantum chemical approach is that mechanistic suggestions can be transformed into energetics. Quantum chemical calculations can be used to estimate the energy barriers connected with different proposed reaction mechanisms for an enzyme. If a computed barrier is too high, then the proposed mechanism has to be rejected.

This approach will be demonstrated for two mechanistic problems for cytochrome *c* oxidase, the terminal enzyme in the respiratory chain. The first problem concerns the O−O bond activation mechanism. New results for the O−O bond cleavage step will be presented, and different mechanisms will be compared. The second problem concerns the mechanism for the coupling between the exergonic reduction of molecular oxygen and the pumping of protons across the mitochondrial membrane. This latter problem is much more difficult to handle within a quantum chemical model, but it will be demonstrated how this can still be done, and how conclusions can be drawn regarding the likelihood of different mechanistic suggestions for this process. A mechanism for the proton pumping will be proposed.

I-D p-4. Characterisation of tyrosine variants from cytochrome *c* oxidase

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Cytochrome *c* oxidase is the terminal enzyme of the respiratory chain. It couples the reduction of oxygen to water with the translocation of protons across the mitochondrial or bacterial membrane. The determination of the three-dimensional structure of the enzyme from *Paracoccus denitrificans* [1] offers the possibility of site directed mutagenesis studies to investigate the relationship between the structure and the catalytic function of the enzyme, but still the mechanism of electron coupled proton transfer is poorly understood.

It has been shown by Raman spectroscopy, that in cytochrome *c* oxidase the PM-intermediate of the catalytic cycle is not a peroxy- but an oxoferryl-state [2]. To generate this state one additional electron is required, that cannot be provided by the metal centers. It is suggested that the missing electron is donated to the binuclear site by a tyrosine residue, that then forms a radical which can be detected in the PM- and F•- intermediates of the catalytic cycle[3].

One possibility to produce PM- and F•- intermediates artificially in cytochrome *c* oxidase is the addition of low hydrogen peroxide concentrations to the fully oxidised enzyme [4]. Using EPR-spectroscopy a radical species is detected is this reaction is assigned to a tyrosine residue. [5,5a] To address the question, which tyrosine residue is the origin of the radical species several tyrosine variants of subunit I are investigated.

These variants are characterised by EPR- and UV/VIS-spectroscopy, by their turnover rates and by their proton-pumping ability. From these experiments it is concluded, that the origin of the radical species appearing in PM- and F•- intermediates produced with hydrogen peroxide is tyrosine 167.

The significance of this finding for the catalytic function of the enzyme is discussed.

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I-D p-5. Non resonance Raman spectroscopic analysis of heme-copper oxidases: characterization of a heme A redox state marker

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The molecular mechanism by which redox energy is converted in proton pumping by cytochrome *c* oxidase is still object of debate as well as the role of the redox active metal center(s) involved in the energy conversion.

This study was aimed to characterize by Raman spectroscopy (ex. at 633 nm) different redox states of the enzyme. The results obtained from a systematic analysis can be summarised as follows:

- The comparison between spectra taken from the fully oxidised (CuA2 + Fea3 + CuB2 + Fea33+) and the fully reduced (CuA1 + Fea2 + CuB1 + Fea32+) purified bovine heart enzyme revealed in the former a Raman shift peaking at around at 1645 cm-1 which was completely absent in the reduced form. Identical results were obtained with purified *P. denitrificans* cyt. *c* oxidase.
- The occurrence of the differential peak at 1645 cm^{-1} in the oxidised form was confirmed in the CN-treated enzyme. Under this conditions the CN-liganded heme a3 is clamped in the oxidised state in the presence of reductant (CuA1 + Fea2 + CuB1 + Fea33+-CN).
- Changes of pH (from 5.5 to 8.0) had no effect on this spectral feature.
- The same analysis carried out on a homologous oxidase purified from the bacterium *Bacillus subtilis* (aa3 quinol oxidase) but lacking the CuA center confirmed the presence of a peak in the 1645 cm^{-1} in the CN-treated fully oxidized state of the enzyme which disappeared in the reduced mixed valence state.
- Differential Raman spectra analysis on cytochrome *c* and myoglobin confirmed the presence of the redox sensitive marker in the former six-coordinated heme c but not in the latter high spin heme.
- The Raman spectra obtained on the pyridine haemochrome extracted from cytochrome *c* oxidase was different from that recorded on the native enzyme and did not show any change between the reduced vs oxidised state.

Taken all together these results indicate that the redox-sensitive (pH-insensitive) Raman shift observed at 1645 cm^{-1} is specifically linked to transition in the valence state of native heme a in heme copper oxidases. Based on its energy features and comparison with spectra of model compounds, the observed Raman signal could be tentatively assigned to a change in the streching mode of a $-N=C-$ double bond thus candidating one of the histidinyl himidazol rings coordinating the low spin heme a iron. This subtle change in the ligation environment of Feheme a, might provide the triggering event for redox Bohr effects (pH-dependent) responsible for the H^+/e^- coupling at heme a.

I-D p-6. The timing of proton pumping in cytochrome *c* oxidase

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The flow-flash technique was used to investigate the timing of proton pumping across the membrane in cytochrome *c* oxidase (CcO) reconstituted into phospholipid vesicles. Studies using a pH-sensitive dye show that between one and two pumped protons per CcO are ejected in the oxidative half of the reaction cycle with a time constant of $\sim$ 1 ms. This is surprising as both the P to F (tau = 100 μs) and the F to O transitions (tau = 1 ms) have been shown to be associated with charge transfer, interpreted as proton pumping (Verkhovsky et al. 1997. Biochim. Biophys. Acta 1318, 6–10). One possible explanation for this discrepancy is that the protein-membrane surface acts as a local buffer trapping the emitted protons. The transmembrane transfer of the protons would then be detected as a charge transfer while the dye would detect the protons only after equilibration with the bulk solution. To determine whether or not pumped protons are taken up from the inside of the vesicles on the 100 μs time scale, CcO was reconstituted in phospholipid vesicles with dye on the inside. A comparison of the kinetics and extent of proton uptake from the inside and release to the outside of the vesicles provides information on the timing of the proton-pumping event. In addition, a comparison of these results to those obtained with CcO in detergent solution provides information on the role of the membrane surface in controlling the kinetics of proton release. Analysis of the results is in progress and will be presented on the poster.

I-D p-7. Nitric oxide reductase (NOR) from *Paracoccus denitrificans* as an O_2-reductase

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Nitric oxide reductase (NOR) from *Paracoccus denitrificans* is a membrane bound cytochrome bc complex and a member of the superfamily of heme-copper oxidases [1]. NOR receives electrons from cytochrome *c* and reduces nitric oxide to nitrous oxide, according to; $2NO + 2\bar{e} + 2H^+ -> N_2O + H_2O$. Moreover the enzyme can reduce molecular oxygen presumably to H_2O: $O_2 + 4H^+ + 4\bar{e} > 2H_2O$.

In the present work the reaction of the reduced enzyme with O_2 was studied using the flow-flash technique. In the reaction with O_2 it is shown that the binding of O_2 to reduced heme b3 is rapid, with a bimolecular rate constant of $4 \times 10^7\ M^{-1}\ s^{-1}$. Following oxygen binding, the hemes are oxidised with a pH dependent rate(the time constant is 20 ms at pH 7.5) that was found to be coupled to proton uptake from solution. Furthermore, kinetic difference spectra determining wich heme groups are oxidised at wich rate will be presented as well as results determining the endproduct (H_2O or H_2O_2) of the reaction.

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I-D p-8. ATR-FTIR spectroscopy and isotope labelling studies of the PM intermediate of *Paracoccus denitrificans* cytochrome *c* oxidase

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The structure of PM intermediate of *Paracoccus denitrificans* cytochrome *c* oxidase was investigated by perfusion-induced attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectroscopy.

Spectra of unlabelled wildtype were compared with universally ^{15}N-labelled and tyrosine ring-d4-labelled versions. Transitions from oxidized to PM state were initiated by perfusion with buffer containing CO/oxygen at pH 9 and extent of conversion was quantitated by simultaneous monitoring of visible absorption changes [Iwaki et al, Biochemistry 2003, 42, 8809]. Some assignments to heme a3 could be made and a shift at 1748/1738 cm^{-1} was observed that has been assigned to a change of environment of the protonated state of a conserved glutamic acid (E278). Further consideration was given to possible changes in the covalent His–Tyr structure that has been found in the catalytic site.

Assignments were made by comparison of data with available spectra of model compounds. In PM minus O spectrum, a trough at 1542 cm^{-1} was downshifted by 15 cm^{-1} upon ^{15}N-labelling. Tyr-d4-labelling caused a decrease in the 1542 cm^{-1} trough together with a new trough at 1438 cm^{-1}. The results strengthen the assignment of a band 1542 cm^{-1} to the protonated state of the His–Tyr in the oxidized state that is lost in PM. In the region around 1100 cm^{-1}, where CN stretching mode of His side chain is expected, two peaks at 1129 and 1106 cm^{-1} in unlabelled enzyme were downshifted by 3–4 cm^{-1} upon 15N labelling but were insensitive to Tyr-d4 labelling. These shifts are consistent with assignment to histidine changes, indicating protonation of metal-ligated His and free imidazolate residues. These findings are discussed in relation to current views of the oxidase catalytic cycle.

I-D p-9. Electronic structure and spectra of purple copper centers in redox proteins

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Abstract: The purple, binuclear CuA centers found in cytochrome oxidases and in nitrous oxide reductase have unique spectroscopic properties. We have studied the electronic structure and spectra of CuA sites by various quantum mechanical methods, with the following conclusions. The Cu(I)−Cu(II) complex has a delocalized spin density, which indicates two weak, opposite Cu−S pi bonds and two weak Cu−S sigma bonds. The high intensity of the absorption is mainly due to transitions from MOs with a symmetric combination of copper xy and (x2−y2) 3d orbitals to a singly occupied one, which is antisymmetric. The unique dinuclear structure, with a very short Cu−Cu bond, is maintained by rack-induced coordination, and it results in a very low reorganization energy for electron transfer.

I-D p-10. Cell free synthesis of functional *Paracoccus denitrificans* cytochrome *c* oxidase

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Cytochrome *c* oxidase is an integral membrane protein and essential component of the respiratory chain. It catalyses the reduction of dioxygen to water coupled with the proton pumping across the membrane to drive ATP synthesis. To unveil the pathway and mechanism of the proton pumping, it is essential to observe ionization and deionization of protonatable groups in the enzyme during catalysis by vibrational spectroscopy. Site-specific labeling of the groups with stable isotopes provides a way to assign them in the enzyme. Cell-free protein synthesis allows us to obtain such labeled proteins. In this study, we have constructed the cell-free expression system for *Paracoccus denitrificans* cytochrome *c* oxidase containing subunits, SI, SII, SIII with two molecules of heme A, three atoms of copper and one atom of magnesium. SI with a C-terminal histidine-tag, SII and SIII were synthesized in the *E. coli* cell-free transcription/translation system supplemented with *E. coli* cell membrane, heme A and Cu2SO4, and, when necessary, with 35S-Met for protein labeling. About 0.5 mg protein was produced per 1 ml of the reaction mixture. Blue native PAGE of the solublized 35S-proteins revealed a protein band of about 100 kDa, which was further fractionated into SI, SII and SIII by SDS-PAGE, indicating the subunit assembly with a 1:1:1 stoichiometry. Incorporation of the proteins into the membrane was assessed by detection of proteinase K resistant segments of 35S-proteins in the membrane fraction. A fraction bound to Ni^{2+}-NTA column exhibited a KCN sensitive ferrocytochrome *c* oxidation activity and showed absorption peak at 604 nm that is characteristics of reduced cytochrome *c* oxidase. The current results indicate that cytochrome *c* oxidase of the three subunits is expressed functionally in the cell-free system.

I-D p-11. Computational study of the chemistry of cytochrome *c* oxidase reaction cycle

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The binuclear centre of cytochrome *c* oxidase is studied by means of quantum mechanical computer simulations [R. Ahlrichs et al; Chem. Phys. Lett. 162, 165-169 (1989)]. The electron and atom structures of approximately 250 atom model-systems of the enzyme at various stages of the catalytic cycle are calculated.

An atom level model for the chemistry of the cytochrome *c* oxidase reaction cycle is suggested. The energetics of the calculated cycle is in agreement with recent experiments [D. Bloch et al. PNAS 101, 529−533 (2004)]. This model has a hydroxide ion at copper in the P state and hydroxide ions at both iron and copper in the O fast state. The optical spectra at some intermediate states of the catalytic cycle are calculated.

I-D p-12. Inhibition of cytochromes ba_3 and aa_3 by nitric oxide

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Mammalian cytochrome *c* oxidase (cytochrome aa3) and Thermus thermophilus cytochrome *c*-552 oxidase (cytochrome ba3) are inhibited in steady state turnover by nitric oxide (formed from NO donors such as proliNONOate). In both cases more than one inhibited species is formed. Mammalian oxidase with cytochrome *c* in an aerobic steady state shows an immediate increased reduction of cytochromes *c* and a upon addition of NO, indicating inhibition of electron transfer at the a->a3 level. Formation of the final inhibited form of the oxidase aa3(2+)NO however lags behind the initial inhibition, suggesting that the latter species is formed secondarily, after NO has initially bound to the fully or partially oxidized binuclear centre, perhaps CuB. The duration of the 'lag' phase is greatest with resting and least with fully activated fast enzyme. Cytochrome ba3 shows a partial reduction of a3 rather than CuB in its aerobic steady state. Nevertheless, NO complexes formed by oxidized cytochrome ba3, during its catalytic steady state and after anaerobiosis, are also different. NO gives a 588 nm species with oxidized ba3 [CuB(2+)NO(+)a3(2+)?] like that formed with cyanide. After ascorbate addition, the b heme is reduced, and a transient a3 species appears spectrally similar to the unligated ferrous form [a3(2+)CuB(2+)NO?]. Upon anaerobiosis the latter slowly changes to a final 600 nm [a3(2+)NOCuB(+)?] form; dithionite returns this latter species to the usual 611 nm reduced a3 form as NO is reductively dissipated. Mechanisms involving intramolecular NO transfer from a3 heme Fe to CuB and vice versa are therefore postulated for both enzymes.

I-D p-13. Dynamic water networks in cytochrome *c* oxidase from *Paracoccus denitrificans* investigated by molecular dynamics simulations

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We present the results of a molecular dynamics study of cytochrome *c* oxidase from *Paracoccus denitrificans* in the fully oxidized state, embedded in a fully hydrated dimyristoylphosphatidylcholine lipid bilayer membrane. Parallel simulations with different levels of protein hydration, 1.125 ns each in length, were carried out under conditions of constant temperature and pressure using three-dimensional periodic boundary conditions and full electrostatics to investigate the distribution and dynamics of water molecules and their corresponding hydrogen-bonded networks inside cytochrome *c* oxidase. The presence of mobile water is indicated in regions of the protein where no water has been found by X-ray crystallography. The majority of the water molecules had residence times shorter than 100 ps, but a few water molecules are fixed inside the protein for up to 1.125 ns. The hydrogen-bonded network in cytochrome *c* oxidase is not uniformly distributed, and the water arrangement is variable. The average number of solvent sites in the proton conducting K- and D-pathways was determined. In contrast to single water files in narrow geometries we observe significant diffusion of individual water molecules along these pathways.

The highly fluctuating hydrogen-bonded networks, combined with the significant diffusion of individual water molecules provide a basis for the transfer of protons in cytochrome *c* oxidase, therefore leading to a better understanding of the mechanism of proton pumping.

I-D p-14. Ultrafast spectroscopy of electron transfer between hemes in bovine heart cytochrome *c* oxidase

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In cytochrome *c* oxidase, reduction of molecular oxygen in the active site requires transfer of electrons from cytochrome *c* towards heme a3. This occurs via a chain of redox intermediates. The dynamics of the final step in this chain, reduction of heme a3 by the chemically identical heme a, cannot be resolved in direct external electron injection experiments. Instead electron redistribution can be monitored upon photodissociation of CO from the mixed-valence (MV) enzyme-CO complex. The published value for the equilibration time in the mitochondrial enzyme is ~ 3 μs [1]. However, a detailed spectral analysis of the microsecond data (by Wikström's group) predicted a substantial additional equilibration phase on a much faster time scale [2]. This prediction was contested (by Brzezinski's group) on the basis of nanosecond experiments [3]. We have performed transient absorption experiments with femtosecond resolution and a time window up to 4 ns under selective excitation of heme a3 in the alpha band (595 nm) starting from the fully reduced ($a^{2+}a_3^{2+}$–CO) and MV ($a^{3+}a_3^{2+}$–CO) CO-complexes. In the MV complex only, a significant spectral evolution with a nanosecond time constant was observed both in alpha and in Soret spectral regions that can be fully ascribed to electron equilibration ($a^{3+}a_3^{2+}a^{2+}a_3^{3+}$). We suggest that the intrinsic time constant of reduction of heme a3 by heme a is on this time scale. The result will be discussed in the framework of conflicting theoretical predictions.

An additional remarkable observation is that the CO-dissociation spectrum from heme a3 depends on the redox state of heme a. This finding adds to the notion that can in principle not be treated as spectrally independent entities. This observation may relate to the strong variation in spectral decompositions using various methods.

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I-D p-15. 1.3 Å resolution structure of the cytochrome *c* domain from *R. marinus* caa_3

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The cytochrome *c* domain of subunit II from the *Rhodothermus marinus* caa3, HiPIP oxygen oxidoreductase, a member of the superfamily of heme Cu containing terminal oxidases, was produced in *E. coli* and characterised. The protein was crystallised and the crystal structure was determined by MAD technique using the anomalous dispersion of the heme iron atom at a resolution of 1.3 Å. The model was refined to the final Rcryst and Rfree values of 13.9% and 16.7% respectively. The structure reveals the insertion of two short antiparallel β-strands forming a small β-sheet, an interesting variation to the classical cytochrome *c* fold. This modification appears to be common to all known caa3 type terminal oxidases, as judged by comparative modelling and by the analyses of the available amino acid sequences for these enzymes. This is the first high-resolution crystal structure reported for a cytochrome *c* domain of caa3 type terminal oxidases. The electrostatic potential map at the molecular surface of this extra c-terminal domain brings new insights into the binding with its redox partner on one side and its interaction with the remaining subunit II on the other side.

I-D p-16. Thermodynamic and kinetic properties of the cbb_3−type cytochrome *c* oxidase from *Rhodobacter sphaeroides*

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The cbb3-type cytochrome *c* oxidase is a distant member of the family of haem-copper oxidases with various distinct characteristics such as high affinity for oxygen. Even though several key structural features are missing from this enzyme the mechanism with which it performs the four-electron reduction of oxygen to water is thought to be the same as in the mitochondrial cytochrome oxidase. Yet little is known about the properties of all cofactors and the way in which electrons are channelled to the active site of the enzyme.

We have characterised cbb3-type cytochrome *c* oxidase from *Rhodobacter sphaeroides* using spectroscopic and electrochemical methods. The midpoint potentials of the five haems in the enzyme were measured and results are in good agreement with previously published data for high potential haems of *Rhodobacter capsulatus* cytochrome cbb3 (Gray et al., Biochemistry 33 (1994) 3120−3127). The electron transfer rates between haems were studied following the backflow of electrons after photolysis of CO-bound enzyme at different redox states using optical spectroscopy. The redistribution of electrons was observed to take place between all haems thereby confirming their importance. Based on this kinetic and thermodynamic data, we propose a model for the electron transfer processes in the enzyme.

I-D p-17. Tryptophan-164 in cytochrome *c* oxidase stabilizes the heme a_3△-propionate-arginine 473 pair in proton translocation

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The mechanism by which cytochrome *c* oxidase translocates protons over the inner mitochondrial or bacterial membrane is still not known. The △-propionate of heme a3 could be the beginning of an exit pathway for protons and is involved in several of the proposed pumping mechanisms (Wikström et al., BBA 1604 (2003) 61−65, Brzezinski and Larsson, BBA 1605 (2003) 1−13). The importance of the -propionate/arginine 473 pair in proton translocation has been shown experimentally (Puustinen and Wikström, PNAS 96 (1999) 35−37). The propionate is stabilized by charge interactions with R473 and R474, and by hydrogen bonds to R473 and W164 (Iwata et al., Nature 376 (1995) 660−669). One proposed pathway of pumped protons leads via an array of water molecules to the -propionate of heme a3.

To study the role of the well-conserved W164 in proton translocation it was mutated to Phe. The activity of the W164F mutant is about half of wild-type enzyme and the proton pumping efficiency decreases. The W164F mutation lowers the Em of heme a3 and electrometric measurements indicate backleak of protons to the binuclear center. These results show the importance of the hydrogen bond between W164 and the heme a3 -propionate for the redox properties of heme a3 and supports the involvement of the area around the heme a3 -propionate in proton exit from the enzyme.

I-D p-18. Structural rigidity of the gating region in cytochrome *c* oxidase requires a specific channel for O_2 delivery to the catalytic site

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Cytochrome *c* oxidase (CcO) is the terminal enzyme of the respiratory chain. It is situated in a membrane where it reduces O_2 to H_2O and pumps protons across the membrane. Oxygen is thought to enter through specific channels starting in the surrounding membrane phase where the solubility of O2 is higher than in the water phase. The catalytic site of CcO, consisting of haem a3 and CuB, can also bind small ligands such as CO and NO. The binding kinetics of these ligands can be used as a probe of the protein dynamics. In CcO from *R. sphaeroides* the residue glycine (I 283), located within an O2 channel ~ 8 Å from heme a3, was replaced by a valine using site directed mutagenesis. In the mutant CcO the binding kinetics of CO was slowed by several orders of magnitude. Once CO was bound it could be photodissociated, but recombined much faster than with the wild-type CcO. These results indicate that the thermal motions of the protein matrix are highly restricted in the region of the protein where O2 enters the catalytic site. The same region is also thought to accommodate the proton-gating machinery, which requires well-defined distances between the intraprotein proton donors and acceptors. The results from this study indicate that the protein motions are restricted in the proton-gating region and that rapid O_2 delivery to the catalytic site requires a gas channel, which is confined within a rigid protein body.

I-D p-19. Mutagenetic evidence for proton transfer through peptide bond in the proton pumping by bovine heart cytochrome *c* oxidase

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Bovine cytochrome *c* oxidase embedded in mitochondrial inner-membrane pumps proton across the membrane coupled to dioxygen reduction to water. A pathway (H-pathway) comprised of the hydrogen-bond network and the water channel located in tandem across the membrane has been proposed to function as the proton-pumping system, based on the X-ray structural analysis (1). A key residue, Asp51, which shows a redox-coupled conformational change suggesting proton release upon reduction of heme a, is not conserved in bacterial and plant cytochrome *c* oxidase. To prove the function of Asp51, we have developed, employing human HeLa cells as a host, an expression system of bovine subunit I as the hybrid with the other human subunits of cytochrome *c* oxidase (2). The hybrid enzyme dominantly expressed over the human enzyme in the mitochondria has the cytochrome *c* oxidation and proton pumping activities almost identical to those of the human enzyme. With our system, the Asp51Asn mutation is found to abolish the proton pumping without impairing the cytochrome *c* oxidation activity.

H-pathway includes a peptide bond between Tyr440 and Ser441 as a part of the proton transfer pathway, which is able to provide the unidirectional active proton-transfer. Protons are likely to be transferred through peptide bond via the imidic acid intermediate (3). However, no functional evidence has been reported thus far. The imidic acid intermediate formed by association of proton to the carbonyl oxygen releases proton, generating the enol-form that immediately tautmerizes to the stable keto-form. The secondary amine of peptide bond is necessary for the proton transfer. Thus, a Ser441 to Pro mutation that generates peptide bond with tertiary amine was carried out for proving the role of the peptide bond in the proton transfer. A double mutation, Val386Leu/Met390Trp was also performed intending to block the water passage through the water channel by bulky side-chains. We constructed each mutant cell line as above expression system, and found the mutations did not lower the cytochrome *c* oxidation activity, whereas they abolished the proton pumping.

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I-D p-20. Distance and orientation studies of the Cu_A fragment of cytochrome *c* oxidase with different cytochrome *c* by pulsed EPR spectroscopy

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Cytochrome *c* oxidase is the terminal enzyme in the respiratory chains of mitochondria and many bacteria, and catalyzes the formation of water by reduction of dioxygen. The first step in the cytochrome oxidase reaction is the bimolecular electron transfer (ET) from cytochrome *c* to the homobinuclear mixed-valence Cu_A centre of subunit II. A binding model was proposed by computer docking algorithms (1). We analysed the interaction between soluble fragments of cytochrome c_{552} and the Cu_A fragment, both derived from *Paracoccus denitrificans* by Pulsed X band and G band (180 GHz) EPR. Partner proteins for the Cu_A (excess negative charge) were (i) the cytochrome c_{552} soluble fragment (positive surface potential), and as controls (ii) the cytochrome c_1 soluble fragment (negative surface charged from bc1 complex) and (iii) the *Thermus thermophilus* cytochrome c_{552} with a mostly hydrophobic surface. The relaxation of the Cu_A centre is enhanced due to dipolar coupling to the faster relaxing heme-Fe in the *P. denitrificans* cytochrome c_{552}. As the relaxation behaviour of cytochrome *c* is known (2), it is possible to extract information about the distance and orientation of the interspin vector by T_2 relaxation measurements. T_2 of Cu_A was measured using a two-pulse echo sequence and recording the signal as a function of time τ between the pulses. Measurements were performed at several magnetic field positions varying temperature between 5 and 30 K. Both the X band and the high-field measurements show the existence of strong relaxation enhancement of the Cu_A by the specific binding of the *P. denitrificans* cytochrome c_{552}. Experimental results and simulations for the different cytochrome *c* partners will be presented.

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I-D p-21. Computational studies of proton pumping mechanism of cytochrome *c* oxidase

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Computer simulations directed toward understanding proton pumping mechanism of cytochrome *c* oxidase described in Refs 1–3, along with the most recent work on the subject, will be discussed.

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I-D p-22. The pH dependence of charge transfer in mixed-valence cytochrome *c* oxidase from *Paracoccus denitrificans*

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Cytochrome *c* oxidase is a transmembrane protein which catalyses reduction of one oxygen molecule to two water molecules. Part of the energy, which is released in the process, is used for vectorial transport of protons across the membrane. The four electrons needed for the reaction are delivered one at a time from cytochrome *c* through CuA and haem a to the binuclear haem a3-CuB center, where oxygen binds. The required protons are provided by two proton conducting channels, the K- and D-pathways. The internal electron transfer reactions and proton transfer processes coupled to them in cytochrome *c* oxidase can be studied by backflow experiments, in which electrons move in the opposite direction compared with normal function. In a backflow experiment, carbon monoxide is dissociated from partly reduced enzyme which leads to lowering of the redox potential of haem a3 and to redistribution of electrons among the redox centers. Upon the dissociation of carbon monoxide first electron transfer from haem a3 to haem a is observed. It has been shown that at high pH this electron transfer is followed by proton release via the K-pathway in the millisecond time range and deprotonation of a water molecule to a hydroxide ion at the binuclear center (M. Brändén et al., Biochemistry (2003) 42, 13178–13184). In this study, the pH dependence of the charge transfer processes after dissociation of carbon monoxide from two-electron-reduced cytochrome *c* oxidase from *Paracoccus denitrificans* was observed by time-resolved optical absorption spectroscopy and electrometry. It was found that the proton transfer takes place in two separate kinetic steps. First, a proton is transferred from tyrosine in the binuclear center (Y280, *P. denitrificans* numbering) through the K-pathway with a time constant of about 150 us at high pH. In a second step, in the millisecond time range, the tyrosine is reprotonated by a water molecule bound to haem a3, and further electron transfer from haem a3 to haem a takes place.

I-D p-23. A third oxygen reductase from the thermohalophilic bacterium *Rhodothermus marinus*

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Rhodothermus marinus, a thermohalophilic Gram-negative bacterium expresses two different oxygen reductases: a caa3 and an cbb3, which have been purified and characterised [M.M.Pereira et al (1999) BBA 1413, 1–13; M.M.Pereira et al (2000) J Bioenerg Biomembr 32, 143–152; M.M.Pereira et al (2000) Biochemistry 39, 6336–6340; M.M.Pereira et al (2001) BBA 1505, 185–208,]. In this study we show evidences for the presence of a third oxygen reductase in this bacterium. This complex is constituted by two subunits, with apparent molecular masses of 48 and 32 kDa. It contains a low spin haem B center and a high spin haem A center which should belong to the catalytic centre of this protein, due to its reactivity with CO. The visible difference spectrum of the reduced minus oxidised protein presents an a- and a Soret bands characteristic of cytochromes *b* and *a*, with maxima at 557 and 427 nm for cytochrome *b* and 600 and 443 nm for cytochrome *a*. A stoichiometry of one cytochrome *a* per cytochrome *b* was obtained by integration of the a-bands and 1 mol of each haem is present per 1 mol of protein. The pyridine hemochrome spectrum corroborates the presence of A and B type haems. Anaerobic potentiometric titrations, followed by visible spectroscopy, showed that the two haem centers reduce together along the titration indicating the presence of a strong interaction between them. The reduction potentials determined at pH 8, were -63 and -64 mV for haems B and A, respectively, and an interaction potential of -47 mV.

I-D p-24. Heme a3–NO interactions in reduced cytochrome *c* oxidases: binding and recombination dynamics

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Cytochrome *c* oxidase (CcO) has a high affinity for nitric oxide (NO), a property in the regulation of respiration [1]. We have previously shown that recombination kinetics of reduced CcO–NO from *P. denitrificans* on the picosecond time scale depend strongly on the NO:enzyme stoichiometry and inferred that more than one NO can be accomodated by the active site [2], already at mildly suprastoichimetric NO concentrations. We have now largely extended these studies by studying rebinding dynamics from the picosecond up to the microsecond time scale, by performing parallel steady-state EPR characterizations under similar conditions as the optical experiments, and comparing them with molecular modeling results. A comparative study was performed on CcO ba3 from T. thermophilus, where two NO molecules cannot be copresent in the active site in steady state due to its NO reductase activity [3]. The kinetic results allow to discriminate between different models of NO-dependent recombination and show that the overall NO-escape probability out of the protein is high when only one NO is bound to aa3, whereas strong rebinding on the 15-ns time scale was observed for ba3. The EPR characterizations show similar results for aa3 at substoichiometries NO:enzyme ratios and for ba3, indicating formation of a six-coordinate heme-NO complex. The presence of a second NO molecule in the aa3 active site strongly modifies the heme-NO EPR spectrum, and can be rationilized by a rotation of the NO with respect to the proximal histidine. This proposal is consistent with molecular modeling studies. The binding properties of the second NO molecule will also be discussed.

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I-D p-25. Cytochrome *c* oxidase as a calcium binding protein

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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 C/P aqueous phase. In the bacterial COX it is occupied by tightly bound Ca^{2+}[1], whereas the mitochondrial oxidase can bind reversibly Ca^{2+} or Na^{+}. The functional role of the site has not yet been established. Replacement of D477A in subunit I of *P. denitrificans* bacterial oxidase converts Ca^{2+}/Na^{+} binding to the reversible mitochondrial type [1]. Using the red shift of heme a absorption spectrum as an indicator of Ca^{2+} binding with the site, we studied characteristics of the reversible cation binding in *P. denitrificans* D477A oxidase in comparison with the bovine oxidase and homologous mutant COX D485A from *Rh. sphaeroides*. The rate constants of Ca^{2+} binding (7×10^{3} M^{-1} s^{-1}) and dissociation from the site (7×10^{-3} s^{-1}) appeared to be similar to those in D485A COX and significantly differed from the values obtained earlier for bovine COX ± kon. 30×10^{3} M^{-1} s^{-1} and koff 0.1 s^{-1}) which indicates sterical difficulties in Ca^{2+} interaction with bacterial enzymes. Determined earlier by equilibrium titrations Kd value of 10^{-6} M for Ca^{2+} binding to COX D477A from *P. denitrificans* [1] is exactly equal to the kon/koff ratio obtained now and the same as in bovine COX [2] while data we had obtained earlier for D485A oxidase from *Rh. sphaeroides*: extremely high affinity towards Ca^{2+} (Kd 6nM) in equilibrium titrations and discrepancy with the kon/koff value 190 nM indicating possible multistage Ca^{2+} binding to COX—might be considered as a peculiar property of the latter enzyme. One calcium competes with two sodium ions for the binding with either the reduced or the oxidized forms of the mammalian COX. In contrast, one calcium is found to compete with only one sodium (Kd = 4 mM) for the binding site in D477A mutant COX from *P. denitrificans*. It is proposed that the binding site for the second sodium ion in bovine oxidase involves D442, homologous to D477 in *P. denitrificans* oxidase.

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I-D p-26. Photoinduced electron transfer in cytochrome *c* and cytochrome *c* oxidase

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The photoactive redox label TUPS (1-thiouredopyrene-3,6,8-trisulfonate) was covalently bound to various native lysine and engineered cysteine residues of horse heart cytochrome *c*. The kinetics of electron transfer from the excited dye to the oxidized heme and back, as well as the kinetics from the reduced heme to the excited dye and back were measured by multichannel and single wavelength spectroscopy following a UV laser pulse. TUPS was thus shown to be a suitable label in initiating both oxidative and reductive pulses in redox proteins. The complex electron transfer kinetics were modeled by assuming redox induced conformational change in cytochrome *c*.

Electron transfer from TUPS labeled cytochrome *c* to bovine cytochrome *c* oxidase was also monitored spectroscopically. Several mutant horse heart cytochromes were screened and one of them, A15C-TUPS proved to be especially suitable for light induced reduction of cytochrome *c* oxidase. Rapid photoinduced reduction of heme c was followed by efficient reduction of heme a of the enzyme. The electron transfer kinetics between the dye and heme c were altered in the complex with cytochrome *c* oxidase, apparently due to the stabilization of the reduced form of heme c by the interaction.

Yeast iso-1 cytochrome *c* with TUPS covalently bound to C102 was also used to drive electron transfer into oxidized bovine cytochrome *c* oxidase. At alkaline pH heme a became markedly less reduced than at neutral pH, relative to the heme of cytochrome *c* when equilibrium was attained at approximately 400 μs. This finding is consistent with the pH dependence of the midpoint potential of heme a as measured earlier under static conditions, and indicates that the Em/pH dependence of heme a may be retained on the turnover time scale of the enzyme and hence is catalytically relevant.

I-D p-27. Proton interactions with heme a_3 in bovine heart cytochrome *c* oxidase

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Department of Biochemistry and Cell Biology, Rice University, 6100 Main, Houston, TX 77005 When the pH of solution with oxidized cytochrome c oxidase(CcO) is rapidly decreased from 8.3 to 5.9 there is a blue shift of Soret band that occurs with two kinetic phases. The first phase proceeds with an apparent rate of 7 s_{-1} and during this phase the Soret maximum is shifted from 424nm to 422nm. From the pH dependence of the position of Soret band, observed at the end of the first kinetic phase, a single protolytic group with a pK_a=6.7 was identified. The lack of pH sensitivity of the Soret band of mixed CcO, in which both heme a_3 and Cu_B are reduced and stabilized by carbon monoxide (MV.CO), implies an interaction of heme a_3 with the protolytic group. However, the pK_a of the group is independent of the redox state of heme a_3 and Cu_B. This is demonstrated by the stoichiometry of proton uptake during the conversion of oxidized CcO both to MV.CO and to fully reduced CcO at pH values of 5.8, 6.8 and 8.2. Conversion of oxidized CcO to MV.CO was accompanied by the consumption of approximately 2 protons and this value was almost independent of pH. Full reduction of oxidized CcO was associated with the uptake of approximately 2 H^+ at basic pH and this value increases with decreasing pH. It is concluded that the group is distant from heme $a^3$and an interaction between these two sites is mediated by the protein. Arguments are presented that the group, postulated to be Glu60 of subunit II located at the entrance of the proton conducting K-channel interacts with the OH group of the hydroxoethyl farnesyl side chain of heme a_3 through a hydrogen bonded network.

Supported by the National Institutes of Health (GM 55807) and the Robert A. Welch Foundation (C-636).

I-E: ATP-Driven Transport Systems

I-E p-1. Structural studies on prokaryotic CPX-ATPases

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Archaea, eubacteria and eukaryotes have primary active transporters, which control the intracellular heavy metal ion concentrations by acting either as uptake or as export transporters. These integral membrane proteins, also named CPX-ATPases due to a conserved sequence motif, belong to the class of heavy-metal translocating P-type ATPases.

We have expressed several archaeal and eubacterial genes coding for putative CPX-ATPases in a variant of the host strain *Escherichia coli* BL21, which previously has been reported to allow high level heterologous expression of membrane proteins [Arechaga, I. et al. (2000) FEBS Letters 482, 215–219]. Using the CPX-ATPase CopB of the thermoacidophilic archaeon *Sulfolobus solfataricus* as a prototype, we devised a purification scheme consisting of the solubilization of cytoplasmic membranes and two subsequent chromatographic steps. We obtained homogenous protein, which was subjected to biochemical and structural characterization.

The CPX-ATPase as isolated from its natural source is a reference material desired for comparative purposes. Therefore we are working on the purification of the hyperthermophilic CopB protein produced by the native host *S. solfataricus*, using the expression system developed by Schleper and coworkers [Jonuscheit et al (2003), Mol. Microbiol. 48, 1241–1252].

In another approach, the soluble catalytic domain CopB-B containing the ATP-binding and phosphorylation sites of CopB [Deigweiher et al (2004) J. Bioenerg. Biomembr. 36, 151–159] was separately expressed, purified and crystallized. Phase assignment was performed by means of multiwavelength anomalous diffraction using crystals of selenomethionine-labeled protein. A structural model of the domain was derived at the resolution of 2.3 Å.

I-E p-2. The ABC-type uptake system Ggt is involved in steady state regulation of the osmolyte content in *Synechocystis* sp. PCC 6803

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The cyanobacterium *Synechocystis* sp. strain PCC 6803 is able to acclimate to levels of salinity ranging from freshwater to twice the seawater concentrations of salt by accumulating the compatible solute glucosylglycerol (GG). It can be synthesized de novo via the key enzyme glucosylglycerol phosphate synthase (GgpS) or taken up from the medium by the ABC-type transport system Ggt. Besides GG also the transport of sucrose and trehalose are facilitated. The Ggt transporter is a close homologue of the maltose transporter Mal from *E. coli*. Especially the ATP-binding subunit GgtA displays the same domain organization consisting of a transport mediating N-terminus and a regulatory active C-terminus like MalK in *E. coli*. However, in *Synechocystis* no transcription regulator like MalT in *E. coli* is present and the expression of transporter subunits is only regulated in relation to external salt concentration. We propose that the Ggt transporter is involved in the regulation of the steady state content of GG in salt acclimated cells. In mutant cells lacking a transporter subunit GG leaks into the medium. Because the internal osmolyte concentration is the same as in wild type cells the loss of the transporter results in an enhanced GgpS activity. Furthermore, the uptake of externally supplied trehalose by salt acclimated wild type cells results in a decrease of the internal GG concentration which is due to a decreased activity of the GGPS enzyme whereas the ggpS gene expression and GgpS protein content stays unchanged. In an in vitro system using overexpressed GgpS protein the lower enzyme activity in protein extracts from trehalose loaded cells is reconstructible.

By expressing single domains of the GgtA subunit in *Synechocystis* we are analysing the significance of this regulatory principle for the interplay between osmolyte synthesis and uptake.

I-F: Quinol Acceptor Oxidoreductases

I-F p-1. A compensatory double mutation of the alanine-86 to leucine mutant located in the hinge region of the iron–sulfur protein of the yeast cytochrome bc_1 complex

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Mutations in the hinge region connecting the membrane anchor to the extramembranous head-group of the iron–sulfur protein can impede proper assembly and function of the cytochrome bc_1 complex. Mutating the conserved alanines, residues 86, 90, and 92, located in the hinge region resulted in a 30–50% decrease in enzymatic activity without loss of the iron–sulfur protein [1]. The loweredenzymatic activity in the A86L mutant was shown to result from steric interference between the side chains of Leu-86 and Leu-89 [2]. Mitochondria isolated from the double mutant A86L:L89A had cytochrome *c* reductase activity similar to that of the wild type compared to the 50% loss of activity in mitochondria observed in the A86L mutant. Western blotting of mitochondrial proteins separatedby SDS-PAGE followed by densitometry revealed that the amount of the iron sulfur protein was not decreased in mutant A86L or the double mutant A86L:L89A. These results suggestthat generation of the double mutant, A86L/L89A, relieved the steric interference caused by the A86L mutation. By contrast, mitochondria isolated from the L89A mutant, constructed as a control for the double mutant,had a 90% decrease in cytochrome *c* reductase activity compared to the wild type. Western blotting revealed that the iron–sulfur protein was present at 17% that of wild type suggesting that the mutant iron–sulfur protein fails to assemble properly into the bc_1 complex. Molecular modeling simulations, performed using the crystal structure for the *S. cerevisiae* bc_1 complex revealed that the head region of the double mutant does not appear to undergo significant structural alteration; however, the hinge region was modified such that the initial loop protruding from the membrane takes on a slightly altered conformation to accommodate the larger side chain of Leu- 86. The residues remain in the same orientation from the transmembrane anchor until residue Thr-83, which is twisted in the double mutant. Leu-86 is rotated approximately 60 compared to the Ala-86 in the wild type, such that the leucine side chain is facing away from the monomer. This alteration leads to a more pronounced loop shape for the mutant at the start of the a-helical region of the hinge region; however, the hinge region in the double mutant appears flexible. Overall, the double mutant exhibits no sign of steric interaction as all bond distances are sufficient to be nonoverlapping.

I-F p-2. Studies on E445A mutant cytochrome bd oxidase suggest the sequence of hemes in intraprotein electron transfer needs to be revised

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Cytochrome bd is one of the two terminal quinol oxidases in the *E. coli* respiratory chain (1). The enzyme catalyzes charge separation across the bacterial membrane during the quinol oxidation by oxygen but does not pump protons (2). In this work, wild-type and E445A mutant cytochrome bd oxidases were studied by time-resolved spectrophotometric and electrometric techniques. It appears that high spin heme b595, a component of the binuclear catalytic site, is not absent in the E445A mutant as initially proposed (3), but remains ferric even in the dithionite-reducing conditions. The E445A mutation seems to block a putative proton channel, delivering H^+ to the surroundings of heme b595 coupled to its reduction, and markedly lowers its midpoint potential. Both enzymes can react with oxygen however the rate of formation of oxoferryl in the wild-type enzyme is about two orders higher than that in the mutant. In contrast, no any difference could be observed during membrane potential generation associated with electron "backflow" reactions. Heme b595 is a major contributor to electron redistribution from heme d after CO photolysis from the enzyme in the one-electron reduced state. However redox changes of heme b595 are not coupled to generation of membrane potential. Meanwhile, electron transfer from heme d to heme b558 is electrogenic and not perturbed by the mutation. These findings contradict the currently accepted sequence of the hemes in intraprotein electron transfer and suggest the revised sequence, in which instead of direct electron transfer from heme b558 to heme b595, it goes through heme d, i.e., b558->d->b595.

This work was supported by Biocentrum Helsinki, the Sigrid Juselius Foundation, the Academy of Finland; Grant 02-04-48314 from the Russian Fund for Basic Research; Howard Hughes Medical Institute International Scholar Award 55000320; Grant HL16101 from the National Institutes of Health.

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I-F p-3. Kinetic studies on the purifed alternative oxidase protein from *Arum maculatum* and its regulation by organic acids

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We have recently reported the purification of an active and stable alternative oxidase (AOX) protein from mitochondria of thermogenic Arum maculatum spadices (Affourtit and Moore 2004, Bioch. Biophys. Acta 1608: 181–189). Our reported development of a reliable spectrophotometric assay to measure AOX activity is now facilitating studies of the kinetics of the purified protein with respect to (1) quinol substrates and oxygen, (2) inhibitors of AOX, (3) regulation of activity by organic acids such as pyruvate. We show that the isolated AOX protein interacts in a redox-sensitive manner similar to that of the enzyme present in isolated mitochondria. Whilst maximum activity of the purified protein depends on the presence of both pyruvate and the detergent EDT-20, the response of purified AOX to some organic acids differs to the situation observed in isolated mitochondria. With respect to pyruvate, we find that the effect of this keto acid on initial AOX rates appears to be independent of pyruvate dose, duroquinol concentration and AOX amount. Loss of pyruvate from the purified enzyme causes irreversible inactivation (t1/2 90s) which does not require substrate turnover (c.f. Hoefnagel et al 1997, Plant Physiol. 115: 1145–1153). Our data suggest that pyruvate is required to stabilise an active conformation of AOX from A. maculatum. Results are discussed in relation to the potential reaction mechanism of AOX and the mechanism by which pyruvate affects activity of the protein.

This work is sponsored by the BBSRC.

I-F p-4. Regulation of the plant alternative oxidase by pyruvate

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In addition to a conventional cytochrome pathway, plant mitochondria contain a second, nonprotonmotive route of electron transfer, comprised of a single ubiquinol:oxygen oxidoreductase, the alternative oxidase (AOX). This enzyme is regulated by two interrelated posttranslational mechanisms. Firstly, reduction of an intermolecular disulphide bond results in an 'activated' noncovalently linked dimer. Secondly, the reduced protein is further stimulated by a-keto acids, most notably pyruvate. This is thought to occur via a-keto acid interaction with a well-conserved cysteine residue. Previously, we have established a system to functionally express the *Sauromatum guttatum* alternative oxidase (Sg-AOX) in the fission yeast *Schizosaccharomyces pombe*. Interestingly, the resulting antimycin-resistant respiratory activity in isolated yeast mitochondria, does not appear to be stimulated by pyruvate.

Here, we report on the expression of both Sg-AOX as well as a second plant isozyme, the *Arabidopsis thaliana* AOX1a protein (At-AOX1a), in the same yeast system. In contrast to Sg-AOX, At-AOX1a-dependent activity can be stimulated by pyruvate $\sim$ 1.4-fold in isolated yeast mitochondria and $\sim$ 4.5-fold in isolated mitochondrial membranes depleted of endogenous pyruvate. Whilst Sg AOX activity is confirmed to be completely independent of pyruvate, its dependence upon the Q-redox poise would suggest that it is in a constitutively active state, comparable to the 'pyruvate-activated' kinetic dependence of At AOX1a. These data indicate that Sg AOX is the first example of a plant enzyme that appears to function without a dependence on organic acids for full activity. This finding is of particular interest, given that both Sg AOX and At AOX1a conserve the cysteine residue believed to interact directly with pyruvate. As both proteins exhibit a very similar primary structure, we have been able to identify structural components, additional to the regulatory cysteine, that may account for the differences in the regulatory behaviour reported here. The implications of such findings are discussed in terms of the proposed structure of AOX.

This work was funded by the School of Life Sciences, University of Sussex.

I-F p-5. The mechanism of oxidation of ubihydroquinone (QH_2) at the Q_o-site of the bc_1 complex

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The modified Q-cycle mechanism accounts for the kinetic and thermodynamic characteristics of the above reaction with a few simple postulates. Structures from crystallography have confirmed the main topological features predicted from the kinetic model and from structural prediction. One new feature revealed has been the mobility of the extrinsic domain of the Rieske iron sulfur protein (ISP). This moves between catalytic interfaces on cyt c_1 and cyt *b*, to transfer electrons from QH_2 to heme c_1. The ISP behaves as a substrate, and participates in formation of an ES-complex on binding to cyt *b*. Formation of the ES-complex requires participation of two substrates (S), QH_2 and ISPox. From the variation of rate with [S], the binding constants for both substrates involved in formation of the complex can be estimated. The configuration of the ES-complex likely involves the dissociated form of the oxidized ISP (ISPox) docked at the b-interface on cyt b, in a complex in which N of His-161 (bovine sequence) forms a H-bond with the QH_2-OH. Detailed parameters for the description of the rate limiting step have been proposed that account for the known properties of the Q_o-site reaction. The reaction mechanism is complex, involving a bifurcation of electron transfer. The first electron transfer from QH_2 to ISP appears to be limiting. The high activation energy and slow rate have to be reconciled with a short distance for transfer of reducing equivalents. The properties can be explained by a H-transfer in which movement of the electron occurs from a configuration for the H^+ that is unfavorable. A simplified treatment of the activation barrier has been developed in terms of a probability function for the H^+-distribution determined by the Brønsted relationship, and a Marcus treatment of the electron transfer step. Incorporation of this relationship into a computer model allows exploration of the energy landscape. A set of parameters including reasonable values for activation energy, reorganization energy, distances between reactants, and driving forces, all consistent with experimental data, explains why the rate is slow, and accounts for the altered kinetics in mutant strains in which the driving force and energy profile are modified by changes in Em and/or pK of ISP or heme bL. The model is also quite consistent with the measured rates of bypass reactions which reflect the low probability of oxidation of the intermediate semiquinone state by alternative pathways.

I-F p-6. The structure of complex II and its quinone-binding site

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Complex II, also referred to as succinate dehydrogenase or succinate:ubiquinone oxidoreductase (SQR), is a four subunit membrane bound respiratory enzyme. The enzyme couples the oxidation of succinate in the mitochondrial matrix (or cytoplasm in bacteria) with the reduction of quinone in the membrane during aerobic respiration. This reaction directly connects the Krebs cycle and the electron transport chain, however SQR does not directly contribute to proton motive force generation. Electron transfer from succinate to quinone is facilitated in SQR by five redox centres; one Flavin-adenine dinucleotide (FAD), three Iron–sulphur clusters and one heme b.

The 3D crystal structure of Complex II from *E. coli* has recently been solved [Yankovskaya et al. 2003] revealing its molecular architecture. The quinone-binding site (Q-site) is of particular interest as mutations at the site result in a number of human disease states. The current progress of the structural analysis of the Q-site will be presented.

I-F p-7. A comparative structure-based analysis of the pH-dependent reduction potentials of Rieske iron–sulphur proteins

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Rieske iron–sulphur proteins carry an [2Fe–2S] cluster coordinated by two histidine and two cysteine residues. Based on their electrochemical properties, Rieske proteins are grouped into two classes: Rieske proteins from hydroquinone-oxidising bc-type cytochrome complexes display high reduction potentials (300–400 mV) that depend on pH in the physiological range. Rieske proteins from bacterial aromatic ring hydroxylating dioxygenase complexes have low reduction potentials (− 150 mV) that show no pHdependence in the physiological range. The difference in pHdependence between bc-type and dioxygenase Rieske proteins is due to the difference in protonation behaviour of the histidine sidechains coordinating the iron–sulphur cluster. Ligand histidine pKa-values lie within the physiological range in bc-type Rieske proteins, but above in dioxygenase Rieske proteins.

In the presented study, structural differences between a bc-type and a dioxygenase Rieske protein were identified that account for the differences in pH dependence of their reduction potentials. Based on available high-resolution structures, histidine pKa-values and relative reduction potentials were calculated by a combined classical electrostatic/quantum chemical approach.

Obtained histidine pKa-values of the wildtype structures agree well with experiment. By introducing in silico mutational changes, differences between the two studied proteins were removed. From an analysis of the reduction potentials and pKa-values of the mutated structures, differences in electrochemical behaviour could be related to structural differences between the two proteins. Presence of hydrogen bonds towards the iron–sulphur cluster together with absence of acidic residues in bc-type Rieske proteins accounts for their higher reduction potentials and lower ligand histidine pKa-values. These structural differences are thus the basis for the pH dependence of reduction potentials in bc-type Rieske proteins.

The pH dependence of reduction potentials allows for coupled reduction and protonation of the Rieske cluster which is of functional importance in bc-type cytochromes: Rieske proteins appear to act not only as electron but also as proton acceptors in the oxidation of hydroquinone. Hydroquinone oxidation is the key reaction in bc-type cytochrome complexes which participate in all major pathways of biological energy conversion.

I-F p-8. Investigation of the Rieske ISP [2Fe–2S] cluster protein environment using high-resolution EPR

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Rieske-type [2Fe–2S] clusters, defined by the mixed ligation of their Fe atoms by two cysteine and two histidine residues, function in many important electron transfer pathways. Their redox potential and protolytic properties have been fine-tuned by evolution to match the specific chemistries in which they participat, through modification of the immediate protein environment. Several hypotheses have been proposed as to the identities of these fine-tuning elements, but experimental evidence for particular interactions is limited. To address this issue further, structural elements around the reduced cluster of the Rieske iron–sulfur protein (ISP) subunit of the bc_1 complex of *Rhodobacter sphaeroides* have been characterized by orientation-selective ESEEM and HYSCORE spectroscopies. Experiments using protein containing ^{14}N at natural abundance, and protein labeled with ^{15}N have provided the hyperfine and nuclear quadropole interaction tensors of coordinated N_δ nitrogens from two histidine ligands. These data were used to model the histidine coordination. Spectroscopic analyses have been aided by the availability of crystal structures for ISPs from bc_1 and b_6f complexes, and other Rieske-type proteins, and by collaborative spectroscopic work on examples from several of the latter. The two distinct sets of data are compared to identify differences between crystals and frozen solution samples in the packing of the protein surrounding the [2Fe–2S] cluster.

HYSCORE data also revealed weak coupling to ^{15}N of histidine N_ε and backbone amide. The latter are likely involved in the formation of H-bonds with the cluster. In addition, protons of H-bonds near the cluster were identified using deuterium-exchange experiments.

The data from the ISP head domain isolated after proteolysis were compared to those from the native bc_1 complex in the presence and absence of various inhibitors, to determine the influence of Q_o-site occupants on the geometry of the histidine ligands to the cluster. Hyperfine and quadrupole couplings of N_δ, N_ε and peptide nitrogens and hyperfine couplings of hydrogen bonded protons, give a detailed picture of the effect that Q_o-site occupants have on the environment of the Rieske cluster.

Parallel work using protein film voltammetry to measure thermodynamic changes in mutants strains, will allow us to identify structural features associated with interactions of particular side chains that modify the thermodynamic properties.

I-F p-9. Remodelling the cytochrome *b* Q_o site of yeast *Saccharomyces cerevisiae*: study of Q_o inhibitor resistance mutations of crop pathogens

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Inhibitors of the bc_1 complex which bind at the cytochrome *b* Qo site (Q_oIs) are widely used in agriculture to control fungal and oomycete plant pathogens. Resistance to these inhibitors has emerged in field populations of the pathogens. The G143A mutation in cytochrome *b* seems to play a central role in the mechanism of resistance. Interestingly the emergence of the mutation varies significantly between pathogens. In some pathogens, G143A has spread widely whereas in others, the mutation has been detected only in a localized geographical area. Other pathogens have not yet shown Q_oI resistance despite their exposure to the fungicides. A factor which might explain differences in the evolution of the resistance could be small variations in the structure of the Q_o binding domain. It might be postulated that some Q_o site variants can not accommodate G143A without loss of bc_1 complex activity. To investigate the possible role of variations in the structure of the Q_o domain on the impact of G143A, we use yeast as a model system. In this work, we modified few residues in the Q_o domain of yeast cytochrome *b* to mimic the Q_o site of four crop pathogens. We then introduced the mutation G143A and monitored its effect on the bc_1 complex activity. Interestingly G143A no effect on the bc_1 activity in one of the pathogen-like variants whereas it had a slightly deleterious effect in the other variants. Based on this observation in the yeast model, it might be anticipated that G143A could affect the fitness of pathogens differentially, which could contribute to differences in the rates of evolution of Q_oI resistance.

I-F p-10. Probing haem propionate involvement in transmembrane proton transfer coupled to electron transfer in dihaemic quinol:fumarate reductase by ^{13}C-labelling and FTIR difference spectroscopy

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Quinol:fumarate reductase (QFR) is the terminal enzyme of anaerobic fumarate respiration. It couples the reduction of fumarate to succinate to the oxidation of a low-potential quinol to quinone. Although electrophysiological experiments demonstrated that the reaction catalysed by the dihaem-containing QFR from *Wolinella succinogenes* is an electroneutral process, the three-dimensional structure of this membrane protein complex, initially solved at 2.2 Å resolution, revealed locations of the active sites that indicate electrogenic catalysis. To reconcile these apparently conflicting experimental observations, the so-called ''E-pathway hypothesis'' has been proposed [C.R.D. Lancaster, Biochim. Biophys. Acta 1565 (2002) 215–231]. According to this, transmembrane electron transfer via the haem groups is strictly coupled to a parallel compensatory transfer of protons via a transiently established pathway, which is inactive in the oxidized state of the enzyme and only opened upon reduction of the haem groups. Possible constituents of the E-pathway have been proposed to be the side chain of Glu C180, and the ring C propionate of the distal haem. The former constituent has already been confirmed to play a crucial role in the function of dihaemic QFR, thus supporting its role as a participant of the E-pathway [C.R.D. Lancaster, U.S. Sauer, R. Groß, A.H. Haas, W. Mäntele, J. Simon, G. Madej, manuscript in preparation; A.H Haas, U.S. Sauer, R. Groß, J. Simon, W. Mäntele, C.R.D. Lancaster, manuscript in preparation]. In this work, we investigate any possible haem-propionate involvement in redox-coupled proton transfer by a combination of specific ^{13}C-haem propionate labelling and FTIR difference spectroscopy. To introduce the labelled carbon atom into the protein, we created a d-aminolevulinate auxotrophic mutant, and supplied the medium with 1-^{13}C-5-aminolevulinate. The effect of the isotopic substitution on the characteristic vibrations of the haem propionates in the midinfrared range, which are sensitive to protonation and environmental changes, will be discussed. A haem-propionate involvement in redox-dependent protonation events would provide further support for the proposed E-pathway hypothesis of coupled transmembrane electron and proton transfer in dihaem-containing QFR.

I-F p-11. Molecular cloning and characterization of the alternative oxidase gene family from *Candida maltosa*

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Cyanide-resistant respiration catalyzed by the nuclear-encoded alternative oxidase (AOX) is induced in the presence of respiratory inhibitors and sulfhydryl compounds in the nonpathogenic yeast *Candida maltosa*. This organism belongs to *Candida albicans* clade, but has been of particular interest for industrial application based on the ability to assimilate *n*-alkanes oxidizing a large quantity of acyl-CoA and thus thought to be rather resistant to oxidative stress. The molecular mechanism by which the specific inhibitors of cytochrome bc_1 complex trigger the signal transduction toward nuclei to express the AOX gene seems to be somewhat different from that in Hansenula (Pichia) anomala, which has been well investigated.

The genomic DNA containing the gene family encoding AOX was cloned by colony hybridization probed with a partial AOX clone, which had been obtained by PCR employing the degenerate primers corresponding the highly conserved sequences among fungal AOXs. The AOX gene family is composed of two members, AOX1a and AOX1b, which encode polypeptides consisting of 371 (1,113 bp) and 349 (1,047 bp) amino acids, respectively. AOX1b is located 540 bp upstream of AOX1a in the same transcriptional direction. Similar tandem arrangement of two AOX genes has been identified in *Candida albicans*. Multiple alignment of the predicted amino acid sequences with those from other fungi demonstrated significant homology throughout most of the polypeptides, while the least conserved areas were at the N-termini, reflecting the degenerate nature of mitochondrial targeting sequences.

To elucidate the physiological roles of AOX, an aox1 mutant strain was created using targeted gene disruption. Due to a homozygous disruption of both AOX1a and AOX1b, mitochondrial cyanide-insensitive, SHAM-sensitive oxygen uptake activity was completely lost in the mutant. To estimate the resistance against oxidative stress, the cells were treated with H_2O_2 and menadione. When glycerol was added as a sole carbon source in the medium that greatly enhances the AOX gene expression, the mutant strain had significantly impaired growth compared with wild-type strain. Further, functional abnormalities were confirmed in the Mito Tracker Green FM-stained mutant cells grown on *n*-decane assimilation.

These data suggest that the alternative oxidase plays a role in the defense mechanism against oxidative stress in *C. maltosa*, regulating the cellular energy metabolism.

I-F p-12. Breaking down the flash-induced turnover of the cytochrome bc_1 complex into separate steps

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In chomatophore vesicles of *Rb. capsulatus*, a flash of light triggers the oxidation of an ubiquinol molecule in center P of the cytochrome bc complex (bc), at the interface between cytochrome *b* and the mobile FeS domain of the Rieske protein. The addition of Zn ions at <100 mkM has no impact on the extent of voltage generation by bc 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 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) [1]. We studied the correlation between these reactions at anaerobiosis, when the ubiquinone pool was half-reduced to imitate the native redox poise. In a wide range of conditions, the reduction of cytochrome *b* was by order of magnitude faster than (i) the cytochrome *c* reduction by ubiquinol and (ii) the voltage generation. We consider these observations as evidence for two functionally different proton paths at center P. First, we suggest that upon cytochrome *b* reduction, protons are pulled inside bc along a transmembrane water chain, already suggested to serve for electrostatic compensation [2]. This compensation might account for the absence of voltage generation matching the cytochrome *b* reduction. Second, we suggest that protons, after being liberated in center P, get out (electrogenically) via the Zn-binding site. The X-ray data showed that the Zn ion binds to a distinct histidine-rich patch next to center P in the mitochondrial bc [3]. Notably, this patch is sheltered by the FeS domain when the latter is docked to cytochrome *b* and is exposed when the FeS domain is close to cytochrome *c*. This feature explains (i) why the reduction of cytochrome *c* is coupled to the voltage generation, (ii) why Zn ions acts concurrently on both these reactions, and (iii) why protons cannot enter through this site upon cytochrome *b* reduction.

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I-F p-13. Determination of phospholipids bound to cytochrome bc_1 complex of *Saccharomyces cerevisiae*

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The cytochrome bc_1 complex (QCR) of *S. cerevisiae* is a multisubunit membrane protein complex located in the inner mitochondrial membrane. It catalyzes the electron transfer from ubiquinol to cytochrome *c* by the Q cycle mechanism (Mitchel 1976, J. Theor. Biol. 62, 327–367) thereby translocating protons from the matrix to the intermembrane space. X-ray structure analysis revealed the presence of six specifically bound phospholipids: cardiolipin (CL), phosphatidylinositol (PI), phosphatidylcholine (PC) and phosphatidylethanolamine (PE)(Lange, C., Nett, J.N., Trumpower, B.L. and Hunte C., 2001, EMBO J. 20, 6591–6600; Palsdottir, H., Lojero, C.G., Trumpower, B.L. and Hunte, C., 2003, J. Biol. Chem.278, 31303–31311; Palsdottir, H. and Hunte, C., Biochim. Biophys. Acta., in press). Their binding positions imply that they contribute to the structural stability as well as to the function of the protein complex. For instance, the headgroup of CL is located close to the quinone reduction site providing a possible entry site for proton uptake.

During purification of QCR the amount of phospholipids retained in the membrane protein complex is progressively lowered as assessed by thin layer chromatography. Already after the first chromatography step the composition of the remaining phospholipids is different from that of the inner mitochondrial membrane indicating a specific binding of the enriched classes PE and CL. The phospholipid species and their fatty acids were qualitatively analyzed by electrospray ionization tandem mass spectrometry (ESI-MS/MS) identifying them by their characteristic fragmentation patterns. The observed phospholipids remaining in the QCR crystals (CL 64:4–72:4, PE 32:2–36:2, PI 34:1 and 36:1) resemble the classes found in the X-ray structure. Specific lipids are essential for the functionality of membrane proteins (Gomez, B. Jr and Robinson, N.C., 1999 Biochemistry, 38, 9031–9038) and their ability to form high quality crystals (Kurisu, G., Zhang, H., Smith, J.L., Cramer, W.A., 2003, Science 302, 1009–1014). To allow routinely monitoring of phospholipids a normal phase HPLC combined with evaporative light scattering detection was developed. The presented results merge qualitative data with quantification of the phospholipids.

I-F p-14. Solubilization of functionally connected cytochrome bc_1 complex and reaction center from *Rb. sphaeroides* R-26.1 chromatophores by beta-dodecylmaltoside

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The procedure for isolating cytochrome bc_1 complex from bovine and yeast mitochondria and from *Rb. capsulatus* and *Rb sphaeroides* Ga photosynthetic membranes [1] was applied on *Rb. sphaeroides* R26.1 chromatophores. The neutral detergent β-dodecylmaltoside was found to selectively solubilize cytochrome bc_1 complex together with reaction center (RC). The preparations were characterized before any chromatographic step. Flash photolysis experiments revealed large QB occupancy with a rather slow RC charge recombination rate of 0.25 s-1. TLC analysis of the lipids retained in the extracts showed the presence of comparable amounts of phosphatydilglycerol (PG), phosphatydilcholine (PC) and phosphatydiletanolamine (PE). Moreover, reduced horse cytochrome *c* added to these preparations was rapidly oxidised revealing the presence of cytochrome *c* oxidases. This was confirmed by addition of sodium azide which proved to reduce the cytochrome *c* oxidation rate. RC multiple turnover measurements at 550 nm in presence of reduced cytochrome *c* indicated the existence of a consistent quinone pool. The complete rereduction in the dark of cytochrome *c* was observed following its photoxidation by actinic illumination. Antymicin A reduced the rate of cytochrome *c* rereduction suggesting a role of bc_1 complex in driving this process. In spite of a probable functional association between RC and bc_1 complex, the extracts showed only a minimal contamination of LH complexes as revealed by optical spectra and SDS-PAGE analysis. This preparation, suitably improved by purification steps, represents a promising system for investigating mechanistic and structural aspects of bacterial photosynthesis in a simplified but comprehensive form.

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I-F p-15. Effect of flutolanil on mitochondrial rhodoquinol-fumarate oxidoreductase from parasitic nematode, *Ascaris suum*

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Parasitic helminth have exploited a variety of energy transducing systems in their adaptation to peculiar habitats in their hosts. The parasitic nematode, Ascaris suum, resides in the host small intestine where oxygen tensions are low, and has exploited unique anaerobic respiratory chain to adapt to its microaerobic habitat, called the NADH-fumarate reductase system.

Electrons from NADH are accepted by rhodoquinone through NADH- rhodoquinone oxidoreductase (NQR) (1), and then transferred to fumarate through rhodoquinol-fumarate reductase (QFR). The anaerobic electron transfer in NQR couples with proton transport across the innermitochondrial membrane, providing ATP even in the absence of oxygen. In this system, rhodoquinol oxidizing QFR serves as the terminal oxidase, producing endproduct succinate.

Considering the A.suum QFR is the member of succinate-quinone oxidorecuctase (SQR) superfamily, on the basis of hemes' and transmenbrane subunits' composition, it is classified into the same type with mammalian SQR which catalyzes reverse reaction of A. suum QFR. There must be the structural features which give efficient fumarate reducing activity in the case of A. suum, or extreme unidirectional activity in the case of mammalian enzyme. The crystallographic approach would help understanding those features.

In this study, we have established the purification procedure of QFR from A. suum mitochondria, and also found Flutolanil which is known as the inhibitor of fungus SQR, is potent inhibitor of the nematode enzyme. Moreover, the kinetic analysis revealed the uniqe effect of Flutolanil, which implied the existence of multiple quinone binding site.

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I-F p-16. Cytochrome bc_1-analogous complexes in extremophilic organisms

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Cytochrome bc_1-analogous complexes are widely distributed in the photosynthetic and respiratory electron transfer chains of the bacteria and eukarya. Consequently, the complexes from these organisms have been intensively studied. Considerably less is known about the homologous complexes from the archaea and organisms thriving under conditions of extreme pH or salinity.

Genomic data indicate the presence of cytochrome bc_1-homologous complexes in some, but not all archaea (Hiller et al. 2003). However, until now, only the soxM- complex from the thermo acidophilic crenarchaeon Sulfolobus acidocaldarius has been isolated and partially characterized (Komorowski et al. 2002). Additional information is available from studies conducted on isolated, or heterologous expressed Rieske iron sulfur proteins, one of the three strictly conserved subunits of these complexes (Schmidt 2004). Being located on the outside of the cytoplasmatic membranes, or the equivalent in the case of mitochondria and chloroplasts, the Rieske proteins are facing special challenges maintaining their structural integrity and stabilizing an acid sensitive cofactor. Based on the available crystallographic and functional data some of the strategies by which this challenge is meet can be identified.

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I-F p-17. Study of electrogenic reactions of cytochrome bf-complexes in model system

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Most of the information concerning the electrogenic events in the cytochrome (cyt) bf-complex has been obtained from analysis of the slow phase of electrochromic shifts of the carotenoid absorption bands that reflect the generation of a transmembrane electric potential gradient (delta psi) in chloroplast thylakoids and in whole plant cells. Assays on thylakoid membranes suggested that the main electrogenic step (70–75%) is due to electron transfer between the two hemes b, whereas the remaining part (25–30%) is associated with cyt b oxidation and/or uptake of stromal protons accompanying the plastoquinone (PQ) reduction. This estimate was based on the extent of attenuation of the slow carotenoid shift in the presence of a PQ reductase (Q_i) site inhibitor 2-*n*-nonyl-4-hydroxyquinoline-*N*-oxide (NQNO) and a PQ oxidase (Q_o) site inhibitor stigmatellin. To examine the mechanisms of charge transfer in the purified bf-complex, an active hybrid model system comprising photosystem I (PS I) and bf-complexes has been constructed (Rich P., Heathcote P., Moss D. Biochim. Biophys. Acta. 1987. V. 892. P. 138–151). The goal of the present work was to study the flash-induced delta psi generation provided by bf-complex turnover coupled with electron transfer via cyt c6, which reduces the photooxidized chlorophyll dimer P700 in PS I. The flash-induced generation of delta psi in this hybrid system has been monitored by a direct electrometric technique in proteoliposomes (PL) containing PS I and cyt bf-complexes from cyanobacteria and spinach. The position of the PS I primary donor P700 and cyt f close to the outside surface of the membrane of PL enabled us to study the electrogenic reactions of the cyt bf-complex in this model system. In the presence of decyl-PQ and cyt c6, besides the fast electrogenic phase due to the primary charge separation in PS I, additional phases appeared in the millisecond time range. The amplitudes of these phases were partially decreased in the presence of NQNO; these phases fully disappeared upon addition of stigmatellin. The kinetics of delta psi generated by the cyt bf-complex were similar to the kinetics of cyt b reduction measured by flash-spectrometry in the hybrid PL. We suppose that the NQNO-sensitive phase is most probably due to PQ reduction and protonation at the Q_i site, while the NQNO-insensitive/stigmatellin-sensitive phase is due to electron transfer between cytochrome *b* hemes and proton ejection accompanying PQH_2 oxidation at the Q.

I-F p-18. Structural analysis of cytochrome *c* bound to the cytochrome bc_1 complex: an interaction critical for electron transfer

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In respiration, the mobile electron carrier cytochrome *c* shuttles electrons from the cytochrome bc_1 complex to cytochrome *c* oxidase. Specific and transient interaction between the redox partners ensures a fast turnover. A crystal structure of the yeast cytochrome bc_1 complex with its bound substrate cytochrome *c* and an FV fragment has been determined at 2.97 Å resolution (Lange and Hunte 2002, Proc. Natl. Acad. Sci. USA 99 2800–2805). The specific interactions of the reaction complex are mainly mediated by nonpolar forces. A central cation–Pi interaction is an important and conserved feature of the interface. Cytochrome *c* binds only to one recognition site of the homodimeric complex. The occupancy of ubiquinone in the Q_i site is higher in the monomer with bound cytochrome *c* suggesting a coordinated binding of cytochrome *c* and ubiquinone. Cryo-conditions have been established for the crystals of the ternary complex and the structure has been refined at a higher resolution, namely at 2.52 Å, allowing to include water and phospholipid molecules and to analyse the Q_i site occupancy.

To address the question of single or multiple binding conformations for the electron transfer complex, structures have been determined at defined redox states and at different ionic strength values. The interaction of cytochrome *c* with the cytochrome bc_1 complex is ionic strength dependent. Cytochrome *c* binding is maximal below an ionic strength of 120 mM. Kinetic data show an ionic strength dependence of cytochrome *c* reduction with a maximum activity at 120 mM, close to the physiological value (Hunte, Solmaz, Lange, 2002, Biochim. Biophys. Acta 1555 21–28). Structures at the defined ionic strength values of 180 mM and 100 mM have been determined and will be presented. Furthermore,structures of the oxidized and ascorbate reduced complex were determined, respectively. Comparison of these structures indicates that the interaction of the electron transfer complex is affected by the redox state. Small shifts in cytochrome *c* position as well as alterations in interacting pairs of side chains have been observed, providing evidence for the dynamic nature of the binding interaction.

I-F p-19. Interrelationships between membrane potential, Q-pool redox poise and oxygen consumption rate in potato and yeast mitochondria

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We have simultaneously determined membrane potential, Q pool redox poise (Qr/Qt) and oxygen consumption rate (vO_2) under different energetic conditions (state 3, state 4 and uncoupled) in potato tuber and yeast (*Schizosaccharomyces pombe*, Sp.o11) mitochondria oxidising externally added NADH. Various steady states were attained by applying subsaturating NADH concentrations using an NADH-regenerating system. The kinetic results obtained following this approach agree well with those obtained with the more commonly used technique of modulating succinate-dependent respiratory activity with malonate.

All three parameters change in parallel, however there are limiting conditions where one parameter is stable and the other two change, e.g., in isolated potato mitochondria during state 3, vO_2 reaches a maximum value before the membrane potential and Qr/Qt reach a steady state.

Recent experiments done with Sp.o11 have different kinetic plots from what we have previously reported [Affourtit, C., Albury, M.S., Krab, K. and Moore, A.L.J.Biol.Chem. 274 (1999), 6212–6218]. We believe this to be due to a better coupling of the mitochondria and we are currently investigating the control of membrane potential on Q-pool kinetics.

After characterizing fully the mitochondrial dynamics of Sp.o11 mitochondria in terms of membrane potential, Qr/Qt and vO_2 under a variety of energetic conditions we are applying the same method on Sp.o11 mitochondria expressing alternative oxidase (AOX). AOX is a nonprotonmotive, cyanide insensitive, terminal oxidase found in plants and a number of fungi, protists and trypanosomes. AOX activity affects the efficiency of oxidative phosphorylation [Affourtit, C., Albury, M.S., Krab, K. and Moore, A.L. J. Biol. Chem. 274 (1999), 6212–6218]. We will present results on how, in Sp.o11 mitochondria, the kinetic curves of the interrelationships of membrane potential, Qr/Qt and vO_2 under state 3, state 4 and uncoupled conditions are dependent an AOX activity.

I-F p-20. Study of proton channels in the quinol oxidase (aa_3) from *Acidianus ambivalens* using theoretical methodologies

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Haem-copper oxidases are membrane bound proteins found in the respiratory chain of aerobic organisms. These proteins catalyse the reduction of oxygen to water in a process coupled with the translocation of protons across the mitochondrial or cytoplasmic membrane. Because the catalytic activity of these proteins occurs well inside their subunit I (at haem cofactors), proton channels must exist in order to allow the protons to reach the catalytic centre for reaction with the oxygen and to be translocated from one side of the membrane to the other. Several different channels have been previously identified using site-directed mutagenesis and structural data. However, due to the high divergence among the haem-copper oxidases superfamily, these proton channels can show remarkable differences and their conservation is a matter of debate. The Quinol Oxidase from *Acidianus ambivalens* is an example of a protein whose proton-channel topology departs from that normally found in haem-copper oxidases. Given that a structural analysis is very important to understand the proton translocation process in these proteins and given that the structure of the Quinol Oxidase has not been yet resolved, we have used comparative modelling techniques in order to derive a 3D model of this protein. Due to the high importance that water molecules have in this proton pumping mechanism, we have combined the generated models of the protein with a newly theoretical methodology in order to identify those regions of the protein where the probability of finding water molecules is higher. From our results we were able to identify two spatial homologous channels with the canonical oxidase from *Paracoccus denitrificans*, and a third channel with a unique structural position among all terminal oxidases with known structure.

I-F p-21. Substrate binding and proton transfer pathways in the mitochondrial cytochrome bc_1 complex

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The yeast cytochrome bc_1 complex (QCR, ubiquinol:cytochrome *c* oxidoreductase) is part of the respiratory chain, which resides in the inner membrane of mitochondria. QCR couples the transfer of electron between ubiquinol and cytochrome *c* to a vectorial proton translocation across the membrane. Proton release and uptake is integral to QCR catalaysis [1]. The enzyme mechanism is decribed by the modified Q-cycle, in which four protons are released at the quinol oxidation site (Q_P site) and two protons are taken up at the quinone reduction site (Q_N site) during the turnover of the enzyme. Proton transfer pathways at the two active sites are still poorly understood. As can be seen from the high resolution structure of yeast QCR, the active sites are located at the boundary of the hydrophobic core of the enzyme. Most importantly, Q_N and Q_P have no direct access to the aqueous solution [2]. On the basis of the structural data, proton transfer pathways for both active sites have been proposed [3, 4]. The suggested proton wires rely on conserved residues as well as on phospholipids bound in the vicinity of the Q_N site [3]. In this study, the proposed proton transfer pathways are challenged by a set of QCR variants, that were constructed by classical site-directed mutagenesis and biolistic bombardment to create variants of the mitochondrially encoded cytochrome *b*. The variants are characterized in respect to enzyme kinetics as well as substrate and inhibitor binding. Changes in the quinone/quinol binding sites are characterized by inhibitor studies as well as with EPR experiments. FTIR studies of the isolated variants are used to investigate redox-dependent protonation states of the key residues for proton transfer at the Q_P site. These data give experimental support for the proposed proton transfer pathways. Thereby, the understanding of the molecular mechanism of the enzyme is improved.

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I-F p-22. Cofactor ligand interaction in the bc_1 complex studied infrared spectroscopically in the spectral range from 2000-500 cm^{-1}

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Ubiquinol–cytochrome *c* oxidoreductase (bc_1 complex) is one of the fundamental components of the respiratory electron transfer chains located in the inner mitochondrial or bacterial cytoplasmic membrane. In this study we present the electrochemically induced FTIR difference spectra of the bc_1 complex from *P. denitrificans* and *S. cerevisiae* in the spectral range from 1800 to 500 cm^{-1}. This method allows to determine the reorganizations in the enzyme perturbed upon the redox reaction. The mid infrared provides information on the reorganization of the backbone, the cofactors and of possible protonation reactions. In the lower frequency range below 800 cm^{-1}, modes from the heme ring and from amino acids liganding the redox centers are discriminated due to spectra on model compounds and of water soluble fragments of the enzyme. On the basis of the detailed analysis of the spectra and available structural information (1–3), the interaction between inhibitors and individual amino acid side chains is presented for the reduced and oxidized form of the complexes. Interaction partners are identified together with side directed mutants.

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I-F p-23. Electron transfer coupled protein in relaxation in reaction center of *Rb. sphaeroides*

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Proteins can control low-exothermic electron transfer via charge redistribution and/or conformational changes [1]. We studied how the replacement of Pro-209 of the L-subunit by Tyr or Glu [2,3] in the *Rb. sphaeroides* reaction center (RC) affected protein relaxation accompanying the flash-induced reduction of the secondary quinone Qb.In native membrane vesicles, we traced electrometrically proton transfer to Qb upon both semiquinone and quinol formation (in response to the first and second flash, respectively). In the mutants, activation energy of the slower component of proton transfer was lower ($\sim$30kJ/mol) than in the wild type ($\sim$60 kJ/mol), both after the first and the second flash. Together with increased inhibitor accessibility of quinone-binding sites in the mutants, this evidence suggested that the quinone pocket in the mutant RCs was less tight than in the wild type Rcs.

The steered MD simulations revealed that in the case of the mutant RC a smaller force was needed to cause a flip motion of the Qb ring between its two functional positions in the pocket, as compared to the wild type. The energy difference was in quantitative agreement with the experimental data. This evidence indicated that the high activation barrier upon the electron/proton transfer at the Qb site might be due to the protein conformational change enabling the twisting movement of the Qb ring between its two functional positions, as suggested previously [1, 4–6].

We conclude that Pro-209 essentially contributes to the tightness of the quinone-binding pocket.

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II-A Mitochondrial Dynamics

II-A p-1. Characterization of HFis1 domains in mitochondrial division

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Mitochondria are dynamic structures that undergo fusion and fission events continually throughout the life a cell (Bereiter-Hahn and Voth 1994). The cellular mechanisms that regulate mitochondrial division and fusion are still poorly understood, even though some of the proteins implicated have been identified in several organisms and may play a role in apoptosis. DRP1 is a cytosolic dynamin-like GTPase which participates in mitochondrial division, together with the integral membrane protein hFis1 (Smirnova, Griparic et al. 2001; James, Parone et al. 2003). Studies in yeast suggest that Dnm1, the yeast homologue of DRP1, is recruited to the mitochondria, and that this recruitment depends on the presence of Fis1 at the outer mitochondrial membrane (Sesaki and Jensen 1999; Mozdy, McCaffery et al. 2000). It has been proposed that the Fis1 protein acts as a docking protein, but it also seems to play a role in the division itself (Shaw and Nunnari 2002). To understand the role played by hFis1, we analysed the effect of overexpressing wt and truncated versions of hFis1 on mitochondrial morphology and cell death. The wt version of hFis1 triggered fragmentation of mitochondria and led to cell death when transfected into Hela cells. We also show that the C terminus contains signals sufficient to target yellow fluorescent protein to the outer mitochondrial membrane and induces an aberrant mitochondrial morphology leading to cell death. The N terminus of hFis1 seems essential for division as removal of the first helix of hFis1 resulted in loss of the fragmented morphology and abolished cell death. Our results suggest the presence of a bipartite signal within hFis1 that may be responsible for its effect on mitochondrial division and cell death.

II-A p-2. On the mechanism of ceramide-dependent release of proapoptotic proteins from mitochondria

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Mitochondria play a key role in apoptosis through the release in the cytosol of several intermembrane proteins including cytochrome *c*, AIF (apoptotic induction factor), endonuclease G, Smac-DIABLO (1). Two general mechanisms for this protein release process have been proposed: i) matrix swelling, with consequent outer membrane rupture, caused by cyclosporin-A (CsA) sensitive mitochondrial permeability transition (MPT) (2); ii) formation of specific conducting channels, provided by Bax, Bad and tBid, which make the outer membrane permeable to cytochrome *c* (3). Several observations have been reported in the last years indicating that ceramide may activate the mitochondrial route of apoptosis (4–6). Addition to isolated mitochondria of cell permeable C_2-ceramide caused inhibition of the electron transfer activity (4), a large increase of ROS generation (4,5) and cytochrome *c* release (4,6). Long-chain natural C16-ceramide caused cytochrome *c* release from heart mitochondria as well, without affecting the membrane potential (4).

Our work was aimed at analysing the effect of both short- and long-chain ceramides on the release of proapoptotic proteins from rat heart mitochondria and the mechanism by which this process takes place.

The results obtained show that on addition of either C_2- or C_{16}-ceramide to mitochondria suspended in a saline medium, release of cytochrome *c* and AIF from the intermembrane space takes place. The release process is Ca^{2+}-independent and is not inhibited by CsA. In order for the protein release process to occurr, the presence of an oxidizable substrate is required. When mitochondria are suspended in sucrose instead of potassium medium, only short chain C_2-ceramide causes cytochrome *c* release, through a Ca^{2+}-dependent and CsA sensitive MPT mechanism. The latter effect appears to be related to the membrane potential dissipating ability exhibited by C2-ceramide.

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II-A p-3. Two mitofusin proteins, mammalian homologues of Fzo, with distinct functions are both required for mitochondrial fusion

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Mitochondria are dynamic organelles undergoing frequent fission and fusion or branching. Although these morphologic changes are considered crucial for cellular functions, the underlying mechanisms remain elusive, especially in mammalian cells. We characterized two rat mitochondrial outer membrane proteins, Mfn1 and Mfn2, with distinct tissue expressions that are homologous to *Drosophila* Fzo, a GTPase involved in mitochondrial fusion. Expression of the GTPase-domain mutant of Mfn2 ($Mfn2^{K109T}$) in HeLa cells induced fragmentation of mitochondria in which $Mfn2^{K109T}$ localized at the restricted domains. Immuno-electronmicroscopy revealed that $Mfn2^{K109T}$ was concentrated at the contact domains between adjacent mitochondria, suggesting that fusion of the outer membrane was arrested at some intermediate step. Mfn1 expression induced highly connected tubular network structures depending on the functional GTPase domain. The Mfn1-induced tubular networks were suppressed by coexpression with Mfn2. Immunoprecipitation experiments revealed that Mfn isoforms could form homooligometric and heterooligomeric complexes in HeLa cells. In vivo depletion of either isoform by RNA interference revealed that both are required to maintain normal mitochondrial morphology. Fusion of differentially labeled mitochondria in HeLa cells that were subjected to depletion of either Mfn isoform and subsequent cell fusion by hemagglutinating virus of Japan revealed that both proteins are essential and have distinct functions in mitochondrial fusion. We conclude that the two Mfn isoforms cooperate in mitochondrial fusion in mammalian cells and that the balance of expression between Mfn_1 and Mfn_2 in tissues might reflect tissue-specific mitochondrial morphology.

II-A p-4. OPA_1, a mitochondrial dynamin related protein mutated in dominant optic atrophy, participates in the mitochondrial pathways of apoptosis

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Mitochondria amplify apoptotic signals by releasing proteic cofactors in the cytosol to induce the competent activation of effector caspases, the proteases that dismantle the dying cell. A profound remodelling of the inner mitochondrial structure, including fusion of the individual mitochondrial cristae and enlargement of the narrow tubular cristae junction, is needed to insure the complete release of cytochrome *c* (1). The shape of the mitochondrial reticulum and of the individual mitochondria is controlled by a family of large GTPases called dynamins. So far, one of these proteins, OPA_1, has been localized to the inner mitochondrial membrane, facing to the intermembrane space (2). Dominant optic atrophy, the most common cause of inherited optic atrophy has been ascribed to loss of function mutations of OPA_1 (3). Preliminary results of our laboratory suggest that OPA_1 might participate in the mitochondrial remodelling pathway during apoptosis. We therefore elected to study whether gain and loss of function of OPA_1 affect the apoptotic response and if this occurs by a direct effect on the mitochondrial pathway(s) of apoptosis. We generated two OPA_1 dominant negative mutants that recapitulate the mutations found in patients with dominant optic atrophy: K301A OPA_1 has a single aminoacid substitution within the GTPase domain of the protein, whereas R905stop is a truncative mutation that generates a protein lacking the coiled-coil domain essential for protein protein interactions. Expression of a wild-type (wt) but not of the inactive, dominant negative K301A or R905stop OPA_1 protects from apoptosis induced by intrinsic stimuli such as staurosporine, H_2O_2 and etoposide. Overexpression of OPA_1 inhibits cytochrome *c* release from mitochondria following H_2O_2 treatment. We are in the progress of dissecting whether OPA_1: (i) interacts with the mitochondrial apoptotic pathways regulated by BCL-2 family members; (ii) acts by interfering with the mitochondrial remodelling pathway or by promoting mitochondrial fusion. Our data will be instrumental in understanding whether OPA_1 participates in apoptosis and its mutations can therefore result in sustained apoptosis of the retinal ganglion cells.

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II-A p-5. Proteomic analysis of calcium-induced phosphorylation events in porcine heart mitochondria

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Posttranslational modification of mitochondrial proteins by phosphorylation or dephosphorylation may play an essential role in signaling pathways involved in the regulation of energy metabolism, as well as in the initial steps of mitochondria-induced apoptosis. The purpose of this study was to evaluate the protein phosphorylation changes occurring in mitochondria due to extramitochondrial Ca^{2+}. Mitochondria isolated from porcine heart left ventricle were exposed to either no calcium or a high physiological free calcium level (100 μM) to induce apoptosis, as confirmed by cytochrome *c* release from the inner mitochondrial membrane. These high Ca^{2+} levels were used for our initial screen to detect potentially both physiological as well as pathophysiological processes. The mitochondrial phosphoproteome was analyzed using two-dimensional gel electrophoresis and fluorescence staining with a novel dye that indiscriminately stains all phosphoproteins. The staining intensity of the fluorescent phosphosensitive dye was normalized by the intensity of a total protein stain to determine the degree of phosphorylation for each spot observed on the gels. Phosphoproteins were identified using MALDI tandem time-of-flight mass spectrometry in conjunction with computer-assisted protein spot matching. Numerous phosphorylated peptides ($\sim$ 30 individual proteins) were observed in steady-state mitochondria not exposed to calcium, including several proteins involved in the mitochondrial electron transport chain. These included: cytochrome oxidase and several site I, II and III subunits. The degree of phosphorylation changed significantly in response to high levels of calcium for many proteins of varying function. Those currently identified include succinate dehydrogenase, citrate synthase, and manganese superoxide dismutase. These results suggest that proteins of the mitochondria are regulated by phosphorylation events triggered by calcium signaling or apoptosis. The functional significance of the phosphorylation events is being investigated for selected proteins or enzyme systems.

II-A p-6. Two mitofusin proteins, Mfn1 and Mfn2, regulate oligomerization states of the mitofusin-containing complex through distinct GTPase activity in mitochondrial fusion reactions

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The mammalian homologues of yeast and *Drosophila* Fzo, Mitofusin (Mfn) 1 and 2, are both essential for mitochondrial fusion and maintenance of mitochondrial morphology. Though the GTPase domain is required for Mfn protein function, the molecular mechanisms of the GTPase-dependent reaction as well as the functional division of the two Mfn proteins are unknown. To examine the function of Mfn proteins, tethering of mitochondrial membranes was measured in vitro by fluorescence microscopy using green fluorescence protein- or red fluorescent protein-tagged and Mfn1-expressing mitochondria, or by immunoprecipitation using mitochondria harboring HA- or FLAG-tagged Mfn1 or Mfn2 proteins. These experiments revealed that Mfn1-harboring mitochondria were efficiently tethered in a GTP-dependent manner, whereas Mfn2-harboring mitochondria were tethered with only low efficiency. Sucrose density gradient centrifugation and coimmunoprecipitation revealed that the GTP-dependent tethering across apposed Mfn1-harboring mitochondria produced oligomerized $\sim$ 230- and $\sim$ 400-kDa complexes. The $\sim$ 400-kDa complex was composed of Mfn_1 from distinct apposing membranes (docking-complex), whereas the $\sim$ 230-kDa complex was composed of Mfn_1 present on the same membrane (*cis*-complex). Mfn1 exhibited higher activity for this reaction than Mfn_2. Purified recombinant Mfn1 exhibited $\sim$ 8-fold higher GTPase activity than Mfn_2. These findings indicate that the two Mfn proteins have clearly distinct activity, and suggest that Mfn1 is mainly responsible for GTP-dependent membrane-tethering.

II-A p-7. A novel mechanism of regulation of cardiac contractility by mitochondrial functional state

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It is generally considered that mitochondria regulate cardiac cell contractility by providing ATP for cellular ATPases and by participating in Ca^{2+} homeostasis. However, besides these well documented functions, other possible mechanisms by which mitochondria can influence contractility have been largely overlooked. Here, we demonstrate that inhibition of the mitochondrial electron-transport chain strongly increases Ca^{2+} dependent and independent isometric force development in rat ventricular fibers with selectively permeabilized sarcolemma. This effect is unrelated to the ATP-generating activity of mitochondria or Ca^{2+} homeostasis. Furthermore, various conditions that increase K^+ accumulation in the mitochondrial matrix (activation of ATP- or Ca^{2+}-dependent K^+ channels as well as inhibition of the K^+ efflux pathway via the K^+/H^+ exchanger) induce a similar mechanical response. Modulators of mitochondrial function that augment isometric force also cause swelling of mitochondria in the vicinity of myofibrils in situ, as shown by confocal microscopy. Osmotic compression of intracellular structures abolishes the effect of mitochondria-induced force modulation, suggesting a mechanical basis for the interaction between the organelles. These findings suggest a novel mechanism for cellular regulation of myofibrillar function, whereby increases in mitochondrial volume can impose mechanical constraints inside the cell, leading to an increase in force developed by myofibrils. The proposed role of mitochondria in the regulation of myofibrillar activity may have important pathophysiological implications. During energetic stress, for example, opening of K^+ channels and/or blocking of the K^+/H^+ exchanger due to mitochondrial deenergization could increase the developed tension and consequently counteract the negative inotropic effect. Agents aimed at controlling transmembrane ionic fluxes through the mitochondrial membrane might thus provide valuable tools for modulating cardiac contractility.

II-A p-8. Quantification of mitochondrial fusion

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Mitochondria divide and fuse continuously. To quantify the rate of mitochondrial fusion in living cells we have constructed a mitochondrial imported form of photoactivatable GFP (mito-PA-GFP) that allows single mitochondria labeling. Following the activation of GFP in individual mitochondria, the measurement of the rate of fluorescence decrease allows a quantification of mitochondrial fusion rates. Using this assay we found that mitochondrial fusion halts during apoptosis, close in time to Bax translocation and SMAC/Diablo release from the mitochondrial intermembrane space. A modified form of this mito-PA-GFP assay has helped in the identification a new candidate protein that is not required for the mitochondrial fusion step but is required for the coupling of the rates of inner and outer mitochondrial membrane scission.

II-A p-9. Ca^{2+} and regulation of mitochondrial respiration in the heart

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In oxidative muscles mitochondria are organized into functional complexes with myofibrils and sarcoplasmic reticulum, forming intracellular energetic units (ICEUs) with very regular arrangement of mitochondria. The relationship between this structural organization and mechanisms of mitochondrial regulation has been studied applying fluorescent confocal microscopy and analysis of kinetics of mitochondrial respiration. This analysis was performed in permeabilized cardiomyocytes (PCM), permeabilized myocardial fibers (PMF) and their ''ghosts'' preparations after selective myosin extraction (GMF) and during calcium-induced contraction by varying free [Ca^{2+}] in the range 0.1–3.0 μM. At 0.1 μM free [Ca^{2+}] myocardial fibers and cardiomyocytes were relaxed and apparent Km' for exogenous ADP was high (300–400 μM) in a line with our previously published data. However, increased free [Ca^{2+}] from 0.1 to 3.0 μM induced, in presence of 2 mM ATP, strong contraction of PCM and PMF with significant changes in mitochondrial arrangement and interactions. Structural changes in PMF were accompanied by significant decrease of apparent Km' for ADP in the kinetics of mitochondrial respiration from 330 ± 50 to 40 ± 20 μM with rather minor changes in Vmax. This decrease in apparent Km' was observed also after inhibition of mitochondrial Ca^{2+} uptake by 10 μM Ruthenium Red. In contrast, no contraction and no changes in mitochondrial localization were seen in GMF due to the absence of myosin. Accordingly, apparent Km' for ADP in this preparation did not change by calcium and kept high (270 ± 90 μM). Measurement of the intensity of TMRE fluorescence demonstrated that in all preparations mitochondrial inner membrane potential was not altered by the increase in free calcium concentration, and that mitochondrial permeability transition is probably not involved in the decrease of apparent Km' for ADP.

Summarizing, calcium-dependent contraction and shortening of sarcomere length causes significant changes in ICEUs structure with parallel increase in apparent affinity of mitochondrial respiration to ADP, thus indicating strong structural and functional interrelations between sarcomere and mitochondria.

II-A p-10. Role of PBR in mitochondria, cellular and heart dysfunctions induced by oxidative stress and ischemia–reperfusion: protective effect of SSR180575, a potent, specific and irreversible PBR ligand

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It is noteworthy that mitochondria many time has been implicated in the biochemical, cellular and physiological dysfunctions induced by cardiac ischemia–reperfusion, ultimately leading to myocardial infarction. In particular, opening of the mitochondrial permeability transition pore (MPTP), a multiproteic complex of the inner mitochondrial membrane, plays a key role in the regulation of apoptosis, in early event occurring during the injury process of ischemia–reperfusion. In this context, the peripheral benzodiazepine receptor (PBR), a mitochondrial protein proposed to be a component of the MPTP, could play a significant role in mitochondrial regulation although its exact function remains unclear.

The aim of this work was to determine the exact role of PBR in ischemia–reperfusion injury and to test the potential beneficial effect of a novel PBR ligand, SSR180575. First, we demonstrated specificity, potency and irreversibility of the PBR ligand on mitochondria isolated from rat heart (Vin et al. 2003). Then, in order to characterize the mitochondrial, cellular and cardiac consequences of ischemia–reperfusion, we examined the effects of SSR180575 in several models of oxidative stress in isolated cardiac mitochondria and cells and in ischemia–reperfusion induced cardiac damage (Leducq et al., 2003). Hydrogen peroxide (H_2O_2) decreased mitochondrial membrane potential, reduced oxidative phosphorylation capacities, and caused cytochrome *c* release, caspase 3 activation and DNA fragmentation. SSR180575 (100nM) prevented all these effects. In perfused rat hearts, SSR180575 (100 nM–1 μM) greatly reduced the contractile dysfunctions associated with ischemia–reperfusion.

In conclusion, we have demonstrated that PBR plays a major role in the regulation of cardiac ischemia–reperfusion injury, and that SSR180575, a novel specific and irreversible PBR ligand, is of potential interest in these indications.

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II-A p-11. Ablation of mitofusin 2 alters cytosolic and mitochondrial Ca^{2+} transients

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Mitochondrial morphology results from the net balance between fusion and fission events. Mitofusin (MFN) 1 and 2 are large GTPases belonging to the family of dynamin-related proteins that mediate fusion of outer mitochondrial membranes in mammalian cells. Loss of function mutations in mfn2 gene cause Charcot-Marie-Tooth type IIa neuropathy (1), while deletion of mfn1 and mfn2 in the mouse results in embryonic lethality (2), suggesting that these proteins are crucial for cell function. Mitochondria participate in Ca^{2+} signalling, modulating the shape and the extent of cytosolic Ca^{2+} transients. We reasoned that disruption of the regulated mitochondrial network might alter Ca^{2+} signalling in MFN-deficient cells. Here we show that cytosolic Ca^{2+} increase in response to passive discharge of intracellular stores was bigger in mfn2 $-/-$ but not in mfn1 $-/-$ mouse embryonic fibroblasts (MEFs) as compared to their wt counterparts. We measured mitochondrial Ca^{2+} uptake with the fluorescent Ca^{2+} indicator rhod-2 and the genetically encoded Ca^{2+} sensor aequorin. Mitochondria of mfn2 $-/-$ cells accumulated more Ca^{2+} than wt and mfn1 $-/-$ cells. Reexpression of MFN2 in mfn2 $-/-$ MEFs corrected cytosolic ($[Ca^{2+}]$cyt) and mitochondrial ($[Ca^{2+}]$mit) Ca^{2+} responses, showing that the effect was a specific consequence of MFN2 deletion. Thus, specific ablation of MFN2 impairs mitochondrial and hence cytosolic Ca^{2+} signalling, a potential pathogenetic mechanism for Charcot–Marie–Tooth type IIa neuropathy.

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II-A p-12. Identification of genes affected by mitochondrial morphology in mammals

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In recent years, the view of mitochondria has changed from that of individual, static organelles to a dynamic, organized network shaped by movements, fission and fusion. Although mitochondrial fission and fusion was proven in yeast a long time ago, it is only recently that we and others confirmed mitochondrial fusion in mammalian cell cultures (Mattenberger et al. FEBS Lett., 2003; Legros et al. Mol Biol Cell, 2002; Santel et al. J Cell Sci, 2003; Eura et al. J Biochem, 2003).

In mammals, the only two well characterized proteins implicated in mitochondrial fusion are mitofusins 1 and 2. Homozygous knock-out of those proteins lead to mitochondrial fragmentation (Mfn1) or partial fragmentation and loss of regular shape (Mfn2) and both of them lead to embryonic lethality in mice (Chen et al. J Cell Biol, 2003).

In order to understand the role of mitochondrial fission and fusion, we used as a model immortalized embryonic fibroblasts isolated from Mfn1 knock-out mice (Chen and al. J Cell Biol, 2003). Although those cells display highly fragmented mitochondrial network, they have no growth defect and they appear to be normal. We hypothesized that those cells survive because they have been able to compensate for deficits induced by the lack of mitochondrial fusion. To identify those compensatory mechanisms or to identify genes altered by impaired fusion, we performed a transcriptional analysis by microarray. Several genes were found to be altered in those cells. We are currently investigating the relevence of those genes in the context of mitochondrial fission and fusion.

II-A p-13. Selective elimination of mitochondria (“mitoptosis”) induced by the inhibitors of respiration and uncouplers

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The damage of mitochondria induced by specific inhibitors of bioenergetic functions in human carcinoma cells (HeLa) and monkey kidney epithelial cells (CV-1) was studied. The inhibitors of respiratory chain and uncouplers (that dissipated mitochondrial membrane potential) caused fragmentation of tubular mitochondria, following formation of small round bodies and swelling of the organelles. The process was fully reversible during 24h after wash out of the inhibitors. Cytochrome *c* was not released into cytosol during fragmentation of mitochondria and no signs of apoptosis were observed.

The prolonged (48–72 h) incubation with the uncouplers in combination with antimycin or myxothiazole (the inhibitors of Complex III) induced clustering of mitochondria around the nuclei followed by almost complete elimination of mitochondria from cells (‘‘mitoptosis’’). Electron microscopy and immunoblot analysis demonstrated that the other organelles (nucleus, Golgi, endoplasmic reticulum etc.) remained intact. During prolonged incubation with the mitochondrial poisons apoptosis was induced in a significant fraction (60–70%) of the cells but the cells depleted of mitochondria remained viable without apoptotic changes in nuclei and plasma membrane (blebbing). It could be suggested that elimination of the damaged mitochondria would prevent overproduction of reactive oxygen species and release of proapoptotic proteins into cytosol thus preventing apoptosis. Probably ‘‘mitoptosis’’ provide a selective advantage for cancer cells under stressful conditions in rapidly growing tumors.

II-A p-14. Large-conductance channel openers modulate skeletal muscle mitochondria function

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Potassium channel openers (KCOs) stimulate ion flux through K^+ channels. They can act on potassium channels localized in plasma membrane and in membranes within the cell. In mitochondrial inner membrane two types of K^+ channels have been identified: mitochondrial ATP-regulated K^+ channels (mitoKATP channels) and Ca^{2+}-activated K^+ channels of big conductance (mitoBKCa channels). The mitoBKCa channel was described in cardiac mitochondria of guinea pig ventricular cells and it has been shown that its activation can protect heart against infraction.

The function mitoBKca channel can be modified by its openers, which activate the channel and stimulate K^+ influx into mitochondrial matrix. The functional role of these activation in mitochondria is still unknown, however. The BKCa activators can be divided into two groups: benzimidazolone derivatives (i.e., NS1619, NS004) and indole carboxylate derivatives (i.e., CGS 7184). These KCOs can modulate the mitochondrial inner membrane potential and influence on respiratory rate in different ionic environment. The influence of these compounds on function of mitochondria isolated from rat skeletal muscle will be presented.

Work supported by Polish State Committee for Scientific Research grants No. 3P04A05025.

II-B Channels in Coupling Membranes

II-B p-1. Bax is not directly involved in the Ca^{2+}-induced mitochondrial permeability transition

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The mitochondrial Permeability Transition Pore (PTP) and Bax are both believed to have a role in the release of proapoptotic proteins from mitochondria in the early stages of apoptosis. The PTP is thought to be formed by a supramolecular complex reportedly including Bax or activated by Bax after its transfer to mitochondria.

We have verified whether Bax influences the formation and/or the properties of the Ca^{2+}/Pi-induced PTP using mitochondria isolated from the isogenic human colon cancer *bax*$^{+}$ and *bax*$^{-}$ HCT116 cell lines (provided by B. Vogelstein). Mitochondria from Bax^{+} cells were enriched in (loosely attached) Bax compared to cell lysate. In swelling and Ca^{2+}-retention as well as patch-clamp experiments on mitoplasts Bax^{+} and Bax^{-} mitochondria were undistinguishable. In both cases the PT was more difficult to induce and to inhibit than in rat liver mitochondria. In electrophysiological experiments ''megachannels'' very similar to those studied in rat liver mitoplasts and identified as the PTP were present in about 15% of patches. In another approx. 30% of experiments we observed activity by 3-700 pS channels whose properties were similar to those of mitochondrial porin, and which might represent ''one-half'' of the putatively dimeric PTP. The same results were obtained with mitochondria isolated from Bax^{+} cells exposed to apoptosis-inducing agents, in which Bax was largely present as an integral membrane protein. Mitochondria from *bax*$^{-}$ cells, into whose membrane Bax had been stably incorporated in vitro by exposure to purified Bax and t-Bid, behaved in the same way. Mitochondria from a *bax*$^{-}$/*bak*$^{-}$ cell line (provided by E. White) also exhibited very similar Ca^{2+}/Pi-inducible swelling. Since no significative difference has been detected between Bax^{+} and Bax^{-} mitochondria/mitoplasts in either suspension or patch-clamp work, we conclude Bax is unlikely to be a major component or a direct regulator of the PTP.

II-B p-2. Thallium (I) induces the mitochondrial permeability transition in Ca^{2+}-loaded rat liver mitochondria

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Swelling and respiration of Ca^{2+}-loaded rat liver mitochondria in the media containing 125 mM nitrates (KNO_3 or $NaNO_3$ or NH_4NO_3) or 250 mM sucrose were studied in the presence of oligomycin. In Ca2+-free experiments, energization of mitochondria by succinate stimulated mitochondrial contraction. In sucrose medium the mitochondrial contraction was observed at all used concentrations of TlNO3 and Ca^{2+}. In the nitrate media the contraction of mitochondria occurred in their swelling with increase of both $TlNO_3$ and Ca^{2+} concentrations; cyclosporin A (CsA) or ADP eliminated the swelling. In the nitrate media containing 100 μM Ca^{2+} and 75 mM $TlNO_3$ respiration of mitochondria in the presence of 2,4-dinitrophenol was decreased, recovery to respiration values of Ca-free experiments took place after CsA or ADP addition. It is concluded that Tl^{1+} induces mitochondrial permeability transition (MPT) only in Ca^{2+}-loaded mitochondria. In contrast to Tl^{1+}, heavy metals (Cd^{2+}, Hg^{2+}, Zn^{2+}) can stimulate MPT without Ca^{2+}-loading of mitochondria. It was suggested that swelling of mitochondria found in experiments with thallium (I) salts in vivo can be due not only to a high permeability of the inner mitochondrial membrane to Tl^{1+}, but also to MPT opening followed by accumulation of other cations besides Tl^{1+} in the matrix of cytoplasm (Doklady Biochem. Biophys. (2003) 392: 247–252).

II-B p-3. K-ATP-dependent channel takes part in transport Tl across the inner membrane of rat liver mitochondria

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Monovalent thallium is used as an analogue of potassium to study ionic transport across biological and artificial membranes. The inner membrane of rat liver mitochondria (RLM), displaying a high passive permeability for Tl^{1+}, represents a barrier for K^+, which is the dominant cation in the cytosol and mitochondrial matrix. However, energized mitochondria are able to transport K^+ which is driven along the potential occuring on the inner mitochondrial membrane in response to energizing. In the presence of permeating cations, this process results in swelling mitochondria in the cases when the influx of K^+ is not compensated with the efflux of proton via K/H antiport. Besides the neutral K/H antiport, specific K-channels are involved in the maintenance of intramitochondrial homeostasis. The group of specific K-channels includes K-ATP-dependent channels. The K^+ flux through these channels is blocked by adenine nucleotides and their analogues, as well as long chain acyl-CoA esters, being completely restored by guanine nucleotides. The treatment of mitochondria with *N*-ethylmaleimide (NEM), which stimulates K^+ influx, increased the extent of inhibition of K^+ transport by adenine nucleotides by 1.5 times. It was interesting to determine whether selective for K, K-ATP-dependent channels in RLM can transport monovalent thallium ions.

In our experiment ATP and palmitoyl-CoA decreased the transport of K^+ across the inner mitochondrial membrane approximately by 50%. Guanidine nucleotides (in particular, GDP) blocked the effect of ATP. These data are in a good agreement with the results obtained by Garlid and coworkers(Beavis A.D., Lu Y. and Garlid K.D. JBC, 1993, v.268, h.997–1004).In case of Tl, ATP and palmitoyl-CoA also inhibited Tl entry into the mitochondria, but with a low extent. This may be related to other, different ways of thallium influx into mitochondrial matrix. An addition of GTP completely eliminated the inhibitory effect of ATP. The treatment of the mitochondrial suspension with NEM caused an approximately 2.5-fold increase in the K^+ influx. Note that the extent of inhibition of K^+ transport with ATP increased to 60%. The transport of Tl^{1+} in the presence of NEM, in contrast to K^+, changed insignificantly due to initially high passive permeability of membrane for Tl^{1+}. ATP inhibited Tl^{1+} transport by 25–30%. All these results led us to conclude that K-ATP-dependent channels may be involved in the transport of monovalent thallium ions across the RLM.

II-B p-4. Regulation of the mitochondrial permeability transition by protein kinase G in rat liver mitochondria

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Background: Mitochondria form high conductance mitochondrial permeability transition (MPT) pores in both necrotic and apoptotic cell death. In simulated ischemia/reperfusion injury, nitric oxide prevents MPT-dependent killing of hepatocytes. This protection is mimicked by the cGMP analog (8-Br-cGMP).

Aim: Here, we investigated the ability for cytosolic liver extracts and cGMP-dependent protein kinase (PKG) to inhibit directly the onset of the calcium induced MPT in isolated liver mitochondria.

Methods: Mitochondria were prepared from rat liver by differential centrifugation and cytosolic extracts from the 100,000 × g supernatant of liver homogenates. Purified PKG was obtained commercially. The MPT was assessed by swelling after 200 μM CaCl2 in Mg^{2+}-free buffer containing 0.5 mg/ml mitochondria.

Results: Cytosolic extract inhibited the MPT in a dose-dependent fashion. Inhibition required ATP (= 25 μM). At low but not high concentrations of cytosolic extract, inhibition of the MPT also required the cGMP analog (8-pCPT-cGMP, 100 μM). Heated extract (80 °C for 30 min) had no effect on the MPT. The specific PKG inhibitor, KT5823 (1 μM), reversed protection against the MPT by cytosolic extracts. Purified PKG, in the absence of liver cytosol, also inhibited onset of the MPT in the presence of at least 25 μM ATP.

Conclusions: PKG directly antagonizes onset of the MPT in liver mitochondria.

II-B p-5. Ceramides form protein permeable channels in outer mitochondrial membranes at physiologically relevant concentrations

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Mitochondria generate ceramide (a sphingosine-based lipid) in the presence of proapoptotic signals and addition of ceramide to isolated mitochondrial suspensions induces cytochrome *c* release. We provide evidence that the ceramide-induced release of proapoptotic proteins from mitochondria during the induction phase of apoptosis is due to its ability to form channels in membranes. Ceramide channels in the outer membranes of isolated mitochondria allow the bidirectional flux of exogenously added cytochrome *c*. This ceramide-induced increase in the permeability of the mitochondrial outer membrane can be reversed upon removal of ceramide with albumin, thus ruling out the possibility for a detergent-like effect of ceramide. Most importantly, ceramide forms protein permeable channels in mitochondrial outer membranes at concentrations of only 4–8 pmol of ceramide per nmole mitochondrial phospholipid, which correlates with levels of mitochondrial ceramide that are achieved during the induction phase of apoptosis. In addition, ceramide channels appear to be specific to the mitochondrial outer membrane as they allow the release of adenylate kinase, but not fumarase from mitochondria. No channel formation was observed in the plasma membranes of erythrocytes even at levels 40 times higher in the membrane than those effective in mitochondria. The channels in the mitochondrial outer membrane achieve an optimal size with a molecular weight cutoff of 60,000 for protein flux. In planar phospholipid membranes ceramide generally forms one permeation pathway in the form of many individual conducting increments. As the conductance grows and stabilizes, both increments and decrements are seen. The large conductance decrements are multiples of a fundamental unit (4nS in 1.0M KCl) strongly favoring the conclusion that ceramide channels are cylindrical barrel-stave channels, and excluding other hypothetical structures. Results in planar phospholipid membranes and isolated mitochondria indicate that ceramide channels are good candidates for the pathway by which proapoptotic proteins are released from the mitochondrial intermembrane space during the induction phase of apoptosis.

II-B p-6. Structural studies on PM28A, an aquaporin from spinach

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Aquaporins are integral membrane proteins found in most cell types and in organisms ranging from bacteria and yeasts to plants and animals. The importance of these proteins is implied by their high abundance; they constitute up to 50% of the total membrane protein content in some membranes (Borgnia et al, 1999, Annu. Rev. Biochem. 68, 425–458). They facilitate the rapid transport of water across the cell membranes in response to osmotic gradients and share the same overall topology with six transmembrane helices (Heymann et al, 1999, News Physiol. Sci. 14, 187–193). Three dimensional structures of two Aquaporins and one closely related Glycerol transporter is currently available (Sui et al, 2001, Nature, vol 414, 872–878; Savage et al, 2003, PLoS Biol. Epub Dec 22; Fu et al 2000, Science, vol 290, 481–486).

One of the major integral proteins in spinach leaf plasma membranes is the aquaporin PM28A. The water channel activity of PM28A is regulated by phosphorylation. The regulatory mechanism is not known but because of the high abundance of PM28A in the membrane it is believed that phosphorylation mediates a direct gating of the channel (Johansson et al 1998, The Plant Cell, vol 10, 451–459).

PM28A can be highly overexpressed in the yeast Pichia pastoris and as such can give up to 25 mg protein per l of culture (Karlsson et al 2003, FEBS Letters, 537, 68–72). He we report the purification and crystallisation of His-tagged PM28A expressed in P. pastoris. The purification is a one-step procedure using affinity chromatography. Crystals belong to the orthorhombic space group P222 with cell dimensions of $a = 66.76$ Å, $b = 104.2$ Å, $c = 181.2$ Å, alpha = beta = gamma = 90. A data set to 3.7Å has been collected and molecular replacement trials with previously structurally determined aquaporins as models are being carried out. Optimisation of the crystals are currently also in progress.

II-B p-7. Characterisation of the pomAB stator complex of the *Vibrio cholerae* flagellar motor

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Vibrio cholerae, the causative agent of cholera disease, is a gram-negative, curved, rod-shaped bacterium, which is highly motile by a single, Na^+-driven polar flagellum. Since motility is discussed to be an important prerequisite during the infection cycle of this organism, studying the operation mode of its flagellar motor is of special interest. Electron microscopic pictures reveal that flagellar motors are assembled from multiple rings (C-, FliG-, MS-, P- and L-ring) that together with the central rod constitute the basal body. This complex is connected to the flagellum via the so-called hook. The basal body is surrounded by 8–14 motor complexes which consist of PomA and PomB, two intrinsic membrane proteins. These PomAB motor complexes convert the sodium motive force into the torque needed to drive flagellar rotation.

In this project we constructed several expression plasmids harboring the pomAB genes of *V. cholerae* and performed expression studies. A *V. cholerae* pomAB deletion mutant which lacks the pomAB genes showed a nonmotile phenotype, whereas the flagellum was normally assembled. Interestingly, the pomAB defect could be complemented by transformation with plasmids harboring the pomAB genes. Furthermore, the *V. cholerae* PomAB complex was overexpressed in the *E. coli* expression strain C41 (DE3) and PomA and PomB were copurified by Strep-Tactin affinity chromatography. We identified PomA and PomB by immuno blotting, MALDI-MS and N-terminal sequencing and showed that the PomAB complex can be labeled by DCCD in a pH-dependent manner. At pH 8.0, Na^+ slightly decreases the modification of PomB by DCCD. Further labeling experiments with a nonmotile PomB mutant (D23N) will indicate whether DCCD specifically binds to the strictly conserved aspartic acid residue at position 23 of PomB.

Several residues within the single transmembrane helix of PomB are highly conserved and were subjected to site-directed mutagenesis. The *V. cholerae* pomAB deletion mutant was transformed with a plasmid harboring either the wildtype or mutant pomAB genes and the resulting phenotypes were tested in swarming assays. Exchange of the strictly conserved aspartic acid residue at position 23 in PomB either to an asparagine or glutamic acid residue resulted in a nonmotile phenotype whereas changing the serine at position 26, conserved in all Na^+-driven flagellar motors, to either alanine or threonine did not affect motility.

II-C Secondary Active Transport Systems

II-C p-1. Identification of yeast and human mitochondrial S-adenosylmethionine transporters

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Introduction: In *S. cerevisiae*, *S*-adenosylmethionine (SAM) is synthesized from ATP and methionine by two synthetases, Sam1p and Sam2p, that are both localized exclusively in the cytosol (Kumar A et al. (2002) Genes Dev., 16, 707–719). Therefore SAM must be transported into the mitochondria where it is required, for methylation reactions of DNA, RNA and proteins. Moreover in yeast SAM is required as essential cofactor in the last step of biotin and lipoic acid biosyntheses both localized in the mitochondria. Here we report the identification and functional characterization of the yeast mitochondrial SAM carrier (Sam5p), encoded by YNL003c, and of its human ortholog named SAMC, encoded by SLC25A26. They have the characteristic features of the mitochondrial carrier family (Palmieri F., Pflugers Arch-Eur J Physiol 2004, 447, 689–709). The corresponding proteins were overexpressed in bacteria, reconstituted into phospholipid vesicles and identified from their transport properties. The subcellular localization of the GFP fused to Sam5p or SAMC, the phenotype of SAM5 knock-out yeast strain, and the tissue distribution of the human SAMC were also investigated.

Results: Recombinant Sam5p and SAMC catalyzed an active influx of [^{3}H]SAM into proteoliposomes in exchange for internal SAM. Sam5p catalyzed also an influx of [^{3}H]SAM into empty proteoliposomes whereas SAMC catalysed virtually only a countertransport. High activities were also found with internal adenosylornithine (sinefungin), S-adenosylcysteine (SAC) and S-adenosylhomocysteine (SAHC). The Km and Vmax values of Sam5p were 75.6 ± 8.8 μM and 105.5 ± 30.2 μmol/min per g protein, respectively. The Km and Vmax of the recombinant purified SAMC were 23.0 ± 2.5 μM and 463 ± 95 μmol/min per g protein, respectively. SAHC, SAC and adenosylornithine inhibited [^{3}H]SAM/SAM exchange competitively. Sam5p-GFP and SAMC-GFP expressing cells showed a green fluorescence of the mitochondrial network. Yeast cells lacking SAM5 were unable to grow on synthetic minimal medium supplemented with glucose in the absence of biotin or on rich medium with nonfermentable carbon sources. Both phenotypes of the knock-out mutant were reversed by expressing the cytosolic SAM synthetase (Sam1p).

II-C p-2. The oligomeric state of the mitochondrial ADP/ATP carrier

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Members of the mitochondrial carrier family transport metabolites and cofactors across the inner mitochondrial membrane to link the biochemical pathways in the cytosol with those in the mitochondrial matrix (Palmieri, F., 2004 Pflugers. Arch. 447, 689–709, and Kunji, E. R. S., 2004 FEBS. Lett. 564, 239–244). An important member of this family, the ADP/ATP carrier, exchanges cytosolic ADP for ATP produced in the matrix by ATP synthase, and thereby replenishes the eukaryotic cell with metabolic energy (Klingenberg, M., 1993 J. Bioenerg. Biomembr. 25, 447–457). The general consensus over the last 30 years is that mitochondrial carriers exist as homodimers as could be inferred from analytical ultracentrifugation, neutron scattering, gel filtration, blue native gel electrophoresis, cross-linking studies, and experiments with differential tags or covalent linkers (references in Kunji, E. R. S., 2004 FEBS. Lett. 564, 239–244). Recently, the projection structure of the yeast ADP/ATP carrier inhibited with atractyloside at 8 Å (Kunji, E. R. S. and Harding, M., 2003 J. Biol. Chem. 278, 36985–36988) and the atomic model of the bovine ADP/ATP carrier inhibited with carboxy-atractyloside at 2.2 Å (Pebay-Peyroula, et al., 2003 Nature. 426, 39–44) were published. Both studies show that the carriers are monomers forming a six trans-membrane alpha-helical bundle. However, they might form a dimer by association of two separate monomers (Kunji, E. R. S. and Harding, M., 2003 J. Biol. Chem. 278, 36985–36988.). In this poster, we will readdress the issue of the oligomeric state of the yeast ADP/ATP carrier in detergent solution by using a variety of different methods, which include differential tagging, size-exclusion chromatography, ultracentrifugation and blue native gel electrophoresis. Our initial findings are that the carrier does not form a stable dimer in detergent regardless of the conformational state or the type of detergent, which is in contradiction with earlier findings.

II-C p-3. A single amino acid of human mitochondrial ADP/ATP carriers is critical for their nucleotide exchange activities in yeast mitochondria

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The three human adenine nucleotide carriers (HAncp) were successfully expressed in *S. cerevisiae* lacking any functional ANC. Although their kinetic activities have been determined, further studies are limited by the slow growth of the yeast transformants. We have isolated yeast transformants with a higher growth capacity. Two mutations on the HANC gene were found, leading to the replacement of valine 181 by methionine or phenylalanine. The resulting proteins presented higher ADP/ATP exchange activities, correlating with the increased growth capacities. Replacement of valine 181 with all other amino acids have shown different patterns of growth rescue depending on the amino acid. Interestingly, introduction of the V181M mutation in nonfunctional HANC1 point mutants is able to restore yeast growth. Together these results demonstrate the importance of position 181 for HAncp function in yeast.

II-C p-4. Identification of the human mitochondrial ATP-Mg/Pi transporter

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The functions of several members of the mitochondrial transporter family found in genome sequences are unknown. At the same time there are other transport activities observed in intact mitochondria that have yet to be associated with specific proteins. An example is the reversible counterexchange of ATP-Mg for Pi that accounts for the net uptake or efflux of ATP-Mg, as Pi recycles rapidly through the membrane via the phosphate carrier.

By screening human ESTs with the sequence of the human ADP/ATP carrier (AAC1) we selected clones encoding proteins of unknown function and containing Ca^{2+}-binding EF-hand motifs in their sequences. The corresponding cDNAs (accession numbers AJ619961, AJ619962 and AJ619963) encode three proteins (named APC1-3) with 66–75% identical amino acids, and with Ca^{2+}-binding motifs in their N-terminal domains and the characteristic features of the mitochondrial carrier family in their C-terminal domains. They were overexpressed in *E. coli*, purified and reconstituted into liposomes. The recombinant proteins APC1 and APC2 transported ATP-Mg, phosphate, ATP, ADP and, less effieciently, AMP in an electroneutral H^+-compensated counterexchange. The APC-mediated transport was inhibited by mercurials, bathophenanthroline, tannic acid and bromocresol purple. Little inhibition was observed with carboxyatractyloside and bonkrekate (powerful inhibitors of the AAC1). The green fluorescence (GFP) protein fused to APC1-3 was found to be targeted to mitochondria. The transport properties of APC1 and APC2 and their targeting to mitochondria demonstrate that they are responsible for the ATP-Mg/Pi exchange described in the past in whole mitochondria. The tissue specificity of the three isoforms shows that at least one isoform is present in all the tissues investigated. By screening the human genome databases with the cDNAs of APC1, APC2 and APC3, the corresponding genes (SLC25A24, SLC25A23 and SLC25A26, respectively) were found. They were located on three chromosomes, 1p13.3, 19p13.3 and 9q34.13; contained10exons separated by nine introns; and all the splicing junctions occurred in the same nucleotide regions, indicating a triplication of a common ancestral gene. The main function of the APC isoforms is probably to catalyze the net uptake or efflux of adenine nucleotides into or from the mitochondria, thus explaining the variation in the matrix adenine nucleotide content, which has been found to change in many physiopathological situations.

II-C p-5. The mitochondrial pyruvate carrier

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The ability of mitochondria to transport pyruvate across their inner mitochondrial membrane is essential for a variety of metabolic processes within cells, from the funnelling of the products of glycolysis to the TCA cycle, to supplying substrate to pyruvate carboxylase for gluconeogenesis and fatty acid synthesis. Although a considerable body of literature has been assembled about this process (including inhibitor and substrate specificity, tissue expression levels and kinetic properties) the carrier protein involved has long remained unidentified. By screening a bank of deletion mutants for loss of inhibitor-sensitive mitochondrial pyruvate uptake, we have identified the YIL006w gene as the carrier responsible for pyruvate transport in the yeast *Saccharomyces cerevisiae* (Hildyard and Halestrap, 2003). YIL006w encodes a protein of 41.9 kDa that is a member of the mitochondrial carrier family (MCF). This family of predominantly inner mitochondrial membrane-located transporters is characterised by possession of a tripartite structure, three tandem repeated homologous sequences of approx 100aa each, carrying two transmembrane helices linked by an extensive matrix facing loop; and also by possession of between one and three copies of the consensus motif P-X-[D/E]-XX-[K/R]-X-[R/K]-X10-30-[E/D]-G-X4-[F/Y/W]-[K/R]-G (Walker and Runswick, 1993). YIL006w possess an N-terminal extension of 66aa of unknown function that is not uncommon among yeast MCF members (Hashimoto et al, 1999), but the sequence from residue 67 onwards shares significant homology at the protein-level with both the yeast mitochondrial flavin carrier (FLX1) and the unannotated YEL006w gene. The closest human homologues are the human mitochondrial folate carrier, and two as yet-unannotated human genes (accession nos. NP_115691 and NP_060625). Progress on identifying which of these proteins may be responsible for pyruvate transport in mammalian mitochondria will be presented.

II-C p-6. Site-directed mutagenesis of the mitochondrial carnitine/acylcarnitine carrier: identification of four vicinal cysteine residues

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The mitochondrial carnitine/acylcarnitine carrier is responsible for the transfer of acylcarnitines into the mitochondrial matrix for the b-oxidation pathway. The structural features of the carnitine/acylcarnitine carrier demonstrate that it belongs to the mitochondrial carrier protein family (1). We have previously investigated the role of the carnitine carrier Cys residues by site-directed mutagenesis. Single and multiple mutants of the carnitine carrier were constructed in which one or more of the six Cys residues were substituted with Ser. Inhibition analysis of single and multiple Cys-mutants with monofunctional SH reagents revealed that each of the six native Cys of the protein can be replaced without substantial variation of the activity and that Cys-136, the major target for SH reagents, is accessible from the cytosolic side of the carrier (2). In the present work we have studied the possibility that some Cys residues are in close proximity to each other in the protein structure. The effects of SH oxidizing, cross-linking and coordinating reagents were evaluated on single and multiple Cys-mutants of the carnitine/acylcarnitine carrier. All the reagents tested inhibited efficiently the wild-type protein. The inhibitory effect was reduced by the substitution C136S and abolished by the double substitution C136/155S. Reduction of inhibition was also observed in double mutants in which Cys-136 together with Cys-23 or Cys-58 were substituted with Ser or, alternatively, Cys-155 and Cys-58 were substituted with Ser. Among the four replacement mutants, i.e., proteins containing two of the six native Cys, only those containing the couples Cys-136/155, Cys-136/58, Cys-136/23 or Cys-155/58 were sensitive to the reagents. To confirm the formation of a S–S bridge in the four replacement mutants containing the couples Cys-136/58 and Cys-136/23, fXa recognition sites between Cys-58 (or 23) and Cys-136 were inserted. Pretreatment of the two proteins with diamide prevented their cleavage by the fXa protease. The results described, revealed that Cys-23, Cys-58, Cys-136 and Cys-155 form a cluster of four vicinal SH groups. From these data, the relationships among three hydrophobic intermembrane segments and two hydrophilic loops are defined.

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II-C p-7. Structure and function of Na^+/H^+ antiporter Nha1p in yeast plasma membrane: a novel membrane protein Cos3p capable of enhancing Nha1p

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Na^+/H^+ antiporter (Nha1p) in the plasma membrane of *Saccharomyces cerevisiae* plays an important role in Na^+ and H^+ homeostasis. Nha1p is composed of an integral membrane and a hydrophilic tail domains. This overall molecular structure is similar to that of the mammalian Na^+/H^+ antiporters, although the primary sequences are very different among them. We are interested in the structure–function relationship of Nha1p based on a comparative aspect of various Na^+/H^+ antiporters (Kamauchi, S., et al. J. Biochem. (Tokyo), (2002) 131, 821). For the integral membrane domain, three functionally essential and important Asp residues were found by systematic replacement of Asp and Glu residues by Asn and Gln, respectively. We also found that the first and 12th trans-membrane domains are functionally important from the results of random mutagenic study. These findings suggested that the arrangement of functionally important residues in Nha1p is different from those of bacterial Na^+/H^+ antiporter NhaA (Tsuboi, Y. et al., J. Biol. Chem. (2003) 278, 21467) and mammalian NHE. We also studied a role of the tail region by a sequential deletion of this domain and found that about 40 amino acid residues (C1 + C2 domain) play a role in the antiporter function and also localization of Nha1p to the plasma membrane (Mitsui, K. et al., J. Biochem. (Tokyo), (2004) 135, 139). We also found that a novel membrane protein Cos3p binds to C1 + C2 domains and enhances salinity resistant cell growth (Mitsui, K. et al., J. Biol. Chem. (2004) 279, 12438). Cos3p was coprecipitated with Nha1p and its hydrophilic domain was capable of binding to C1 + C2 in vitro. Although Cos3p-GFP localizes to vacuolar membrane predominantly, overexpression of Nha1p caused shift of Cos3p-GFP localization to the plasma membrane. These results suggested that Cos3p is a novel factor enhancing the Nha1p activity. Cos3p is a member of 11 COS family proteins which functions have not been described. We currently study the function of other Cos family proteins besides Cos3p with Nha1p.

II-C p-8. Structural analysis of the yeast mitochondrial ADP/ATP carrier in two-dimensional crystals

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The mitochondrial ADP/ATP carrier replenishes the eukaryotic cell with metabolic energy by transporting ATP, which is synthesised in the matrix by the ATP synthase, across the inner mitochondrial in exchange for ADP from the cytosol. Structural studies of the yeast ADP/ATP carrier by electron crystallography and the bovine carrier by X-ray crystallography show that the carrier is a bundle of six transmembrane alpha-helices [Kunji E.R.S. and Harding, M. (2003) J. Biol. Chem. 278, 36985–36988; Pebay-Peyroula E, et al. (2003) Nature 426, 39–44]. Electron crystallography gives the opportunity to study the structure and conformational changes of membrane proteins in the membrane. We describe computational procedures for modeling projection data from electron microscopy and analyse the molecular packing of the yeast ADP/ATP carrier in two-dimensional crystals.

II-C p-9. A novel biological function for the *Echerichia coli* multidrug transporter MdfA in pH homeostasis

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MdfA is an *Escherichia (E.) coli* multidrug (Mdr) transporter. Cells expressing MdfA from a multicopy plasmid exhibit multidrug resistance against a diverse group of toxic compounds due to active export. In this presentation we show that in addition to its role in multidrug resistance, MdfA confers alkaline pH resistance and allows growth of transformed cells under conditions close to those normally used by alkalophiles (up to pH 10). mdfA deleted *E. coli* cells are sensitive even to mild alkaline pH conditions, and the phenotype is fully restored by MdfA expressed from a plasmid, but not by MdfA mutants defective in Mdr transport. This novel activity of MdfA requires Na^+ or K^+, and MdfA-mediated drug transport is inhibited by ions. Fluorescent studies with inverted membrane vesicles demonstrate that MdfA catalyzes Na^+ or K^+ dependent dissipation of ΔpH, and experiments with reconstituted proteoliposomes confirm that MdfA is solely responsible for this phenomenon. Taken together the results demonstrate the capacity of a single protein to turn *E. coli* from an obligatory neutrophile into a facultative alkalophile, and suggest a novel physiological role for MdfA in pH homeostasis. This function and the Mdr transport activity of MdfA seem to be coupled.

II-C p-10. Crystallization attempts with the ADP/ATP carrier

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ATP fuels in energy most of the biological processes in the cell. Its synthesis takes place in the mitochondrial matrix. The ADP/ATP carrier is an inner mitochondrial membrane protein that drives both the export of ATP into the cytoplasm and the import of ADP into this matrix.

This protein belongs to the Mitchondrial Carrier Family and is encoded by a gene of the nucleus. It is composed of three motives of about 100 residues each, arising from the triplication of an ancient gene. It contains the signature sequence (RRRMMM) of nucleotide carriers.

The X-ray structure at 2,2 Å of the bovine protein in complex with an inhibitor [1] [2], carboxyactractyloside, reveals a deep pocket opened to the intermembrane space, in which the inhibitor is tightly bound.

In order to understand the binding and transport mechanism of adenine nucleotides, we undertake efforts in cristallizing the ADP/ATP carrier in a different conformation and/or in the presence of nucleotides. Because membrane protein crystallization remains a difficult field, we explore various crystallization approaches, in particular using lipids—indeed the structure shows that endogenous lipids interact strongly with the carrier. Among them, crystallization in lipidic cubic phases were successful in some membrane protein crystallization [3] and are interesting to investigate. In order to reduce the amount of protein necessary for screening the crystallization conditions, we also work on the miniaturization of the setups.

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II-C p-11. Reconstitution into liposomes of the glutamine transporter from renal cell plasma membrane

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One of the major role of the amino acid glutamine in mammals is the shuttling of ammonia among the different organs and tissues. To perform this function, transport systems are necessary to allow reabsorption of glutamine in kidney and its trafficking from glutamine-delivering tissues, like brain, and glutamine-accepting tissues, like liver. In the last decade, the genes coding for the transport systems have been identified and the gene products have been characterized in expressing cell systems; however, several aspects of the transporter function are still unclear or unraveled (1). As in the case of other transport systems of mitochondrial or plasma membrane, reconstitution into liposomes gives some help in clarifying functional properties since this approach allows to control experimental parameters like the composition of the internal compartment that cannot be handled in cell systems.

Here we describe a procedure for the reconstitution into liposomes of a renal glutamine transporter. The transporter has been solubilized from rat renal apical plasma membrane (brush-border) with $C_{12}E_8$ and reconstituted by removing the detergent from mixed micelles with Amberlite XAD-4. The reconstitution has been optimised with respect to the protein concentration, the detergent/phospholipid ratio and the interaction with the Amberlite resin. The reconstituted glutamine transporter catalyses a first-order antiport reaction (^{3}H-glutamine/glutamine or ^{3}H-glutamine/amino acid) stimulated by extraliposomal Na^+, (not K^+ and Li^+), which is 1:1 cotransported with glutamine. Nor Na^+, neither K^+ has any effect on the glutamine antiport when present inside the proteoliposomes, thus excluding a bidirectional movement of the cations. Optimal activity is found at pH 7.0–7.5. Amino acids inhibit the transport; the most effective are alanine, serine, threonine, cysteine and asparagine; glycine, proline, histidine, glutamate and methylaminoisobutyrrate have low or any effect. The Km for glutamine on the external and internal transporter sides are 0.4 and 13 mM respectively; they are independent on the countersubstrate type and concentration. The transporter is asymmetrical and it is unidirectionally inserted into the liposomal membrane with an orientation corresponding to that of the native membrane. On the basis of some functional properties and of the source of the reconstituted transporter, it can be identified as the ASCT2 (ATB°).

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II-C p-12. Functional properties of the carnitine transporter from kidney apical plasma membrane reconstituted into liposomes

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Plasma membrane carnitine transport systems mediate uptake, distribution and reabsorption of carnitine (1). The transporters constitute a protein subfamily known as OCTN; OCTN1-3 have been cloned from human and rat tissues; however, there is still incomplete knowledge about the function and regulation of OCTNs, investigated in cell systems (2). Very recently, we have pointed out a procedure for reconstitution of the renal carnitine transporter into liposomes. The reconstituted transporter has been identified as the OCTN2 on the basis of functional properties like the Na^+-dependence and substrate specificity. The transport protein is functionally asymmetrical and it is inserted in the liposomal membrane in the same orientation as in the native membrane (3).Properties of this transporter have been here revealed by means of the reconstituted system. ^{3}H-carnitine uptake or efflux from proteoliposomes requires countersubstrates as carnitine, carnitine acyl esters of various length, betaine or TEA; transport is negligible in the absence of countersubstrate or in the presence of creatine, coline, arginine, trimethyl-lysine as countersubstrates. External, not internal, Na^+ is required for the transport; thus, the transporter catalyses a specific Na^+-dependent substrate antiport; the substrates can be translocated in both the directions, whereas Na^+ is translocated only from outside to inside. Betaine and acylcarnitines behave as competitive inhibitors of the carnitine transport, whereas TEA leads to mixed or noncompetitive inhibition, suggesting the presence of two different substrate binding domains on the protein. The carnitine antiport is strongly dependent on external acidic pH, but is independent of the internal pH. SH-reagents inhibit the carnitine antiport; the IC50 are 1.5–3 μM for the membrane impermeant MTSES, mersalyl and p-CMBS, 300 μM for the membrane permeant NEM, suggesting that some of the four Cys of the large external loop of the OCTN2 may be important for the transport. This transporter should play a role in the reabsorption of carnitine from the tubular lumen in exchange with carnitine acyl esters or other metabolites.

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II-C p-13. The mitochondrial adenine nucleotide carrier from *S. cerevisiae*: structural and functional properties of each monomer within the functional dimer

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The adenine nucleotide carrier (Ancp) is a mitochondrial protein located in the inner membrane. Ancp allows the exchange of ADP vs. ATP between matrix and cytoplasm. Numerous studies have shown the endogenous Ancp to be a dimer in vivo.To study the structural folding and functional properties of each monomer within the dimer, we have constructed genes encoding two types of covalent tandem heterodimers. In these chimeras, one of the subunits is of the wild type and the other is either an inactive mutant of ANC2 (op1) or a member of the mitochondrial carrier family, namely the phosphate carrier (PiC).

The ANC and the PiC/ANC constructs were respectively introduced into a *Δ*anc2 and a double deleted *Δ*pic, *Δ*pic2 mutant strain then the restoration of growth on nonfermentable carbon sources studied. Results indicate that ANC2-op1 and op1-ANC2 were as efficient as ANC2. Moreover, ANC2-PiC and PiC-ANC2 were able to simultaneously restore both the ADP/ATP and Pi transport functions. In vivo, expression of the dimeric genes led to synthesis of stable 64 kDa proteins. Binding properties of [^{3}H]ATR, a specific inhibitor of Ancp, and ADP/ATP exchange kinetics were compared with those of Anc2p. Results indicate that protomers of the op1/Anc2p chimera cross talk to each other during the nucleotide exchange. In contrast, the properties of PiC chimera show that Pic and Anc protomers are independent. Nevertheless, surprisingly CATR was now able to inhibit both the ADP/ATP and Pi transport.

II-C p-14. Towards structure determination of NhaA, the Na^+/H^+ antiporter of *Escherichia coli*

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Na^+/H^+ antiporters are a family of ubiquitous membrane proteins present in cytoplasmic and organelle membranes of plants, animals, and microorganisms. These antiporters play a major role in pH and sodium homeostasis. NhaA, the main Na^+/H^+ antiporter of *Escherichia coli*, is an electrogenic antiporter with a stoichiometry of $2H^+/Na^+$. It is required for growth at high Na^+ concentration or high pH (Padan, E., Venturi, M., Gerchman, Y., Dover, N.(2000). Biochim. et Biophys. Acta 1105, 144–157.). His-tagged NhaA is produced by homologous overexpression (TA16/pAXH). Efficient overexpression, affinity purification and functional reconstitution of NhaA-(His)6 have opened the way to structural studies of NhaA.

Well ordered three-dimensional protein crystals are required for atomic structure determination. Crystallization of membrane proteins for high-resolution structural studies is difficult because of the amphipatic surface of the molecules and their relatively small hydrophilic domains. A strategy to increase the probability of obtainig well-ordered crystals is the enlargement of the polar surface of the protein, most generally by attaching polar domains with specifically binding antibody fragments (Hunte, C., Michel, H. (2002). Curr Opin Struct Biol. 12, 503–508.). Monoclonal antibodies specific for native NhaA have been selected by a special His-tag-ELISA and derived recombinant fragments have been constructed. Four mAbs that recognize NhaA in its native conformation have been obtained (1F6, 2C5, 5H4, 6F9) (Padan, E., Venturi, M., Michel, H., and Hunte, C. (1998). FEBS Lett. 441, 53–58.). For cocrystallization with NhaA, both the Fv and Fab fragments of the mAb 2C5 and 5H4 have been cloned and overexpressed in *E. coli* (Venturi, M., Seifert, C., and Hunte, C.(2002). J Mol Biol 315, 1–8.). To speed up protein purification for screening of crystallization conditions, a copurification of NhaA with bound antibody fragments has been devised. In addition it has been found that binding of the 2C5 Fv fragment to the protein is strongly pH dependent.

So far, three types of 3D crystals of NhaA are available: the first type is composed of NhaA alone; the other two are cocrystals with the fragments of the NhaA specific conformational mAbs: NhaA-Fv 2C5 and NhaA-Fab 2C5. These are the first diffracting 3D crystals obtained and the first significant step towards atomic resolution of NhaA.

II-C p-15. Crystallographic analysis of AcrB

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Multiple drug resistance in *Escherichia coli* is often associated with the overexpression of the acrA, acrB and tolC genes encoding the membrane fusion factor AcrA, the RND family type efflux pump AcrB and the outer membrane channel-tunnel TolC, respectively. This system confers resistance towards a variety of lipophilic and amphiphilic drugs, dyes and detergent molecules. The substrate specificity of the AcrAB-TolC system is determined by the inner membrane RND component AcrB. This membrane protein consists of 1049 amino acids and contains 12 transmembrane spanning helices. Located between helix I and II, as well as VII and VIII is a large (> 300 amino acids) periplasmic loop. We crystallized AcrB in three different space groups (R32, P321 and R3) and obtained experimental MAD-phasing maps from selenomethionine substituted AcrB R32 crystals to 3.5 Å [1,2]. Further data sets of native and substrate soaked crystals up to a resolution of 2.7 Å were collected and provided good quality electron density maps, which allowed us to complement the published AcrB structure (PDB code 1iwg [3]).

Introduction of amino acids 860–865 and 868 lacking in the 1iwg structure and deletion of a highly disordered region (amino acids 669–678)improved Rfree and average B factors in the 2.7 A model. Wecould not identify significant densities indicating specific antibiotic binding sites in the AcrB R32 space group data sets under the soaking conditions tested.

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II-C p-16. Analysis of functional coupling: mitochondrial creatine kinase and adenine nucleotide translocase

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In muscle and brain cells, phosphocreatine and adenylate kinase shuttles provide a link between ATP producing and ATP consuming sites. As a part of phosphocreatine shuttle, the functional coupling between mitochondrial creatine kinase (MiCK) and adenine nucleotide translocase (ANT) has been identified by stimulating oxidative phosphorylation (OxPhos) with creatine. Recently, it has been shown that the coupling plays an important role in preventing the opening of permeability transition pore. However, regardless of the large amount of experimental data on functional coupling between MiCK and ANT, dating back to the 1970s, the intimate mechanism of the interaction between the proteins is still not clear.

The aim of the present work is to identify the simplest mechanism that is able to reproduce the available experimental data on functional coupling between MiCK and ANT. The following experimental results were analyzed by the mathematical models: changes in the apparent kinetic properties of the MiCK reaction when coupled to OxPhos; competition between MiCK-activated mitochondrial respiration by competitive ATP-regenerating system; and studies of radioactively labeled adenine nucleotide uptake by mitochondria in presence of MiCK activity.

Two alternative mechanisms are studied: (1) dynamic compartmentation of ATP and ADP, which assumes the differences in concentrations of the substrates between intermembrane space and surrounding solution due to some diffusion restriction; (2) direct transfer of the substrates between MiCK and ANT. The mathematical models based on these possible mechanisms were composed and simulation results were compared with the available experimental data. The first model, based on dynamic compartmentation mechanism, was not sufficient to reproduce the measured values of apparent dissociation constants of MiCK reaction coupled to oxidative phosphorylation. The second model which assumes the direct transfer of substrates between MiCK and ANT is shown to be in good agreement with experiments. Namely, the second model reproduced the measured constants and the estimated ADP flux, entering mitochondria after MiCK reaction. This model is thermodynamically consistent, utilizing the free energy profiles of reactions. The analysis revealed the minimal changes in free energy profile of MiCK-ANT interaction required to reproduce the experimental data. Possible free energy profile of the coupled ANT-MiCK system is presented.

II-C p-17. The novel alkalitolerant strain of the *Yarrowia lipolytica* yeast is an exceptionally useful model for dissecting properties and regulation of plasma membrane Na^+-coupled phosphate transporters

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Phosphorus is an essential element for all living organisms. However, inorganic phosphate (Pi) is the least accessible nutrient in many ecosystems, where it is often present in low, micromolar concentrations. Yeasts, like other organisms, have evolved complex mechanisms to satisfy their demands for this indispensable element under conditions of its fluctuating availability. In *Saccharomyces cerevisiae*, uptake of Pi from outside the cell is mediated by a number of plasma membrane H^+- or Na^+-coupled cotransport systems (for a review see Persson et al., Curr. Genet., 43(4): 225–244), concentrating Pi against its thermodynamic gradient. However, the activity of the Na^+-coupled transporter in *S. cerevisiae* was rather low, thus casting some doubt on the physiological significance of this Pi transporter. Obviously, *S. cerevisiae*, thriving under acidic conditions, is not the best model organism for studying yeast Na^+-coupled transporters, active predominantly at alkaline conditions. The newly isolated osmo-, salt, and alkalitolerant strain of *Yarrowia lipolytica*, perfectly adapted to extreme growth conditions is an excellent model for characterization of Na^+-coupled Pi transporters in yeast cells grown at alkaline conditions. The utility of this strain lies in its unique (for yeast cells) capacity to grow at alkaline pH values (pH 9.5–10.0, the highest pH growth limit for yeasts reported so far), while preserving under these extreme conditions the full viability, high growth rates, high respiratory activity and metabolically active, tightly coupled mitochondria harboring the fully competent respiratory chain. A method was elaborated allowing cell growth on solidified buffered media at rigorously maintained pH values. In *Y. lipolytica* cells grown at pH 9.5–10, Pi uptake is accommodated by several kinetically discrete Na^+-dependent systems that are specifically activated by Na^+ ions and insensitive to the protonophore CCCP. One of these, a low-affinity transporter, with a Km value of 30 M for monophosphate anion, at pH 9.5, operates at high Pi concentrations. It was, to our knowledge, kinetically characterized in the first time. The other two very active (up to 7–8 $\mu mol^{-1}\ min^{-1}$ g $cells^{-1}$, dry weight) high-affinity systems, with Km values of 0.5 and 4 μM for monophosphate anion, respectively, function during Pi-starvation, and are under the dual control of the prevailing pH values and extracellular Pi concentrations. In cells grown at pH 9.7, Na^+-coupled P_i transport systems provide most, it not all, P_i accumulation.

This work was supported by research grants from the Russian Foundation for Basic Research (Grant 03-04-48388a), from the Russian Academy of Sciences (Grant on Molecular Biology), and the Swedish Natural Science Research Council.

II-C p-18. Characterisation of electrogenic bromosulfophthalein transport in carnation petal microsomes and its inhibition by anti-bilitranslocase antibodies

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Bilitranslocase is a rat liver plasma membrane carrier, displaying a high-affinity binding site for bilirubin [1]. It is competitively inhibited by grape anthocyanins, including aglycones and their mono- and di-glycosylated derivatives [2]. In plant cells, anthocyanins are synthesised in the cytoplasm and then translocated into the central vacuole, by a so-far uncharacterised mechanism. The aim of this work was to find whether a homologue of rat liver bilitranslocase is expressed in plants, where it might play a role in the vacuolar transport of anthocyanins. By using two anti-sequence anti-bilitranslocase antibodies for developing immunoblots, a homologue of rat liver bilitranslocase was identified in microsomal, plasma membrane and tonoplast fractions isolated and purified from carnation petals. Furthermore, the bromosulfophthalein-based assay of rat liver bilitranslocase transport activity was implemented in these sub-cellular membrane fractions, allowing to identify a bromosulfophthaleine carrier (K_M=5.3 μM), which is competitively inhibited by cyanidin 3-monoglucoside (K_i=51.6 μM) and mainly non-competitively by cyanidin (K_i=88.3 μM). Both anti-sequence anti-bilitranslocase antibodies inhibited this carrier. One antibody could be shown to target a bilirubin-binding site (K_d=1.7 nM), similarly to that found for liver bilitranslocase. The other antibody was shown to target a high-affinity binding site for cyanidin 3-monoglucoside (K_d=1.7 μM) in the carnation carrier (but not in the liver one) and a high-affinity bilirubin-binding site (K_d=0.33 nM) in the liver carrier (but not in the carnation one). It is concluded that carnation petals express a homologue of rat liver bilitranslocase, with a putative function in the membrane transport of secondary metabolites.

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II-C p-19. Direct evidence of bilirubin uptake into liver cells by bilitranslocase

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Bilirubin (Br), the product of heme catabolism, is formed in all cells and shed into the blood, where it is transported by albumin to the liver. At this level, its is taken up, glucurono-conjugated and excreted into the bile by an ATP-dependent efflux pump. Uptake of Br from the blood into the liver has been proposed to be mediated by OATP-2(*SLC21A6*), with a K_M for Br of about 160 nM [1]. Later, this Br transport function has been questioned [2].

Bilitranslocase (BTL) is a plasma membrane organic anion carrier that binds Br with high affinity (K_d= 2 nM) [3]. The aim of this work was to test directly the Br transport capacity of BTL in liver cells. A cell transport assay was set up, based on the measurement of the time-dependent disappearance of Br from a medium bathing a monolayer of cultured human liver cells (HepG2). The involvement of BTL was investigated by testing the effect of anti-sequence antibody on the kinetics of Br disappearance.

The medium containing Br was a simple phosphate buffered saline solution (pH 7.4). Under these conditions, its solubility is < 70 nM, i.e. high enough to saturate BTL, but far too low to be detected by radioactive counting or conventional UV-V is spectroscopy. The samples were therefore assayed by thermal lens spectrometry [4], a technique that enabled to measure Br concentrations in the range 2-50 nM, avoiding the confounding presence of albumin.

Br uptake was found to be a quite fast phenomenon, that was abolished not only by the anti-sequence anti-BTL antibody, but also by nicotinic acid, that binds to BTL with high affinity (K_d = 11 nM) at the same level as Br [3]. The serine reagent PMSF that binds to the Br- and nicotinic acid binding site of BTL [5], abolished Br uptake into cells as well. Taurocholate and digoxin, two OATP reference substrates, did not affect Br uptake in our assay. Thus, BTL is a Br carrier, as also accepted by the Transport Classification Database (http://tcdb.ucsd.edu/).

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II-D Physiology and Pathology of Energy Dissipation

II-D p-1. Phosphorylation on tyrosine residues of mitochondrial oxphos complexes: effect of H_2O_2 and tissue difference

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As tyrosine phosphorylation regulates enzyme activities and as the normal balance between tyrosine kinases and phosphatases is disturbed in various pathologies, the aim of this work was to study tyrosine phosphorylation in mitochondria, using a phosphotyrosine antibody. We examined changes in the tyrosine phosphorylation of brain mitochondria after in vitro addition of ATP, and found that phosphorylation of several proteins increased, especially in the presence of a tyrosine phosphatase inhibitor. As tyrosine phosphatases are sensitive to oxidants, we studied the sensitivity of the tyrosine phosphorylation profile upon in vitro addition of H_2O_2. Moreover, the effect of H_2O_2 was studied on the respiration rates and results showed that H_2O_2 induced a significant decrease in state 3 respiration and in ATP synthesis. Finally, we studied the tyrosine phosphorylation pattern of each isolated complex of the respiratory chain and the ATP synthase by BN-PAGE followed by 2nd dimension SDS-PAGE. Western blotting and LC-MS/MS on parallel samples identified the alpha- and gamma-chain of the ATP synthase, the flavoprotein of complex II, the core protein II of complex III, and the subunit II of cytochrome *c* oxidase at positions of tyrosine phosphorylated bands. The tyrosine phosphorylation of the ATP synthase alpha-chain had been confirmed by antiphosphotyrosine immunoprecipitation. We also found evidence that a protein at about 75 kDa whose tyrosine phosphorylation is sensitive to H_2O_2 could be HSP75. We found that muscle mitochondria contained already tyrosine phosphorylated proteins in basal state compared to brain mitochondria, suggesting a tissue specificity. Finally, we ran computer-assisted predictions of tyrosine phosphorylation sites, tyrosine kinases phosphorylating the tyrosine sites, and proteins binding to the phosphorylated sites.

II-D p-2. Role of the metabolic network attenuation in the biochemical threshold effect

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The study of mitochondrial bioenergetics and related pathologies has shown the possibility to inhibit considerably the activity of a respiratory chain complex, up to a critical value, without affecting the rate of mitochondrial respiration or ATP synthesis. This phenomenon was called the ‘‘Biochemical Threshold Effect’’. The molecular bases for this phenomenon remain unclear. However, one possible mechanism is the concept of ‘‘metabolic network attenuation (MNA)’’ that derives from the Metabolic Control Analysis (MCA).

MNA considers the compensation of a perturbation in the activity of a particular enzyme on the overall flux, through the metabolic network. Accordingly, a moderate inhibition in the activity of a given respiratory chain complex could lead to a variation in intermediate metabolites concentration (coenzyme Q, cytochrome *c*, electrochemical gradient of protons) which in turn could modulate the activity of the other enzyme complexes, to finally keep the respiratory flux unchanged, or few. We questioned here how the importance of such attenuation depends on both the initial concentration of intermediary metabolites and the quantity, composition and kinetics of each enzyme complex, as well as their arrangement within the metabolic network and the supercomplexes. For this, we studied the importance of each of these parameters in determining the biochemical threshold value and the MNA, in a comparative analysis on mitochondria isolated from rat muscle, heart, liver, kidney and brain, where we previously determined the threshlod value for each respiratory chain complex. In these mitochondria we analyzed the steady-state of respiration (flux value), the structure of the metabolic network (organization, composition), the concentration of intermediate substrates, and the kinetic parameters (Vmax) of the different respiratory chain complexes.

We show how variations in these parameters between different tissues can explain in part the tissue specificity observed in the biochemical threshold values. Finally, to visualize MNA, we followed the variations in reduced cytochrome *c* concentration, at steady-state, following flux titrations with antimycin A or KCN.

In this work, we describe how the oxidative phosphorylation metabolic network can compensate for a significant enzyme complex inhibition to maintain to respiratory flux unchanged. This provides a kinetic explanation for the biochemical threshold effect and validates the prediction supported.

II-D p-3. Improved insulin sensitivity and reduced adipose tissue mass in relation to mitochondrial function, energy state and PPAR activation

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Much data indicates that lowering of plasma triglyceride levels by hypolipidemic agents is caused by a shift in the liver cellular metabolism, resulting in increased peroxisome proliferator activated receptor (PPAR) alpha regulated fatty acid catabolism in mitochondria. Feeding rats with tetradecylthioacetic acid (TTA) leads to hypolipidemia, and our results suggest that a TTA-induced increase in hepatic fatty acid oxidation and ketogenesis drains fatty acids from blood and extrahepatic tissues and that this contributes significantly to the beneficial effects of TTA on fat mass accumulation and peripheral insulin sensitivity (Berge, R.K., Skorve, J., Tronstad, K.J., et.al. (2002). Curr Opin Lipidol 13, 295–304.). These effects are associated with altered energy state parameters of the liver at the tissue-, cellular, and mitochondrial level (Grav, H.J., Tronstad, K.J., Gudbrandsen, O.A., et.al. (2003). J Biol Chem 278, 30525–30533). The hepatic phosphate potential, energy charge, and respiratory control coefficients were lowered, while rates of oxygen uptake and oxidation of pyridine nucleotide redox pairs were elevated. This is compatible with the mild uncoupling observed in liver mitochondria after TTA administration, confirming increased proton conductance of the inner membrane. TTA treatment induced expression of hepatic uncoupling protein 2 (UCP-2) in rat as well as in wild type and PPAR alpha-deficient mice. However, it might be that TTA-mediated activation of other PPARs (e.g., PPAR delta)can compensate for deficiency of PPAR alpha.

II-D p-4. Identification of a functioning mitochondrial uncoupling protein 1 in rat thymus

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We present evidence that rat thymus contains a functioning mitochondrial uncoupling protein (UCP 1). Using reverse transcriptase-polymerase chain reaction we showed that rat thymus contains RNA transcripts for UCP 1. Antipeptide antibodies specific for UCP 1 detected UCP 1 protein in mitochondria isolated from thymus. No significant difference was found in the amount of UCP 1 protein expressed in thymus tissue taken from fed or fasted animals. Evidence for functional UCP 1 in rat thymus mitochondria was obtained by a comparative analysis with the kinetics of GDP binding in mitochondria from brown adipose tissue. Both tissues showed equivalent BMAX and KD values. In addition, a large component of the nonphosphorylating oxygen consumption by thymus mitochondria was inhibited by GDP and subsequently stimulated by addition of nanomolar concentrations of palmitate. We purified UCP 1 from thymus mitochondria by hydroxyapatite chromatography and identified the isolated protein by peptide mass mapping and tandem mass spectrometry using MALDI-TOF and LC-MS/MS, respectively. We conclude that rat thymus contains a functioning UCP 1 that has the capacity to regulate metabolic flux and production of reactive oxygen-containing molecules in the thymus.

II-D p-5. Inhibitors of mitochondrial bioenergetics in studies on structure of mitochondria in the cell, oxidative stress and apoptosis

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The effects of the specific inhibitors of respiratory chain, F0F1ATP synthase and uncouplers of oxidative phosphorylation on survival of carcinoma HeLa cells and on structure of mitochondria in the cells were studied. The inhibitors of respiration (piericidine, antimycine, myxothiazole), F1-component of ATP synthase (aurovertin) and uncouplers (DNP, FCCP) did not affect viability of HeLa cells, apoptosis induced by TNF or staurosporin and antiapoptotic action of Bcl-2. Apoptosis was induced by combine action of respiratory inhibitors and uncouplers indicating the possible proapoptotic action of ROS generated by mitochondria.

Short-term incubation of Hela cells with the mitochondrial inhibitors and 2-deoxyglucose(DOG) followed by 24–48 h recovery resulted in massive apoptosis. Apoptosis correlated to transient (3–4 h) and limited (60–70%) depletion of ATP. More prolonged or more complete transient ATP depletion induced pronounced necrosis. In this model combination of respiratory inhibitors and uncouplers accelerated apoptosis in the period of recovery. The signs of oxidative stress was observed at the early steps of apoptosis induced by ATP depletion.

The inhibitors of respiration and uncouplers induced fragmentation of tubular mitochondria and formation of small round bodies followed by their swelling. These transitions were not accompanied with release of cytochrome *c* into cytosol and were fully reversible. The combined effect of respiratory inhibitors and uncouplers developed more rapidly indicating possible involvement of ROS generated by mitochondria. More prolonged (24–48 h) incubation with this combination of inhibitors caused clustering and degradation of mitochondria.

II-D p-6. The change in photosynthesis and superoxide dismutase activity under the stress of low temperature and high irradiation in a desiccation-tolerant chlorella

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At low temperature algae or plants performed a decreased photosynthetic efficiency whereas in high light environment algae or plants absorb energy that exceeds the consumed by photosynthetic processes. We intend to clarify the relationship between the growth and the activity of superoxide dismutase (SOD) which is the first line of cellular defense against reactive oxygen species under the stress of low temperature and high irradiation.

Green alga Chlorella which has a chloroplast structure comparable to that of higher plants was used in this study. Chlorella DT and 8b are almost alike in their physiology, but DT strain is a desiccation-tolerant strain. With optimal illumination of 120 μmol per m^2 per s, the cell growth rate and chlorophyll fluorescence Fv/Fm value were nearly the same between DT and 8b at optimal temperature. Upon exposure to the temperature below 15oC, DT exhibited higher survival rate and significantly increased SOD activity than that of 8b. Furthermore, with illumination of 240 μmol per m^2 per s, Fv/Fm ratios of DT decreased more greatly than those of 8b in the early period of low temperature acclimation. Under the combined stress of low temperature and high light more SOD isoenzymes were induced in DT with the endurance of acclimation, as compared to those of 8b. From the results, we found that SOD activity was highly correlated to the photosynthesis and cell growth in desiccation-tolerant Chlorella DT.

II-D p-7. Activation of alternative oxidase and uncoupling protein decreases reactive oxygen species generation in amoeba A. castellanii mitochondria

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Amoeba Acanthamoeba castellanii mitochondria possess two free energy-dissipating systems, diverting energy from ATP synthesis, i.e., uncoupling protein and alternative oxidase. Uncoupling protein mediates a free fatty acid-activated, purine nucleotide-inhibited proton reuptake dissipating the proton electrochemical gradient built up by the respiratory chain. A. castellanii alternative oxidase is an antimycin-resistant purine nucleotide-stimulated oxidase that consumes mitochondrial reducing power without energy conservation into the proton electrochemical gradient. The aim of this study was to determine the role of the two energy-dissipating systems in unicellular organisms, such as amoeba A. castellanii, possibly as an antioxidant systems preventing damage of the cell at the level of energy production. It is shown that in isolated amoeba mitochondria oxidizing succinate, the activation of uncoupling protein by externally added free fatty acid (such as linoleic acid) resulted in decrease in H_2O_2 production. On the other hand, the inhibition of the linoleic acid-induced uncoupling protein activity by GTP enhanced production of H_2O_2. Similar results were obtained during activation and inhibition of the alternative oxidase activity in amoeba mitochondria. The activation of antimycin-resistant alternative oxidase-mediated respiration by GMP significantly lowered H_2O_2 production. Addition of benzohydroxamic acid (an inhibitor of alternative oxidase) cancelled the GMP-induced effect on H_2O_2 generation level. These results suggest that in A. castellanii mitochondria, alternative oxidase and uncoupling protein can be considered as additional endogenous antioxidant systems decreasing harmful reactive oxygen species production.

This work is supported by the Polish State Committeee for Scientific Research (KBN-029/P04/2003/25).

II-D p-8. Is bird longevity linked to the presence of highly active uncoupling proteins?

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Despite their high metabolic rates, birds have a much higher maximum longevity compared to mammals of similar body size, and thus represent ideal models for identifying longevity characteristics not linked to low metabolic rates. In this study we show that the isolated mitochondria from canary birds‘ myocardium have functionally active Uncoupling Proteins (UCPs).

The resting respiratory rate of isolated myocardial mitochondria was strongly stimulated by 10 μM Palmitic Acid (PA) (increased by 150%). ADP/O ratio determined during ADP pulse in absence of BSA, GTP and PA was 1.45. The same ratio decreased up to 1.1 with 10 μM PA. No effect of GTP on PA-induced resting respiration mediated in FFA depleted mitochondria was observed. However, inhibition by GTP significantly increased to 40% and 60% upon 24 and 48 h starvation stress, respectively.

These results, together with those previously obtained in birds and in various mammalian species, suggest that the rate of low mitochondrial free radical production in the canary birds muscle could be due to the presence of highly active UCPs. Overexpression of UCP2 and three are currently under the investigation in our laboratory.

II-D p-9. In situ modular control analysis of bioenergetic transduction in intact beating heart

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As compared to skeletal muscle, the energy balance [1] in heart bioenergetics is characterized by an improved homeostasis of all energetic intermediates [2, 3]. Indeed, increase in contractile activity in heart is not accompanied by an important decrease in phosphocreatine (PCr) content, by contrast with what is observed in muscle. How this homeostasis is achieved is still unclear. Therefore we decided to apply modular control analysis (top-down[4]) on perfused beating heart in order to study the distribution of control in muscle bioenergetics by using noninvasive techniques, including 31P NMR spectroscopy.

The control scheme of heart bioenergetics was determined by using PCr as the obligatory energetic intermediate (ATP was constant under all conditions studied), and considering PCr producer (mitochondria) and consumer (ATP consumption linked to contraction). In perfused rat heart, we could demonstrate that mitochondria exert almost no control (5–10% only) on energy flux during contraction, in a wide range of contractile activity [2, 3]. However, recent results obtained using mouse perfused hearts showed an almost perfect shared control between energy production and consumption over contraction. The mechanisms responsible for these differences are currently under investigation.

These results show that in situ modular control analysis could be used to study intact heart energetics under different working conditions or following drug action. This approach may fruitfully be applied to pathological situations or therapeutic strategies. Recent developments give also access to the study of transgenic mice.

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II-D p-10. Secondary structure of purified rat uncoupling protein 1 expressed in *Saccharomyces cerevisiae*

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The uncoupling protein 1 (UCP_1)s a member of the mitochondrial anion carrier family that mediates energy dissipation into heat by catalysing the reentry of protons of the electrochemical gradient. Free fatty acids strongly activate UCP_1, purine nucleotides, such as GTP, are inhibitors and it seems that ubiquinone could be a cofactor. Here, we report the expression and purification of a recombinant form of UCP_1 with the purpose of biophysical characterization by circular dichroism (CD) spectroscopy. To obtain a homogenous UCP_1, we have introduced a C-terminus polyhistidine tail with the aim of affinity purification step. Despite the presence of the his-tag, the UCP_1 product is efficiently targeted to the mitochondrial inner membrane and retains its function as evidenced on isolated mitochondria. We have purified the recombinant tagged UCP_1 to homogeneity (>95%) in a two-steps process using a hydroxyapatite column and an additional nickel-chelating affinity step. Fluorescence resonance energy transfer experiments show that *n*-dodecyl-b-D-maltoside-solubilized UCP_1-His6 retains its purine nucleotide binding capacity. The far UV CD spectrum of the functional protein indicates that a-helices represent ~ 68% of entire secondary structure content. Moreover, the helical content remains unchanged in free and purine nucleotide-loaded UCP_1 indicating that purine nucleotide binding mechanism does not involve significant secondary structure rearrangement. Homology modelling of the first repeat of UCP_1 based on the X-ray-solved ADP/ATP carrier and CD experimental data provide evidence that UCP_1 shares similar secondary structure characteristics with the ADP/ATP carrier, at least for the first repeat.

This work was supported by grants of the Belgian Fonds de la Recherche Fondamentale Collective and Pôles d'Attraction Interuniversitaires. R.N. was recipient of FRIA fellowship. P.D. is recipient of FRIA fellowship.

II-D p-11. Modulation of purine nucleotide inhibition of amoeba A. castellanii UCP by the redox state of endogenous ubiquinone in isolated mitochondria

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Amoeba Acanthamoeba castellanii mitochondria possess a free fatty acid-activated, purine nucleotide-inhibited uncoupling protein (AcUCP) that mediates a proton reuptake driven by the mitochondrial electrochemical gradient. We show that AcUCP activity diverts energy from ATP synthesis during state 3 respiration in a fatty acid-dependent way. The efficiency of AcUCP in mitochondrial uncoupling increases when the state 3 respiratory rate decreases. The inhibition of the linoleic acid-induced uncoupling by GTP is observed only during titration of respiration by decreasing substrate availability. Thus, the inhibitory effect of GTP is only observed when the reduced sate of ubiquinone is decreased. Conversely, when ubiquinone is shifted to a more reduced state favoring the production of superoxide anion, AcUCP is switched on likely to decrease reactive oxygen species production.

This work is supported by the Polish State Committee for Scientific Research (KBN-029/P04/2003/25).

II-D p-12. Kinetics of intracellular free calcium during ischemia and reperfusion in myoglobin-deficient mouse myocardium

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Intracellular calcium loading is considered to be a major cause of ischemia–reperfusion damage in heart muscle. In excitable tissues where rapids changes in cytosolic free calcium occur, methods capable of rapid response are needed, and fluorescent probes have been obtained wide acceptance. These are typically calcium chelators with a dissociation constant (Kd) within the range of cytosolic free calcium concentration. An aspect mostly overlooked in earlier investigations is the pH sensitivity of the apparent Kd of calcium binding to the probe and the influence of cellular acidification due to anaerobic glycolysis in ischemia. Another source of error in fluorometry of heart muscle is the variation in an internal filter effect due to large absorbance spectrum changes of myoglobin because of deoxygenation and reoxygenation during ischemia and reperfusion.In the present case we monitored simultaneously the fluorescence changes in intracellular pH and calcium probes in a model which does not suffer from myoglobin interference. Isolated hearts from myoglobin gene knock-out mice [Gödecke A, Flogel U, Zanger K, Ding Z, Hirchenhain J, Decking UK, Schrader J. Proc Natl Acad Sci U S A 1999; 96:10495–10500]. were perfused and loaded with the pH probe BCECF-AM and the calcium probe Fura-2-AM, and fluorescence of the probes was monitored simultaneously by the dual excitation method at a sampling cycle of 20 ms, so that pH correction could be applied for every data point. The pH response of BCECF was calibrated against the NMR chemical shift of phosphate in separate experiments. In other experiments Fura-2 was replaced by Rhod-2 AM, a more mitochondrion-specific calcium probe.

It was found that ischemia caused a decrease in Fura-2 fluorescence which in the absence of Kd changes would indicate calcium decrease, but when the temporal pattern of pH and concomitant Kd changes were taken into account, an extensive, almost linear intracellular free calcium increase was observed, reaching 600–1000 nM in 20 min of global ischemia. The calcium concentration returned rapidly to the basal level upon reperfusion with a half time of 18 s. Behaviour of Rhod-2 fluorescence during calcium paradox, which permeabilized the sarcolemma, indicated that a major part of the Rhod-2 signal originates from mitochondria.

The results show that in spite of extensive calcium accumulation during ischemia the recovery upon reperfusion is fast without initial calcium spiking.

II-D p-13. Relationship between early apoptosis and induction of UCP_2 in rat hepatocytes

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Uncoupling protein 2 (UCP_2) is virtually absent in liver parenchymal cells under normal physiological conditions. Its expression can be induced by lipopolysaccharide (LPS) as a complex cross-talk response between hepatocytes and liver macrophages—Kupffer cells, leading namely to macrophage production of tumor necrosis factor a (TNFa), prostaglandines and ROS. Some of these effectors may induce UCP_2 expression, but also may trigger apoptosis. To exclude the apoptotic origin of the observed partial dissipation of mitochondrial membrane potential in hepatocytes isolated from LPS-treated rats, we provide the following studies. First we confirmed that hepatocytes isolated from LPS-treated rats contained up to 2.5-times higher UCP_2 mRNA levels (quantified by real-time PCR) and that exhibit lower mitochondrial membrane potential (detected by fluorescent voltage-sensitive dye JC-1). However, these changes did not correlate with the early apoptosis marker (detected by fluorescent Annexin V conjugate). Moreover, mitochondria isolated from LPS-treated rats displayed a slightly lower membrane potential than controls suggesting a negligible loss of cytochrome *c*, another marker of apoptosis. The small decrease matches the maximum uncoupling capacity of UCP_2. We conclude that increase in UCP_2 expression is properly reflected by the appropriate mitochondrial membrane potential decrease and is not associated with early apoptosis in hepatocytes. (Supported by GACR grant 301/02/1215 and MSM grant 151100003).

II-D p-14. Mammalian uncoupling protein 2 and 3: modulation of purine nucleotide inhibition by the redox state of endogenous coenzyme Q

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The uncoupling proteins 2 (UCP_2) and 3 (UCP_3) are two isoforms of the uncoupling protein subfamily that are expressed in skeletal and heart muscles at various levels. UCP_2 and UCP_3 have been shown to be activated by free fatty acids and inhibited by purine nucleotides in reconstituted systems. On the contrary in isolated mitochondria, the protonophoretic action of muscle UCPs has failed to be demonstrated. Here we show for the first time that skeletal and heart muscle UCPs are activated by linoleic acid in state 3 respiration and dissipate energy from oxidative phosphorylation by decreasing the ADP/O ratio. The efficiency of UCPs in mitochondrial uncoupling increases when the state 3 respiratory rate decreases. The inhibition of the linoleic acid-induced uncoupling by a purine nucleotide (GTP) is not observed in state 4 respiration in the presence of *N*-ethylmaleimide, atractylate and glutamate, in uninhibited state 3 respiration as well as in state 3 respiration inhibited by complex III inhibitors. On the contrary, the progressive inhibition of state 3 respiration by *n*-butyl malonate, which inhibits the uptake of oxidizable substrate, leads to a full inhibitory effect of GTP. Therefore, as the inhibitory effect of GTP is only observed when the reduced state of coenzyme Q is decreased, we propose that the coenzyme Q redox state could be a metabolic sensor that modulates the purine nucleotide inhibition of UCPs in skeletal and heart muscle mitochondria.

This work was supported by grant of the Belgian Fonds de la Recherche Fondamentale Collective. R.N. was recipient of FRIA fellowship. P.D. is recipient of FRIA fellowship.

II-D p-15. Decreased affinity to oxygen of cytochrome *c* oxidase in Leigh syndrome caused by SURF1 mutations

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Mutations in SURF1 gene prevent synthesis of specific assembly protein of cytochrome *c* oxidase (COX) and result in a fatal neurological disorder, Leigh syndrome [1, 2]. Because this severe COX deficiency presents with hardly detectable changes of cellular respiratory rates at normoxic conditions [3], we analyzed the respiratory response to low oxygen in cultured fibroblasts harboring SURF1 mutations using high-resolution respirometry [4]. The oxygen kinetics was quantified by the p50 (the pO2 at half-maximal respiration rate) in intact coupled cells and in digitonin-permeabilized uncoupled cells. In both cases, the p50 in patients was 2.1- and 3.3-fold elevated, respectively, indicating decreased affinity of COX to oxygen. These results suggest that the depressed oxygen affinity may in vivo lead to limitations of respiration because of physiologically low intracellular pO2, resulting in impaired energy provision in Leigh syndrome patients.

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II-D p-16. Growth yield homeostasis in yeast under respiratory conditions: role of the Ras/cAMP pathway

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It is well documented that the growth yield of microorganisms depends on the fraction of ATP utilized for biomass synthesis per se compared to that used for cell maintenance. During aerobic growth, the growth yield may also be a function of the yield of ATP synthesis by oxidative phosphorylation (i.e., ATP/O ratio), a parameter which can vary according to the functional steady state of mitochondria. This steady state is controlled in situ by the adaptation of the energy producing system to energy consumption. In this respect, the enthalpic growth yield of yeast aerobic cultures has been assessed by direct microcalorimetry during the transition from exponential growth to stationary phase. Under these conditions, the ATP turnover largely decreased whereas, the growth yield remained quite constant. We have shown that this steady yield requires a strict dependency between mitochondrial content within a cell and energy demand throughout the transition period. This shows that as long as the cells grow, they are able to adapt their mitochondrial content in such a way that the growth yield is maintained. Under reciprocal conditions, i.e., when one is able to modulate the mitochondrial content, this strict dependency between mitochondrial content and energy demand (growth rate) also applies. This point out a tight regulation of energy demand and energy supply in such a way that the growth yield remains constant in a great variety of physiological situations. The Ras/cAMP/PKA signaling pathway is known to regulate adaptations of yeast cells upon environmental and metabolic transitions. We have shown that this pathway is involved in the regulation of this process. Overactivation of this pathway can lead to situations where the relationship between growth rate and mitochondrial amount is modified. This leads to a decrease in growth yield.

II-D p-17. Mitochondrial threshold effects

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The study of mitochondrial diseases has revealed dramatic variability in the phenotypic presentation of mitochondrial genetic defects. To attempt to understand this variability, different authors have studied energy metabolism in trans-mitochondrial cell lines carrying different proportions of various pathogenic mutations in their mitochondrial DNA. The same kinds of experiments have been performed on isolated mitochondria and on tissue biopsies taken from patients with mitochondrial diseases. The results have shown that, in most cases, phenotypic manifestation of the genetic defect occurs only when a threshold level is exceeded, and this phenomenon has been named the ‘ phenotypic threshold effect ’. Subsequently, several authors showed that it was possible to inhibit considerably the activity of a respiratory chain complex, up to a critical value, without affecting the rate of mitochondrial respiration or ATP synthesis. This phenomenon was called the ‘biochemical threshold effect’. More recently, quantitative analysis of the effects of various mutations in mitochondrial DNA on the rate of mitochondrial protein synthesis has revealed the existence of a ‘translational threshold effect’. We review here several evidences for the existence of these different mitochondrial threshold effects along with their molecular bases and the roles that they play in the presentation of mitochondrial diseases. We report also a new mechanism to explain the biochemical threshold effect, based on a reserve of enzymes not initially participating in the respiratoryrate that can be activated either to respond to a flux increase, or to compensate for a defect induced by a mutation. We show that this mobilization occurs through (i) the assembly ofinactive adenine nucleotide translocator isoform 1 (ANT1) subunits into oligomeric active carriers or (ii) conformational changes in the ANT1 in a PTP-like structure. We discuss howthese transitions are sensitive to the steady-state of OXPHOS functioning, or tissue, and analyze their consequences on the threshold effect.

II-D p-18. Interactions of long-chain fatty acids with the inner mitochondrial membrane-activation of the inner membrane anion channel (IMAC)

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We previously found that long-chain fatty acids release endogenous Mg^{2+} from rat liver mitochondria when they were suspended in slightly alkaline KCl medium [1,2]. Since Mg^{2+} depletion is known to activate ion-conducting pathways in the inner mitochondrial membrane [3,4], the question arose: can fatty acids stimulate anion permeation through the inner membrane anion channel (IMAC) ? For examination, we studied the effects of various fatty acids on the permeability of the inner membrane (IM) to Cl^-.Here we report results ontained with swelling measurements on rat liver mitochondria, and with the patch-clamp technique applied to mitoplasts (mitochondria without outer membrane). We found that (i) low nonlytic concentrations of fatty acids (<30 μM, corresponding to 30 nmol per mg mitochondrial protein) increase dramatically the permeation of Cl^- in mitochondria in slightly alkaline KCl medium (supplemented with the potassium ionophore valinomycin to ensure rapid equilibration of K^+ across IM). Myristic and palmitic acid were more active than lauric, stearic and phytanic acid. (ii) The rates of Cl^- permeation (measured as swelling rates) correlate well with the potency of the applied fatty acids to release endogenous Mg^{2+} from mitochondria. (iii) Myristic acid failed to increase the permeability of IM to gluconate, an anion which is known as bad substrate of IMAC. (iv) Cl^- permeation across IM is blocked by low concentrations of tributyltin chloride (IC50 = 1.5 nmol per mg mitochondrial protein), the most potent inhibitor of IMAC.(v) Myristic acid activated an ion current in patch-clamped mitoplasts exposed to alkaline KCl medium which is best ascribed by the opening of an ion channel with a single-channel conductance of 108 pS. On the basis of these results we conclude that fatty acids stimulate Cl^- permeation across IM by the opening of IMAC.This opening is probably initiated by the withdrawal of Mg^{2+} from intrinsic binding sites at IMAC.

Keywords: Swelling; Fatty acid; Cl^- perneation, Mitochondria; Mg^{2+} depletion; inner membrane anion channel (IMAC);-patch-clamped mitoplasts

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II-D p-19. A genetic modification results in metabolic bypass of the yeast mitochondrial ATP synthase by increasing the intramitochondrial substrate-level phosphorylation flux

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In a previous study, we have identified Fmc1p, a mitochondrial protein involved in the assembly/stability of the yeast F1-ATP synthase at elevated temperature. The deletion fmc1 mutant was shown to exhibit a severe phenotype of very slow growth on respiratory substrates at 37 C. Using a screen based on this phenotype, we have isolated ODC1 as a multicopy suppressor of the fmc1 mutation, this suppression being gene-dosage dependent. ODC1 expression level was estimated to be approximately 10 times higher in mitochondria isolated from the fmc1/ODC1 transformant as compared to wild type mitochondria. Interestingly, ODC1 encodes an oxodicarboxylate carrier which transports 2-oxoglutarate and 2-oxoadipate in a counter-exchange through the inner mitochondrial membrane. A model of bypass suppression supported by in vitro analysis by Rigoulet et al, in 1985 was tested. Surprinsingly enough, a growth defect due to ATP synthase alteration could be partially suppressed by an increase in mitochondrial TCA cycle-dependent ATP synthesis together with an increase in the oxoglutarate oxidative decarboxylation as a result of an increase in the flux of oxoglutarate from the cytosol into the mitochondria. Biochemical data correlate this hypothesis. We show that the suppression of the respiratory-growth deficient fmc1 by the overexpressed ODC1 was not due to a restored stable ATP synthase. Furthermore, respiration and ATP synthesis rates proved to be in agreement with the hypothesis of an increase of the intramitochondrial ATP synthesis via substrate-level phosphorylation. This mechanism of metabolic bypass of a defective ATP synthase unravels the physiological importance of intramitochondrial substrate-level phosphorylations.

II-D p-20. Regulation of uncoupling protein 1 activity elucidated from studying brown fat-mitochondria lacking this protein

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Elucidation of the regulation of UCP_1 activity in its native environment, i.e., the inner membrane of brown-fat mitochondria, has been hampered by the presence of UCP_1-independent, quantitatively unresolved effects of investigated regulators on the brown-fat mitochondria themselves. We have here utilized the availability of UCP1-ablated mice to dissect UCP_1-dependent and UCP_1-independent effects of regulators. Using a complex-I-linked substrate (pyruvate) in GDP-inhibited mitochondria with fatty acids apparently acting as activators, we found that UCP_1 can mediate a fourfold increase in thermogenesis.

After demonstrating that it is in their free form that the fatty acids act, we found that UCP_1 increased fatty acid sensitivity about 30-fold (as compared to the 1.5-fold increase earlier reported based on nominal fatty acid values). By identifying the UCP_1-mediated fraction of the response, we could conclude that the interaction between purine nucleotides (GDP) and fatty acids (oleate) unexpectedly displayed simple competitive kinetics. Only a model in which UCP_1 is inherently active (i.e., ''activating'' fatty acids cannot be included in the model), where GDP functions as an inhibitor with a Km of 0.05 mM, and where oleate only functions as a competitive antagonist for the GDP effect (with a Ki of 5 nM), can fit all experimental data.

We have analyzed the structural requirements for compounds that stimulate UCP_1 in GDP-inhibited brown fat-mitochondria. We have found that a wide range of different structures, only vaguely resembling fatty acids, are able to initiate UCP1-dependent (re)activation of mitochondrial thermogenesis. The limitation for fatty acid chain length is 8–10 carbonyl groups. Increase of unsaturation does not influence activation. Fatty aldehydes (4-hydroxy-2-nonenal, trans-2-nonenal and all trans retinal) are not active; therefore, the carboxyl group of the activators is absolutely required for their function. Introduction of several carboxyl groups, as in beta,beta‘-methyl-substituted alfa,omega-dioic fatty acids (MEDICA 16 and 14), does not significantly diminish their activity. Noticeably, flip-flop-incapable fatty acids (12-hydroxylauric, dodecanedicarboxylic, benzenehexanoic acid) demonstrate UCP1-dependent function as thermogenic (re)activators.Thus, when examined in its native environment, UCP1 functions as a proton (equivalent) carrier, and fatty acid-like molecules reactivate by overcoming GDP inhibition in a competitive way.

II-D p-21. Bioenergetic and mitochondrial ultrastructure morphology changes associated with work overload in the nonischemic part of the dog's myocardium subjected to partial left ventricular ischemia.

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Uncoupling proteins (UCPs) uncouple respiration from oxidative phosphorylation and may negatively regulate reactive oxygen species (ROS) generation. We studied the activity of UCPs in acute myocardial ischemic animal model without reperfusion. Male mongrel dogs underwent left anterior descending coronary artery ligation for subsequent occlusion to induce 35–65% of ischemia on the anterior wall of the left ventricle. After 6 and 24 h of continuous ischemia without reperfusion, samples from nonischemic posterior wall of the left ventricle were collected for bioenergetic and morphologoical studies. State 4 respiratory rate of the isolated and FFA depleted myocardial mitochondria obtained from the nonischemic posterior wall after 24 h of ischemic insult on anterior wall was almost doubled by adding 10 μM Palmitic Acid (PA). ADP/O ratio, in the presence of PA, decreased progressively with time from 0.99 to 0.92 and 0.77 after 6 and 24 h of continuous ischemia, respectively. UCP activity assessed by GTP inhibition of PA-induced resting respiration mediated by UCP in FFA depleted mitochondria. GTP inhibition of PA-induced respiration was increased by 7% and 35% after 6 and 24 h of continuous ischemia, respectively. Strong correlation between the size of ischemic area and UCP activity has been observed. TEM ultrastructural studies showed transition of mitochondrial cristae from lamellar into zig-zag form (>80%) after 24 h of ischemic injury. Collectively, our data suggest that increased activity, and possibly, overexpression of UCPs in the nonischemic myocardium may represent an early protective mechanism against possibly ROS over production as a consequent of increased workload of the nonischemic part of the heart.

II-D p-22. Platelets peroxynitrite levels in patients with migraine during headache-free period

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Our aim in this study was to evalue peroxynitrite production and nitric oxide synthase expression in platelets of migraine patients.

Peroxynitrite ($ONOO^-$) has been implicated in the pathophysiology of a variety of diseases including inflammation, atherosclerosis, arthritis, ischemia–reperfusion injury, acute respiratory distress syndrome and neurodegenerative disorders.

Peroxynitrite is a reactive oxidant produced from nitric oxide (NO) and superoxide which reacts with proteins, lipids, carbohydrates and DNA. The initiation of lipids peroxidation, the direct inhibition of mitocondrial respiratory chain enzymes, the inhibition of membrane N^+/K^+ ATPase activity, the inactivation of membrane sodium channels contribute to the cytotossic effect of peroxynitrite.

Thirty migraine patients admitted to the Headache Center of our Departement were included in the study. The control group consisted of 30 age and sex-matched healthy volunteers. All patients gave informed consent prior to participate to this project and the study was approved by the local ethics committee. Exclusion criteria included a previous history of chronic liver disease, chronic renal failure or hematologic or autoimmune disease, systemic hypertension.

Venous blood of patients outside attacks and controls was mixed with ACD tubes and immediately used for the determination. Platelets peroxynitrite levels and the effect on membrane's enzyme regulation, i.e., Na^+/K^+ ATPasi, Ca^{++} ATPasi and on membrane's fluidity were estimated.

The platelets peroxynitrite levels were significantly higher in patient with migraine compared with controls (7.4 ± 0.058 vs. 1.85 ± 0.018; $p < 0.001$). Membrane Na^+/K^+-ATPase activity was significantly lower in patients (0.035 ± 0.0012 mmol Pi/mg prot/h) than in controls (0.220 ± 0.013 mmol Pi/mg prot/h)Platelet membrane fluidity evaluated by TMA-DPH anisotropy was significantly decreased in patients (0.2515 ± 0.038) compared with controls (0.2301 ± 0.0137; $p < 0.001$).

In these study, we observed an increase of the level of platelets peroxynitrite and of its cytotossic effects in patients with migraine.

These results indicate that in patients suffering from migraine the peroxynitrite is an important cytotossic mediator of the oxidative stress and that it could be involved in the increased risk of vascular disease described in patients with migraine.

II-D p-23. Alteration of mitochondrial membrane potential in fibroblasts with defects in oxphos enzymes

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Measurements of mitochondrial membrane potential ΔΨm) by fluorescent slow potentiometric probes in different cell types are crucial for investigation of various pathophysiological conditions. We have developed a method that allows measuring the changes in ΔΨm based on cytofluorometric measurements of tetramethylrhodamine methyl ester (TMRM) fluorescence intensity in permeabilized fibroblasts [1]. The data obtained from such measurements can be evaluated either qualitatively as relative changes in fluorescent intensities or quantitatively in terms of ΔΨm changes in millivolts obtained from calculation involving a logarithmic transformation of Nernst-equation controlled fluorescence intensity of TMRM accumulated in mitochondria leading to a linear scale for ΔΨm.

This method was applied to detect differences in ΔΨm in fibroblasts from patients with defects of oxidative phosphorylation (OXPHOS), especially deficiencies of cytochrome *c* oxidase (COX) and ATP synthase (ATPase). The fibroblasts with mutation in nuclear gene SURF1 encoding the Surf1 protein essential for COX assembly [2, 3] and fibroblasts with various mutations in mitochondrial DNA ATP6 gene encoding subunit a of ATPase [4] were studied. The fibroblasts with mutations in SURF1 gene revealed normal ΔΨm in state 4 but were more sensitive to functional load (simulated by titration with uncoupler) indicating that COX H+-translocating activity is impaired. Fibroblasts from patients with ATPase defects also showed normal values of ΔΨm in state 4 but lower discharge of ΔΨm at state 3 in accordance with dysfunction of ATP synthase. We can conclude that the method presents a relatively simple and reliable approach to assess ΔΨm changes in fibroblasts with different defects in OXPHOS enzymes.

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II-D p-24. Comparison of nonenergy-conversing bypass in summer bamboo *Bambusa oldhamii* and winter bamboo *Phyllostachys edulis*

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Bamboo is an important economical corps in Asia. The young bamboo shoots are taken as food for they contain rich nutrients, low fat, and high fiber. Bamboo is one of the fastest growing plants on this planet as a new shoot sprouting from the parent plant's rhizomes may grow rapidly over 3 ft per day. However, the mechanism of this rapid growth remains unclear. In order to understand how the energy metabolizes in bamboo shoots, we intend to work on the mitochondria where the energy is mainly produced.

The control of nonenergy-conversing bypass in mitochondria isolated from edible young bamboo shoot of summer bamboo *Bambusa oldhamii* and winter bamboo *Phyllostachys edulis* was investigated. The isolated mitochondria from *B. oldhamii* exhibited a lower respiratory control ratio than those from *P. edulis*. In both species, the mitochondrial nonphosphorylation respiration rates were decreased to 80% with addition of oligomycin; they were increased about twofold with addition of FCCP. The mitochondrial nonphosphorylation respiration rate of *P. edulis* was greatly stimulated by linoleic acid and palmitic acid which mediate uncoupling protein but that of *B. oldhamii* was less affected. When SHAM, an inhibitor of alternative oxidase, was added, the nonphosphorylation respiration rates of both species did not significantly change. Additionally the winter bamboo *P. edulis* contained superoxide dismutase (Mn-SOD) activity whereas *B. oldhamii* contained two Mn-SOD isoenzymes. 2-D blue-native electrophoresis of bamboo mitochondrial respiratory chain proteins was also carried out. In summary, winter bamboo *P. edulis* performed more coupled mitochondrial activity than summer bamboo *B. oldhamii*.

II-E: Reactive Nitrogen Species and Reactive Oxygen Species in Mitochondria

II-E p-1. Short-term nondesensitizing AMPA receptor activation causes mitochondrial impairment and increases peroxynitrite levels

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In this work, we examined the activity of mitochondrial respiratory complexes (MRC) in an experimental paradigm that involves nondesensitizing activation of AMPA receptors and production of nitric oxide (NO) in cultured rat hippocampal neurons. In a previous study, we have shown that an exposure to kainate (KA; 100 μM) plus cyclothiazide (CTZ; 30 μM) for 5 min leads to delayed neurotoxicity and decrease of the intracellular ATP levels 24 h later (Araújo et al., 2003, J. Neurochem. 85: 791–800). Now we evaluated whether the MRC activity was altered following exposure to KA, in nondesensitizing conditions. We found that the activity of complexes I, II/III and IV were decreased to 85.4%, 81.3% and 75.0% of the control ($p < 0.01$), respectively. This effect was prevented by 7-nitroindazole (7-NI; 100 μM), a selective neuronal nitric oxide synthase (nNOS) inhibitor only at the level of complex I, suggesting that it might be an effect mediated by NO. We observed a significant increase in cyclic GMP levels in neurons exposed to KA plus CTZ for 5 min, from 459.4 ± 87.9 fmol/106 cells (untreated cultures) to 814.2 ± 35.8 fmol/106 cells ($p < 0.01$). This increase was prevented by L-NAME (500 μM), a broad NOS inhibitor, and by ODQ (50 μM), a guanylyl cyclase inhibitor. Since inhibition at the level of complex I has been described to be due to peroxynitrite rather than NO itself, we performed immunocytochemistry against 3-nitrotyrosine (3-NT), a biomarker for protein nitration by peroxynitrite, to check whether peroxynitrite levels were elevated following nondesensitizing activation of AMPA receptors. We found increased 3-NT labelling in cultures treated with KA plus CTZ, in contrast to control cultures or cultures pretreated with 7-NI. Taken together, our data suggest that activation of nondesensitizing AMPA receptors leads to NO production, which can be converted to peroxynitrite and cause inhibition of mitochondrial activity at the level of complex I.

(Supported by FCT, Portugal: Grants SFRH/BD/3120/2000, Project POCTI/1999/NSE/35875).

II-E p-2. S-nitrosylation: a key mechanism regulating redox-dependent mitochondrial membrane permeabilization and cell death

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Recently it has been shown that the biological effects of nitric oxide (NO) are mediated by S-nitrosylation of a number of specific proteins although a global role for S-nitrosylation in cellular fate has thus far not been demonstrated. Here we show here that NO blocks glutathione (GSH) redox-dependent mitochondrial membrane permeabilization (MMP) and cell death mediated by respiratory complex III (cytochrome bc_1). The mechanism of this protection depends on the inhibition of oxidation and crosslinking of mitochondrial protein vicinal thiols (PVT) by S-nitrosylation since 1) NO blocks cytochrome bc_1 dependent intracellular oxidation mediated by cytochrome bc_1 after GSH depletion and preserves both mitochondrial transmembrane potential, and mitochondrial ultrastructure, 2) NO preserves the redox status of mitochondrial PVT after GSH redox modulation and 3) NO protected cells and their mitochondrial fractions increased S-nitrosylated cysteine residues. This study shows that S-nitrosylation is a key mechanism regulating mitochondrial integrity, function and mitochondrial-dependent cell death.

II-E p-3. Opening of the mitochondrial permeability transition pore induces reactive oxygen species production at the level of the respiratory chain complex I

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We have investigated the consequences of Permeability Transition Pore (PTP) opening on the rate of production of reactive oxygen species in isolated rat liver mitochondria. We found that PTP opening fully inhibited H_2O_2 production both when mitochondria were energized with complex I or II substrates. Since PTP opening led to mitochondrial pyridine nucleotides depletion, H_2O_2 production was measured again in the presence of various amount of NADH. PTP-opening-induced H_2O_2 production began when NADH concentration was higher than 50 μM and reached a maximum at over 300 μM. At such concentrations of NADH, the maximal H_2O_2 production was fourfold higher than that observed when mitochondria were permeabilized with the channel-forming antibiotic alamethicin, indicating that the PTP opening-induced H_2O_2 production was not due to antioxidant depletion. Moreover, PTP opening decreased rotenone-sensitive NADH−Ubiquinone reductase activity, whereas it did not affect the NADH-FeCN reductase activity. We conclude that PTP opening induces a specific conformational change of complex I that (i) dramatically increases H_2O_2 production so long as electrons are provided to complex I, and (ii) inhibit the physiological pathway of electrons inside complex I. These data allowed the identification of a novel consequence of permeability transition that may partly account for the mechanism by which PTP opening induces cell death.

II-E p-4. Modulation of free radical production by respiratory chain in different kinds of cells

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The mitochondrial respiratory chain is a powerful source of reactive oxigen species (ROS), considered as a major pathogenic agent of most diseases and of ageing (Diplock, AT., Mol. Aspects Med 1994, 15:293).

The major sites of superoxide formation in the respiratory chain are respiratory Complexes I and III, however also other respiratory enzymes are sources of oxygen radicals.

The effect of respiratory inhibitors appears to be the best way to discriminate between mitochondrial and nonmitochondrial ROS, however their effect is ambiguous due to the mulplicity of ROS production sites.

Although antimycin is usually found to stimulate ROS production in intact cells, as it does in mitochondria, the effect of rotenone is contradictory, since different studies reported either decrease or increase of ROS production with rotenone addition (Bernacchia et al; Toxicol.: Mech. Meth. 2004, 14:1).

ROS production in intact cells, in presence of inhibitors and uncouplers of the respiratory chain, was measured by following the fluorescence increase of the probe dichlorodihydrofluorescein diacetate (DCFDA) (Degli Esposti and Mc Lennan., FEBS Lett. 1998, 430:338) that becomes fluorescent upon deacylation and oxidation by intracellular H_2O_2.

We found that lymphocytes and HL60 cells produce less peroxide in the presence of rotenone than in noninhibited conditions, wherease papillary thyroid carcinoma cells and osteosarcoma 143 B cells produce more peroxide under the same conditions. This discrepancy may be due to the relative contribution of Complex I and III to total ROS production.

Since ROS production by reverse flux of electrons is decreased by rotenone (Kushnareva et al., Biochem.J. 2002, 368: 545) another critical point may be represented by membrane potential and the contribution of reverse electron transfer in Complex I. Mitochondrial ROS production is enhanced in State 4 and when the rate of electron transfer is lowered (Skulachev, V.P., Q.Rev.Biophys. 1996, 29:169). The rationale is in a more reduced state of respiratory carriers capable of donating electrons to oxygen. To this purpose uncoupling and release of excessive membrane proton potential may protect mitochondria from damage due to excessive free radical production. A decrease ROS production was observed by uncoupling with FCCP in all types of cells examinated.

Addition of quinones (Q_1, Q_{10}) in general induced decreased ROS production, suggesting their rapid reduction in the cells.

II-E p-5. Interactions of nitric oxide and S-nitrosothiols with mitochondria relevant to cell death

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Nitric oxide (NO) is cytotoxic, and has been implicated in a wide range of inflammatory, ischaemic and neurodegenerative diseases, as well as in host defence against pathogens. Mitochondria may be involved in NO-induced cell death, as NO has three relevant actions on mitochondria: NO inhibits mitochondrial respiration; NO stimulates the production of reactive oxygen species by mitochondria; and reactive nitrogen and oxygen species can stimulate mitochondrial permeability transition (MPT). In many pathological situations there is also a large increase in cellular calcium which may have damaging effects on mitochondria such as activation of MPT or inhibition of the respiratory chain. We investigated whether NO itself or synergistically with elevated concentrations of calcium can cause irreversible damage to mitochondria and whether NO or S-nitrosothiols can induce mitochondria-mediated apoptosis in perfused heart.We found that NO and calcium synergistically stimulated production of peroxynitrite by mitochondria and inhibited mitochondrial respiration. NO plus calcium-induced suppression of mitochondrial respiration was partially caused by inhibition of complex I (due to S-nitrosation or Fe-nitrosylation) and partially by the loss of cytochrome *c* from mitochondria.S-nitrosothiols in isolated mitochondria induced MPT-related cytochrome *c* release, nitrosation and inhibition of complex I and stimulated production of hydrogen peroxide. We also found that perfusion of rat hearts with a physiological S-nitrosothiol GSNO caused the release of cytochrome *c* from mitochondria, inhibition of mitochondrial respiratory chain and caspase activation. Inhibited respiratory chain activity was restored when exogenous cytochrome *c* was added to mitochondria, indicating that respiratory inhibition was caused by lack of cytochrome *c* in mitochondria. Release of cytochrome *c*, respiratory inhibition and caspase activation were prevented when hearts were preperfused with cyclosporin A, suggesting that MTP was involved. In contrast, perfusion of the hearts with ‘pure’ NO donor DETA/NO, releasing similar levels of NO to the GSNO, had no measurable effect on the heart. The data suggest that *S*-nitrosothiols (but not NO) are potent inducers of apoptosis in the heart and that *S*-nitrosothiol-induced apoptosis is mediated by MTP.

II-E p-6. Interaction of cytochrome *c* with NO studied by time-resolved raman and absorption spectroscopy

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Recently, it has been shown that cytochrome *c* (cyt *c*) released during apoptosis is nitrosylated in mitochondria, the first case of its nitrosylation in vivo (Schonhoff, C. M., Gaston, B., and Mannick, J. B. (2003) J. Biol. Chem., 278, 18265). In native cyt c, the heme-iron is ligated to the protein through two axial bonds, involving histidine and methionine. The nitrosylation of cyt c during apoptosis led to the hypothesis of its activation due to nitric oxide (NO) binding which would induce a structural change of cyt c. Similarly to guanylate cyclase this conformational change may be triggered by the breaking of Fe^{2+}-methionine bond. For NO binding to occur, the cleavage of the Fe-methionine bond must transiently occur. Consequently, an equilibrium between the following forms should exist with a low dissociation energy of this bond:

$$[\mathrm{Met}-\mathrm{Fe}-\mathrm{His}] \leftarrow\rightarrow [\mathrm{Met}\ \mathrm{Fe}-\mathrm{His}]-\mathrm{NO} \rightarrow [\mathrm{Met}\ \mathrm{NO}-\mathrm{Fe}-\mathrm{His}]$$

This hypothesis is strengthened by the observation that the Fe–Met bond is cleaved already upon a small temperature increase.

To investigate the properties of the transient species involved in this scheme, we used flash photolysis and ultrafast detection techniques to generate and characterize the relevant 5-coordinate species. We characterized the ferric and ferrous cyt c NO adducts by steady state Raman spectroscopy and we used ultrafast time-resolved absorption and Raman spectroscopy. TRRR allows to measure the evolution of vibrational frequencies related to structural changes with a time resolution of 0.6×10^{-12} s.

After photoexcitation of nitrosylated ferrous cyt c we observed two relaxation times ($t_1 = 2$ ps and $t_2 = 8$ ps) attributed respectively to the vibrational relaxation of the five-coordinate hot species and to the recombination of the NO ligand. These times are of the same order of magnitude as we observe for recombination of methionine ligand in native ferrous cyt c with different relaxation times ($t_1 = 1.8$ ps and $t_2 = 5.5$ ps). The time dependence of Raman spectra of ferrous cyt c and ferrous cyt c NO adducts compared with the stationary spectra show clearly that a conformational change is induced during the ligand release in the case of nitrosylated ferrous cyt c with respect to what observed for native ferrous cyt c. Correlation between this conformational change and the biological activity of the protein will be discussed.

II-E p-7. Hydrogen peroxide production in liver mitochondria from lipopolysaccharide-treated rats is activated by GDP

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Mitochondria belong to major sites of production of reactive oxygen species (ROS). Accelerated electron transport is associated with lower ROS production. Hence, regulated mild uncoupling may attenuate excessive ROS production in mitochondria. Hepatocytes contain negligible amount of mitochondrial uncoupling protein UCP_2 under normal physiological conditions. However, UCP_2 expression in hepatocytes is induced by endotoxin [1] (lipopolysaccharide, LPS) via activation of Kupffer cells leading to expression of cytokines, which subsequently upregulate UCP_2 transcription. Physiological meaning of such activation could be viewed as a protective role of UCP_2 against oxidative stress.

The goal of this work was to examine the rates of hydrogen peroxide generation in liver mitochondria from LPS-treated rats. H_2O_2 production was detected fluorometrically by oxidation of scopoletin in the presence of horseradish peroxidase. In liver mitochondria from untreated control rats, respiring with succinate under the state 4 conditions, H_2O_2 production was unchanged in the presence of UCP_2 inhibitors (GDP, GTP). However, in liver mitochondria from LPS-treated rats the rate of H_2O_2 production increased upon GDP addition, in the absence or presence of added linoleic acid. Similar effect was observed with GTP. We also determined that the activation constant AC_{50} for GDP dose–response in liver mitochondria of LPS-treated is about 90 μM (when half maximum rate was considered between limits of maximum activation by 2.5 mM GDP and no activation in the absence of GDP). All these data support the interpretation, that GDP-sensitive UCP_2-mediated uncoupling is responsible for suppression of H_2O_2 production in liver mitochondria from LPS-treated rats. When UCP_2 is inhibited by GDP (GTP) the H_2O_2 production returns back to the unprotected high rate. (Supported by GACR grant 301/02/1215 to PJ).

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II-E p-8. Formation of reactive oxygen species (ROS), mitochondrial dysfunction and cell death in H_9c_2 cardiomyoblasts

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To study the relationship between oxidative stress and cell death, we exposed H_9c_2 cells to three different protocols: (i) necrosis induced by 2 uM A23187, a Ca^{2+} ionophore; (ii) apoptosis induced by the addition of 7 uM arachidonic acid; (iii) oxidative stress induced by 100 uM H_2O_2. Cells were loaded with the fluorescent probes Mitotracker Red, CMH_2XRos, or TMRM to monitor ROS production or mitochondrial membrane potential in situ, respectively. Cell viability was assessed by means of propidium iodide staining or resazurin reduction.

Upon the addition of A23 and arachidonic acid, Mitotracker Red fluorescence increased 1.6 and 4.0-fold, respectively. These changes in fluorescence, reflecting the formation of ROS, were prevented by preincubating the cells with pargyline, an inhibitor of monoamino oxidases (MAO), enzymes located on the outer mitochondrial membrane that produce H_2O_2 as a byproduct of biological amines deamination. This prevention can be obtained also with cyclosporin A (CsA), an inhibitor of the mitochondrial permeability transition pore (PTP), a voltage gated channel located in the inner mitochondrial membrane. Thus, ROS production appears to be a consequence, rather then a cause, of PTP opening. Treatment of cells with the oxidative stress protocol results in a twofold increase in Mitotracker fluorescence. Interestingly, also this increase was blocked by both pargyline and CsA. This result suggests that the exogenous H_2O_2 triggers the formation of reactive oxygen species at the mitochondrial level, rather than acting as an oxidant per se.

Furthermore, A23187 and arachidonic acid induced mitochondrial depolarization which was followed by cell death. Both these events were significantly reduced by pargyline and CsA. In conclusion, the present findings suggest that (i) MAO activity is an important site of ROS production that can prompt a sequence of events including PTP opening, mitochondrial dysfunction and cell death; (ii) the mitochondrial respiratory chain might act as a ROS scavenging system rather than a site of ROS production.

II-E p-9. Glycerophosphate-dependent hydrogen peroxide production by mitochondria from hamster brown adipose tissue, rat liver and human-term placenta

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In our previous studies (1) we found that mGPDH represents a new site in the mitochondrial respiratory chain where ROS can be generated. In the present communication we describe that the glycerophosphate (GP)-dependent hydrogen peroxide production can be detected not only in brown fat mitochondria where its activity is extremely high, but also in other tissues with relatively high mGPDH expression (placenta), or in tissue where mGPDH biogenesis can be hormonally stimulated (liver).

Hydrogen peroxide generation was detected fluorometrically using DCDFA, luminometrically using luminol + peroxidase and polarographically as a KCN-insensitive oxygen uptake.

We extend our previous findings on brown adipose tissue mitochondria by demonstration that mitochondrial cytochrome *c* has an important role as endogenous electron scavenger and that the damage of mitochondrial membranes highly activates GP-dependent hydrogen peroxide generation.

Mitochondria from human-term placenta have mGPDH activity that represents about 50% of succinate dehydrogenase or rotenone insensitive NADH dehydrogenase activities. Using these mitochondria we found GP-dependent hydrogen peroxide production, which was much higher than succinate- or NADH-dependent peroxide production. In rat liver we tested at first induction of mRNA transcript and mGPDH activity after a single dose of triiodothyronine. We observed maximal increase of mRNA 12 h after hormone application and maximal increase of mGPDH activity after 24 h that correspond well with immunochemical detection of the mGPDH protein. In mitochondria from triodothyronine-treated animals, the increase of GP-dependent hydrogen peroxide production was proportional to the increase of mGPDH activity.

Our data thus demonstrate that mGPDH represents for a cell a potential risk in ROS generation evidently due to a fact that the leak of electrons during the transfer to the CoQ pool seems to be less protected than that from Complex I and II. This fact in connection with recent findings on ROS regulatory function (2) could be also interpreted as a regulatory role of GP-dependent ROS production, because, e.g., in brown fat it could participate in activation of tissue apoptotic process, which starts when the thermogenic function of brown adipose tissue is no more required (3).

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II-E p-10. Mechanisms of cytochrome *c* oxidase inhibition by NO

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Nitric oxide (NO) acts as potent, rapid and reversible inhibitor of cytochrome *c* oxidase (CcOX), the terminal complex of the respiratory chain, by binding in competition with oxygen to the active site of CcOX, heme a_3-Cu_B (1). In particular, two mechanisms of CcOX inhibition by NO have been identified, leading to formation of either a light-sensitive nitrosyl [a_3^{2+}-NO] derivative or a nitrite–bound [$a_3^{3+}NO_2^-$] derivative, at high and low electron flux respectively. Both inhibited states, although with different kinetics, recover full activity by dissociation of either NO (high electron flux) or nitrite (low electron flux) from the active site (2,3). The reaction of CcOX with NO, because of its emerging physiological relevance, is intensively investigated. Interestingly, it has been recently suggested that at high electron flux, upon reversal of inhibition, NO is not released into the bulk as such, but as nitrite via transient formation of $ONOO^-$ in the active site of the enzyme. A discussion of these issues is presented.

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II-E p-11. Implication of oxidative stress arising from mitochondria in oncogenic RAS2(Val19) cells

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Aberrant RAS protein has been revealed to trigger neoplastic transformation or premature senescence by not yet known mechanism. Cells of *Saccharomyces cerevisiae* expressing this oncogenic homolog, RAS2Val19, exhibit a severe oxidative load when grown in glucose-repressed conditions. Oxidation management can be linked to two independent pathways: the constitutively active cAMP/PKA system which effectively reduces expression of some major antioxidant enzymes and a PKA-independent pathway predominantly locking the respiratory state close to nonphosphorylating mode. A link between Ras pathways and mitochondrial function (as the major source of free radicals) has been suggested previously, yet, it is not fully understood how the membrane-located Ras protein initiates switch of respiratory mode and how this will influence function of other intermembrane carriers. We propose the model based on experimental data trying to elucidate mechanism of Ras regulation of oncogenic RAS2Val19 mitochondrial function, thus the induction of oxidative stress. Moreover, the expression of mammalian uncoupling protein, UCP1, recovered many of the mitochondrially connected phenotypes otherwise leading to increased oxidative stress. However, it was shown that the restricted replicative potential of RAS2Val19 yeast cells is linked to their overactive PKA pathway rather than their mode of respiration.

II-E p-12. Differences of superoxide and hydrogen peroxide generation in isolated mouse brain and skeletal muscle mitochondria

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Mitochondria have been suggested to be the most important source of oxygen radicals occurring as byproduct of respiratory chain function. Although different sites at complex I, the bound semiquinone at center 'o' of bc1 complex and the electron transfer flavoprotein of the *β*-oxidation pathway have been implicated to generate superoxide their quantitative contribution to mitochondrial superoxide production remains to be elucidated. In this report we have quantified the superoxide and H_2O_2 production rates of intact mouse brain and skeletal muscle mitochondria under condition of oxygen saturation applying *p*-hydroxyphenylacetate as fluorescent probe for H_2O_2 generation and hydroethidine / and adrenaline oxidation as probes for superoxide formation. At comparable respiration rates and quality of mouse brain and skeletal muscle mitochondria with glutamate + malate and succinate as substrates we observed considerable difference in the H_2O_2 and superoxide generation rates. In mouse brain mitochondria we detected a high complex I-dependent H_2O_2 generation (in the presence of glutamate + malate + rotenone or succinate alone) while a significant bc1-complex-dependent H_2O_2 generation was almost absent (in the presence of succinate + antimycin). Moreover, mouse brain mitochondria had a low complex I-dependent superoxide generation while the bc1-complex-dependent superoxide production in the presence of succinate + antimycin was considerable. This result is consistent with the location of the superoxide producing site in complex I, being very likely the FMN moiety, at the inner side of mitochondrial membrane. In contrast, mouse skeletal muscle mitochondria had a reduced complex I-dependent H_2O_2 generation but a considerably higher bc1-complex-dependent H_2O_2 generation. Additionally, we detected in mouse skeletal muscle mitochondria under all conditions studied elevated superoxide production rates. Our data can be explained by the following facts. (i) The superoxide generation site in complex I is localized at the inner side of inner membrane but the bc1-complex-dependent superoxide generation is localized at the outer side of inner membrane. (ii) Muscle mitochondria seem to generate at comparable maximal rates of respiration much larger amounts of superoxide at center 'o' of the bc1 complex. (iii) And finally, the high endogenous superoxide production of muscle mitochondria is very likely related to the β-oxidation pathway.

II-E p-13. Expression and activity of plant uncoupling mitochondrial protein (ZmPUMP1) in maize seedlings under salt stress

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In plants, environmental stress such as low temperature and high salinity has been shown to induce oxidative stress. Attenuation of the oxidative stress can be elicited by suppression of mitochondrial production of reactive oxygen species (ROS) mediated by a weak uncoupling [1]. The plant uncoupling mitochondrial protein (PUMP) would be hypothetically able to attenuate excessive ROS formation, since its constitutive and small amount would be sufficient for this role. The enhanced PUMP expression and/or activity, induced by oxidative stress, should be then expected, if PUMP plays an antioxidant role. Since almost nothing is known about transcriptional upregulations for PUMP, we attempted to study the effect of salt stress on the expression and activity of the plant uncoupling mitochondrial protein (ZmPUMP1) in seedlings of maize (Zea mays, cv.).

We found that treatment with 250 mM NaCl for 24 h induced lipid peroxidation and decreased phosphorylation efficiency in shoot mitochondria. A twofold increase in ZmPUMP1 mRNA (quantified by real time RT-PCR on a LightCycler) was found in both shoots and roots subjected to salt stress corresponding to an average twofold increase in the number of 3H-GTP binding sites in isolated mitochondria. Moreover, shoots contained nearly seven times less PUMP (3H-GTP binding sites or ZmPUMP1 mRNA) than roots. Our results suggest a possible protective role for maize uncoupling protein (ZmPUMP1) during the oxidative stress induced by salt exposure. The increased ZmPUMP1 expression and activity may serve to suppression of elevated production of reactive oxygen species in mitochondria.

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II-E p-14. Alterations in the antioxidant system and mitochondrial bioenergetics of *Orechromis niloticus* induced by oxyfluorfen

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Environmental contamination due to the extensive use of pesticides is a great problem that needs our attention. Herbicides are considered one of the main groups of the pesticides which are used in many parts of the world to control wide variety of grass weeds. Unfortunately, these pesticides ultimately find their way into rivers, lakes and ponds creating hazardous effects to the aquatic fauna including fish1.

Oxyfluorfen are known to generate reactive oxygen species (ROS)2 which induce a shift of the balance between prooxidative and antioxidative reactions. Superoxide dismutase, catalase, glutathione reductase are major antioxidative enzymes that contribute to the oxidative stress response in animals.

Our study deals with the alterations of mitochondrial bioenergetics and enzymatic antioxidants in the liver fish *Orechromis niloticus* following exposure to the herbicide oxyfluorfen (Goal). Fishes were exposed to different concentrations (0.3 and 0.75 mg/l) of oxyfluorfen for 7, 14, 21 and 28 days. Effect of continuous exposure of two sublethal concentrations of superoxide dismutase, catalase, glutathione reductase, and glutathione S-transferase activities were investigated. These antioxidative enzyme activities were used as biomarkers in the present study to evaluate the toxic effect of oxyfluorfen.

Mitochondrial membrane potential dissipation and state 4 O_2 consumption of succinate-supported respiration was not significantly affected for the tested concentrations. However antioxidative enzymes were significantly affected by oxyfluorfen.

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II-E p-15. Mitochondria are not the major oxygen consumers and ROS producers in hematopoietic stem cells

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This study was aimed to characterise mitochondrial and extra-mitochondrial oxidative metabolism in human CD34+ hematopoietic stem cell HSC. The cells were immunoselected from the peripheral blood following mobilization from bone marrow upon G-CSF conditioning. It was shown that the mitochondrial oxidative phosphorylation capacity of the CD34+ haematopoietic stem cell was very low (0.05 nmoles O2/min/106 cells), when compared with that of other cell types. The hypoxic condition in the bone marrow stromal stem cell niche, could be a factor controlling the expression of the mitochondrial oxidative phosphorylation system. It was, however, noticed, by confocal microscopy analysis, that mitochondria were able to generate and maintain a transmembrane potential. This was particularly evident in a sub-population of the HSCs cells expressing lower level of CD34 (whose progressive lost is indicative of commitment), suggesting a role of the mitochondrial oxidative metabolism in the early stage of HSCs differentiation. The unexpected novelty emerged from this study was the discovery of the occurrence of a NAD(P)H oxidase activity in HSCs (never reported before). Although both the catalytic and regulatory subunits of the plasma membrane NAD(P)H oxidase were shown to be expressed and assembled, its DPI-sensitive activity was much lower than that of macrophagic cells, where the NAD(P)H oxidase serves as a powerful oxygen producing bactericide system. Low active isoforms of NAD(P)H oxidase have, however, been reported in other non phagocyting cells and suggested to be involved in oxygen sensing and oxygen radical-mediated intracellular signalling. We present a model proposing that, following activation by external stimuli, the mobilization of the HSCs from the extreme hypoxic bone marrow microenvironment into a normoxic milieu, is sensed by the NAD(P)H oxidase; this might result in a ROS-mediated intracellular signalling, leading (or contributing) to mitochondria proliferation and/or cell differentiation. The nature of the external stimuli, the type of oxygen reactive species, the targets of the activated intracellular system are under investigation to validate our hypothesis as well as the biochemical features of a stage, occurring in the commitment program, still allowing repopulation of bone marrow.

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III-A Mitochondria in Ageing and Degenerative Diseases

III-A p-1. Collagen VI muscular dystrophies from animal models to human therapy: (II) mitochondrial bioenergetics in myoblast cultures from Ullrich patients

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Collagen VI (CVI) is an ECM protein forming a microfilamentous network with a broad distribution in several organs, including skeletal muscle. Inherited mutations of COL6 genes in humans cause two forms of muscular dystrophies: Bethlem myopathy (BM) and Ullrich scleroatonic muscular dystrophy (UCMD). $Col_6a_1 -/-$ mice lack CVI and they have an early onset myopathic phenotype strongly resembling human CVI disorders and (1). We discovered a latent mitochondrial dysfunction due to increased opening of the permeability transition pore (PTP) in $Col_6a_1 -/-$ mice (2), but the pathogenic mechanisms involved in BM and UCMD are still unknown, and there is currently no effective treatment for patients affected by these disorders. We initiated an investigation of mitochondrial bioenergetics in myoblasts cultures from UCMD patients by epifluorescence microscopy and imaging with TMRM. The response of the mitochondrial membrane potential to oligomycin and/or rotenone revealed a latent mitochondrial dysfunction. Indeed, unlike in cultures from normal subjects, addition of oligomycin or rotenone to UCMD cultures caused mitochondrial depolarization. Cyclosporin A, a selective inhibitor of the mitochondrial permeability transition pore (PTP), reverted the patients' phenotype, suggesting that inappropriate PTP opening may play a role in the pathogenetic mechanism of human CVI myopathies. These results may open new perspectives for pharmacological intervention in human CVI deficiencies.

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III-A p-2. Bioenergetic changes associated with the T8993G mutation of the mtDNA ATP 6 gene

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Mutations in the mtDNA ATP-6 gene cause different neurological disorders, including neurogenic muscle weakness, ataxia and retinitis pigmentosa (NARP) and maternally inherited Leigh's syndrome (MILS). The product of this gene is ATPase-6 (subunit a in *E. coli*), a membrane domain subunit of the F_1F_0-ATPase (ATP synthase) complex that is directly involved in proton translocation. The T8993G mutation changes the highly conserved Leu-156 to Arg, and mitochondria isolated from cells of patients carrying the mutation result in a greatly reduced ATP synthesis rate, as measured in vitro. At variance, the ATPase activity is only slightly reduced, with no statistical significance [Baracca et al. (2000) J. Biol. Chem. 275, 4177–4182]. Therefore, the mutated enzyme might contribute to the energy depletion of the cell. This might be relevant in determining the severity of the clinical phenotype and for setting an appropriate therapy for NARP and MILS patients. To clarify this point and to find molecules possibly capable of reducing ATP hydrolysis, but not ATP synthesis in mitochondria of patients cells, we assayed the ATP synthesis rate by measuring $NADP^+$ reduction as a result of coupling ATP production (by OXPHOS) with glucose phosphorylation and the glucose-6-phosphate dehydrogenase reaction in order to trap ATP once synthesized by residual OXPHOS in cells carrying 80–90% of mutant mtDNA.Furthermore, experiments to test the effects of oligomycin, a specific inhibitor of the F_1F_0–ATPase complex, are in progress both in patient and control fibroblasts as well as in a cybrid cellular model, where only the mutant mitochondria from the patient's cells are transferred in a perennial host cell line, previously depleted of mtDNA. Preliminary results indicate that low concentrations of the inhibitor could differently affect the ATP synthesis rate of wild-type and mutated F_1F_0–ATPase. This concurs with the observations of Manfredi et al. [(1999) J. Biol. Chem. 274, 9386–9391] who reported the possibility of reducing the mutation load of NARP fibroblasts by growing the cells in the presence of oligomycin. The present findings will be discussed in relation with the catalytic properties of the mutated enzyme, and with the possible molecular mechanism that causes the dramatic impairment of the ATP synthesis rate of the F_1F_0–ATPase complex carrying the Leu-156 to Arg change.

(This work was supported by a grant from Telethon Fondazione Onlus, Rome (GP0280/01)).

III-A p-3. Maternally inherited Leigh syndrome-related mutations render cells apoptosis-prone

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The key role of mitochondria in the apoptotic process is well understood, but no much data are available regarding the specific role of mitochondrial DNA mutations in determining cell fate. We investigated whether two mitochondrial DNA mutations (L217R and L156R) associated with maternally inherited Leigh syndrome may play a specific role in triggering the apoptotic cascade.Maternally inherited Leigh syndrome (MILS) is the most frequent neurodegenerative disorder affecting OXPHOS in children. Mutations in the mitochondrially encoded ATPase 6 subunit are a frequent genetic cause of MILS. The common L217R and L156R ATPase6 mutations have been linked to a profound defect in ATP synthesis, which plays a role in the pathophysiology of this disorder. In the present work, we analyzed the susceptibility to apoptosis of osteosarcoma-derived cybrids carrying either the L217R or the L156R mutation.

Analysis of mitochondrial features in these cybrids indicated that both mitochondrial DNA mutations produced evidence of biochemical, functional, and ultrastructural modifications of mitochondria, and that these modifications were associated with an increased apoptotic proneness. Cybrids were highly susceptible to two different apoptotic stimuli, Tumor Necrosis Factor-α and Staurosporin. The mechanism involved was the mitochondrial ''intrinsic'' pathway, i.e., the caspase 9-driven cascade. More importantly, our results also indicated that the polarization state of the mitochondrial membrane, i.e., a constitutive hyperpolarization detected in cybrid clones, played a specific role. This work provides the first evidence that hyperpolarization of mitochondria may be a ''risk factor'' for cells with a deep ATPase dysfunction, such as cells from patients with maternally inherited Leigh syndrome.

III-A p-4. Age dependent changes of mitochondrial properties in heart and m. soleus of Fisher rats

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Effect of aging on functional properties of mitochondria of left heart ventricle and m. soleus was investigated in Fisher rats at age of 4 and 26 months. Mitochondria were respirometrically investigated in saponine skinned muscle fibers and enzyme activities were measured in tissue homogenates. Mitochondrial content as measured by citrate synthase was significantly diminished (− 17% in heart and − 25% in m. soleus). Pyruvate-dependent state 3 respiration scaled on citrate synthase activity decreased with aging by 47% in heart and by 34% in m. soleus. Multiple substrate inhibitor titration was applied to estimate relations between complex I (pyruvate/malate) and complex II (succinate)- dependent fluxes in the same fibers (Succinate Respiration scaled Pyruvate Respiration; SRPR). SRPR significantly decreased from 152 ± 8% to 127 ± 27% in hearts and from 204 ± 20% to 152 ± 36% in m. soleus. This was caused by a significant decrease of complex I activity as it can be seen from the ratio of complex I + III / complex II + III which decreased in heart from 109 ± 36% to 77 ± 30% and from 85 ± 36% to and 61 ± 30% in m. soleus.

Also the CS scaled activities of complex I (− 26%, − 39%) and complex I + III (− 38%, − 42%) were decreased, whereas CS scaled activities of complex III (+ 15%, 0%), II + III (+ 5%, + 7%) and COX (+ 16%, + 3%) remained constant or were even increased in heart and skeletal muscle respectively. The tissue content of coenzyme Q10 did not change during aging.

It is concluded that the mitochondrial content as well as the RCI decrease in heart and m. soleus of aging rats. The oxidation of complex I-dependent substrates is relatively more affected than succinate oxidation due to a specific decrease if complex I. Multiple substrate inhibitor titration in combination with skinned fiber technique is useful tool for detection of complex I-dependent functional deficits in mitochondria.

III-A p-5. Neuroprotection by alkalized extracts of the Corydalis ternata following transient brain ischemia through glutamate-metabolizing enzymes

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Recent studies on glutamate neurotoxicity have established a central role of mitochondria in neuron death, although the participation of mitochondria in their injury is not fully understood. In the present work, we have showed the protective effects of alkalized extracts of the Corydalis ternata in transient brain ischemia. When treated with the alkalized extracts, almost 80% of neurons in ischemia-affected brain area were saved and elevated level of excitatory glutamate dramatically reduced in the model of 5 min gerbil brain ischemia. This protective effect was only observed when the alkalized extracts were infused immediately upon reperfusion. In addition, the alkalized extracts increased gene expression of glutamine synthetase and glutamate dehydrogenase up to 2.2-fold and 5.5-fold, respectively. Ischemic alterations to astrocyte energy metabolism and the uptake and metabolism of the excitatory amino acid transmitter glutamate may be particularly important. Our results provide an insight into the mechanism of neuroprotective action of the alkalized extracts through enzymes in glutamate-glutamine metabolism. The possible roles for glutamate-metabolizing enzymes in astrocyte mitochondria to serve as targets of neuroprotective interventions will be presented.

III-A p-6. Alterations on liver mitochondria of diabetic Goto-Kakizaki (GK) rats

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Several studies have been carried out to evaluate the alterations in mitochondrial functions of diabetic rats. The purpose of this study was to evaluate the metabolic changes in liver mitochondria, during mild hyperglycaemia in Goto-Kakizaki (GK) rats, an animal model of type 2 diabetes, both at initial and at advanced states of the disease.

Liver mitochondria from GK rats presented alterations on respiratory chain activity. In fact, GK rats with 6 months presented increased respiratory ratios. In order to localize which respiratory complexes are affected by diabetes, enzymatic respiratory chain activities were evaluated. Succinate dehydrogenase and cytochrome *c* oxidase activities were significantly augmented in GK rats with 6 months of age as compared to controls. Moreover, H^+-ATPase activity was also significantly increased in these rats.

We observed that GK rats presented a decreased susceptibility of liver mitochondria to the induction of permeability transition (MPT). Apparently, there is a positive correlation between the severity of diabetes mellitus (and duration of the disease) and the decline in the susceptibility to MPT induction. We found that liver mitochondria isolated from diabetic rats presented some metabolic adaptations, such as an increase in coenzyme Q and cardiolipin contents, that can be responsible for the observed decrease in the susceptibility to MPTP opening.

This work was supported by Fundação para a Ciência e a Tecnologia (FCT; Portuguese Research Council).

III-A p-7. Effect of IGF-II treatment low doses on oxidative metabolism of aging rat liver

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The observations that the level of IGF-II declines continuously with aging (J Clin Endocrinol Metab 1998;83:499–502) and mitochondrial dysfunctions may be involved as underlying events (Gerontology 2002;48:343–353) led us to study the effect of IGF-II low dose treatment on liver bionergetics of aging rats.

Young adult (1month) and old (23month) male wistar rats were studied. The old group was divided in two,respectively receiving vehicle alone or IGF-II (1 μ/100 g body wt/day) for 3 weeks. Serum cholesterol,glucose and triglyceride were measured by standard laboratory procedures and liver homogenate MDA by a colorimetric method.Liver mitochondria were isolated by standard procedure. Mitochondrial $\Delta\Psi$ and ROS levels were evaluated by flow cytometry using Rh123 and DihydroRh123,repectivley. Mitochondrial oxygen uptake was measured by a Clark-type oxygen electrode as well as proton leak kinetics (by titration of complex II with malonate as inhibitor in St4 respiration in the presence of oligomycin) and Adenosine Nucleotide Transporter(ANT) activity (by titration with atractyloside as ANT inhibitor). ATP synthesis measurement was performed by a Sigma commercial kit.

Aging showed increased trigliceridemia,glycemia and cholesterolemia and the IGF-II treatment significantly decreased these parameters. The very same patterns resulted for MDA levels in the livers. ADP/O and ATP synthesis appeared significantly lower in aging group and,once again, IGF-II increased the ATP synthesis.This energy waste was evident evaluating $\Delta\Psi$ and proton leak kinetics, i.e., aging rats shown a lower St4 $\Delta\Psi$ and an increased proton leak in comparison with young rats.Once again IGF-II treatment reverted $\Delta\Psi$ to the young group values and made leak to fall by half: The huge ROS production by aging rat mitochondria and its significant drop induced by IGF-II here revealed may account for the above results. The ANT evaluation in aging rats showed a surprising behaviour, i.e., instead of the expected atractyloside concentration dependent progressive inhibition, a St3 respiration rate increase appeared, probably indicating the presence of a MPT(ANT is possibly oxidated, causing a further source of ROS that could damage DNA, RNA, lipids and cell proteins. The IGF-II reverted this effect.

As a conclusion it is possible to maintain that IGF-II at low doses improves glucose and lipid metabolism,minimizing liver oxidative damage and improving mitochondrial functions in aging rats. We thank Lilly for providig IGF-II.

III-A p-8. Neuroprotective strategies in the LHON cybrids galactose model

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Leber's hereditary optic neuropathy (LHON) is a maternally inherited disease associated with point mutations in mitochondrial DNA. The pathology is characterized by selective death of ganglion cells in the retina leading to optic atrophy. The pathogenic mtDNA point mutations for LHON affect complex I with the double effect of lowering ATP synthesis driven by complex I substrates and increasing oxidative stress chronically.

Two different lines of therapeutic intervention can be identified for LHON: (a) to increase respiratory activity and ATP synthesis and (b) to titrate the excess oxidative stress. In patients, therapy is based on the use of CoQ10 or its short-chain derivative idebenone, aiming to restore electron flow as well as increase antioxidant defenses.

In this study we utilized the experimental model of cybrids bearing one of the three pathogenic mutations (11778/ND4, 3460/ND1 and 14484/ND6) incubated in galactose medium, to, i.e., forced to rely on oxidative metabolism only for energy supply. Under this condition, cybrids with LHON mutations, but not control cybrids, undergo apoptotic cell death (Ghelli et al., 2003, JBC 278, 4145). Preincubation with idebenone (0.1–1 μM) failed to protect LHON cybrids from death in galactose medium, whereas at 5 μM this drug was toxic also in glucose medium. The ketone body beta-hydroxy-butyrate (1–4mM), previously shown to correct defects in mitochondrial energy generation bypassing the block of complex I in cultured neurons (Kashiwaya et al., 2000, PNAS 97, 5440), also failed to reduce LHON cybrids death in galactose medium. Cyclosporine A, an inhibitor of mitochondrial permeability transition pore, was also ineffective. The new antioxidant MitoQ, an analogue of CoQ covalently bound to an alkyltriphenylphosphonium cation, specifically targeted into mitochondria, was recently reported to exert a protective effect in Friedreich Ataxia fibroblasts exposed to oxidative stress (Jauslin et al. 2003, FASEB J. 17, 1972). Surprisingly, preincubation with MitoQ (0.1–100 nM) increased the loss of viability of LHON but not control cybrids in galactose medium. At the same concentrations, untargeted decylubiquinone and $TPMP^+$ were also toxic. Only preincubation with reduced glutathione (10mM) promoted a weak, but significant cell survival of LHON cybrids. These results suggest reduced gluthatione as an effective neuroprotective drug for LHON patients treatment.

Supported by Telethon grant n GGP02323.

III-A p-9. Estradiol protects heart mitochondria from the release of cytochrome *c* from mitochondria after ischemia

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Estrogens are known to play a cardioprotective role in global myocardial ischemia–reperfusion, however the mechanism is unclear. We previously reported that estradiol can protect heart mitochondria from the loss of cytochrome *c* induced by high calcium. In this study investigated whether estradiol can prevent release of cytochrome *c* from mitochondria and subsequent inhibition of respiration after stop-flow ischemia in Langendorff-perfused rat female hearts. We found that preperfusion of hearts with 100 nM of estradiol (high physiological concentration) prevented the loss of cytochrome *c* from mitochondria and subsequent inhibition of mitochondrial respiration. After 30 and 60 min of ischemia the mitochondrial level of cytochrome *c* decreased by 21% and 29% respectively, compared to control level. However, in mitochondria from ischemic hearts preperfused with 100 nM estradiol the content of cytochrome *c* was not significantly different from control mitochondria. Cytochrome *c*, released from mitochondria during ischemia, accumulated in cytosol and this was also prevented by estrogen. Cytochrome *c* concentration in cytosolic extracts from 60 min ischemic hearts increased by 56%, compared to control, but was not significantly different from control in cytosols from hearts preperfused with estradiol. Together these results show that estradiol prevents ischemia-induced release of cytochrome *c* from mitochondria to cytosol. These data suggest that cardioprotective effect of estrogens against ischemic damage might be partly related to their direct effect on mitochondria.

III-A p-10. Dominant effects of adPEO-associated ANT1 mutations in *Saccharomyces cerevisiae*

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Autosomal dominant external progressive ophthalmoplegia (adPEO) is a mitochondrial disorder associated with the presence of multiple deletions of mtDNA (Zeviani et al. (1989) Neurol. Clin., 7, 123–156). Four adPEO-associated missense mutations have been identified in the ANT1 gene encoding the muscle-heart specific isoform of the mitochondrial adenine nucleotide transporter [Kaukonen et al. (2000) Science, 289, 782–785; Napoli et al (2001) Neurology, 57, 2295–2298; Komaki et al. (2002) Annals of Neurology, 51, 645–648]. Since ANT1 is not expressed in cultured fibroblasts or myoblasts, to investigate their effects at the protein and at the cellular levels, we introduced three adPEO-associated ANT1 mutations at equivalent positions in AAC_2, the yeast orthologue of human ANT1. Insertion of the aac2 mutant alleles in combination with the endogenous wild-type AAC_2 gene, caused a significant reduction in cytochrome content and/or increased mtDNA instability [Fontanesi et al. (2004) Hum. Mol. Genet. 13, 923–934]. In all cases, the heteroallelic strains displayed an overall Aac2p content comparable to that of the wild-type strain and normal membrane potential. Upon solubilization from mitochondrial membranes and reconstitution into liposomes, the adenine nucleotide transporter activity was found to be variably affected by the different AAC_2 mutations. The analysis of petite mutants in the heteroallelic strains AAC_2/aac2A128P and AAC_2/aac2S303M showed that all of them were rho- indicating that neither the synthesis of mtDNA nor its segregation was inhibited. The increase in the rate of petite colonies observed in these mutant strains can account for the slow, progressive accumulation of multiple deletions in human mtDNA which is proposed to lead to adPEO. Interestingly, at variance with AAC_2/aac2A128P, the AAC_2/ aac2S303M strain showed normal cytochrome profile and cellular respiration compared to a control strain. Furthermore, significant alterations of the cytochrome content were found in the AAC_2/aac2M114P strain but no increase of mtDNA mutability, indicating that the two phenotypes can arise independently. These results shed light on the pathogenesis of adPEO and validate the yeast *Saccharomyces cerevisiae* as a suitable in vivo model to further study the early biochemical and cellular consequences of adPEO-associated ANT1 mutations.

III-A p-11. Mitochondrial dysfunction in ischemic/reperfused rat heart: role of ROS and cardiolipin

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Reactive oxygen species (ROS) are considered an important factor in ischemia–reperfusion injury to cardiac myocytes. It has been demonstrated that hearts subjected to postischemic reperfusion produce high level of superoxide anion. Mitochondrial respiration is an important source of ROS production and hence a potential contributor to cardiac reperfusion injury. The complexes of respiratory chain and phospholipid constituents are particularly susceptible to ROS attack (1, 2). Cardiolipin is a phospholipid rich in unsatured fatty acids, localized almost exclusively in the inner mitochondrial membrane, near the site of ROS production. It has been reported that cardiolipin is important for the full activity of the mitochondrial complex I. In vitro experiments have shown that mitochondrial ROS production induces oxidative damage to cardiolipin which, in turn, results in a defect of complex I activity (3). We have examined the effect of ischemia and ischemia followed by reperfusion of rat hearts on various parameters related to mitochondrial function, such as complex I activity, oxygen consumption, ROS production and cardiolipin content. The activity of complex I was reduced by 25 and 48% in mitochondria isolated from ischemic and reperfused rat heart respectively, compared to the controls. These changes in complex I activity were associated to parallel changes in state 3 respiration. In addition, the capacity of mitochondria to produce H_2O_2, in state 4 respiration, increased upon reperfusion. The mitochondrial content of cardiolipin, decreased by 28 % and by 50 % as function of ischemia and reperfusion respectively. The lower complex I activity in mitochondria from reperfused rat heart could be completely restored to the level of normal heart by exogenous added cardiolipin. This effect of cardiolipin could not be replaced by other phospholipids nor by peroxidized cardiolipin. These results indicate that reperfusion of ischemic heart induces an increase in the mitochondrial ROS production which, in turn, causes oxidative alteration to cardiolipin and consequent decrease in the complex I activity. The defect in complex I activity, in addition to that of complex III and IV previously reported, may lead to a decrease in oxidative phosphorylation and to heart failure on reperfusion.

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III-A p-12. NMD of mutated and alternatively spliced transcripts in human complex I deficiency

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Complex I (CxI) of the mammalian mitochondrial (mt) respiratory chain consists of at least 46 subunits encoded both by nuclear and mt genomes. The function of most of these subunits is unknown. The essential role of the18 kDa subunit, encoded by the nuclear NDUFS4 gene, has been established by the finding that its phosphorylation promotes complex I activity (1–4). In some severe forms of encephalopathies, such as Leigh syndrome (LS) associated to CxI deficiency, it has been found that the mutations affecting the expression of the NDUFS4 gene prevents the assembly of a normal functional complex and produce the appearance of defective subcomplexes (5). Mutational analysis of genes encoding for CxI subunits has revealed that the NDUFS4 gene is particularly prone to mutations (6, 7) among the genes so far associated with the disease (8). Investigations on the pathogenic mechanisms of three different homozygous NDUFS4 mutations in LS patients have revealed that each mutation, although in the same gene, may act by a different mechanism (5). A nonsense mutation introducing a premature stop codon in one of the patients elicited a cell surveillance mechanism of mRNA degradation known as Nonsense Mediated Decay (NMD). In the other two patients, the steady-state level of the transcript was normal, although in one of them, an alternative transcript appeared. We are investigating on the possibility that NMD could also be involved in regulating the expression of natural proteins eliminating transcripts that are generated in error by alternative splicing. Translation of such transcripts might, in fact, generate aberrant and potential harmful proteins. On the other hand, we are also exploring whether some of the alternative transcripts of NDUFS4 gene could generate isoforms of the NDUFS4 protein. All together, our results are consistent with the occurrence of a multistep quality control system in the cell and are revealing novel aspects of the posttranscriptional and posttranslational level of gene expression.

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III-A p-13. Caspase-independent death of cybrids with LHON mutations: role of mitochondrial apoptogenic factors

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Leber's hereditary optic neuropathy (LHON), characterized by a rapid loss of central vision due to a selective death of retinal ganglion cells, is caused by three mtDNA point mutations at positions 11778/ND4, 3460/ND1, 14484/ND6 of ND subunits of complex I. The pathogenic role of complex I dysfunction remains unclear, since it cannot be correlated with a biochemical alteration common to all mutations. We previously reported that cybrids harboring one of LHON mutations, but not control cybrids, undergo apoptosis when galactose replaces glucose in the medium, i.e., when cells are forced to rely on oxidative metabolism only for energy supply (Ghelli et al., 2003, J.Biol.Chem. 278, 4145). Furthermore, we showed that the ATP content of LHON and control cybrids was similar in glucose medium, but once in galactose only LHON cybrids suffered a profound ATP depletion (Zanna et al., 2003, Ann. New York Acad. Sci. 1010, 213). We show here that despite the significant release of cytochrome *c* from mitochondria, caspase-3 was not activated and the pan-caspase inhibitor z-VAD.fmk failed to promote cell survival. The presence of nuclear fragmentation in the absence of caspase activation raised the possibility that other pathways of cell death are activated during galactose incubation.

The aim of this study is the identification of the caspase-independent cell death pathway triggered in LHON cybrids by metabolic stress. The involvement of lysosomal proteases was ruled out by the finding that specific inhibitors of various cathepsins (pepstatin A, E-64d, etc.) failed to promote cell survival. Conversely, western blot as well as immunofluorescence analyses showed that, in addition to cytochrome *c*, the other apoptogenic factors Endonuclease G and Apoptosis Inducing Factor (AIF) were released from mitochondria in LHON cybrids incubated in galactose, but not in control cybrids. Experiments are in progress to test whether AIF and Endonuclease G play a direct role in the regulation of the caspase-independent cell death in galactose medium. The results of these studies will be discussed in the framework of LHON pathophysiology, considering that caspase-independent processes have been demonstrated to play a prominent role in mediating cell death in neurodegenerative diseases.

Supported by grants from Telethon n GGP02323 and from progetto ''Meccanismi e segnali molecolari della sopravvivenza cellulare'', Università di Bologna.

III-A p-14. Cyclosporin A prevents TNF-induced hepatitis desensitizing the mitochondrial apoptotic pathway through the permeability transition pore

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We have studied the effects of cyclosporin A (CsA) administration to Albino Wistar rats (i) on the properties of the permeability transition pore (PTP) in mitochondria subsequently isolated from the liver; and (ii) on the susceptibility of the animals to hepatotoxicity induced by lipopolysaccharide of *E. coli* (LPS) plus D-Galactosamine (DGalN). At 5 mg $\times$ kg^{-1} body weight (which was determined to be the optimal dose) CsA exerted a marked PTP inhibition ex vivo, with an effect that peaked between 2 and 9 h of drug treatment and decayed with an apparent half-time of about 13 h. Administration of 20 μg LPS plus 700 mg DGalN $\times$ kg^{-1} body weight to naïve rats caused the expected increased serum levels of tumor necrosis factor (TNF)-α;, liver inflammation with cleavage of BID, activation of caspase 3, and liver damage with appearance of terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL)-positive nuclei and release of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) into the bloodstream. Treatment with CsA prior to, or within 5 h of the administration of LPS + DGalN protected rats from hepatotoxicity despite the normal increase of serum TNF-α; and BID cleavage. These results indicate that CsA prevents the hepatotoxic effects of TNF-α; by blocking the mitochondrial proapoptotic pathway through inhibition of the PTP, and provide a viable strategy for the treatment of liver diseases that depend on increased production and/or liver sensitization to TNF-α.

III-A p-15. Isoform 2 of the adenine nucleotide translocator is required for the glycolytic metabolism of cancer cells

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The adenine nucleotide translocator (ANT), which exchanges ADP and ATP through the mitochondrial inner membrane, plays a crucial role in energy metabolism. Three isoforms from three different genes (*ANT1–3*) are differentially expressed in Human, in relation to tissue-specific energy requirements: ANT1 (muscular) and ANT3 (ubiquitous) export ATP produced by mitochondrial oxidative phosphorylation. The ANT2?isoform is specifically expressed in proliferative cells with a mainly glycolytic metabolism. Transformed cells and their rho° derivatives without mitochondrial DNA, in contrast to differentiated tissue cells, sustain a high rate of glycolysis under aerobic conditions, so called ‘‘aerobic glycolysis’’. In such glycolytic conditions, the ANT2 isoform is over expressed and its proposed function is to uptake glycolytic ATP into mitochondria. By quantitative real-time RT-PCR, we compared the transcription pattern of both ANT2 and ANT3 isoforms in several human transformed cell lines and in different states of their cell cycle. A higher *ANT2* transcript level was observed in 143B cells (from osteosarcoma) than that in HepG2 cells (from hepatocarcinoma). The comparison of metabolic characteristics of these cell lines showed levels of glycolytic ATP production in accordance with their glycolytic or oxidative metabolic background. The sensitivity of their mitochondrial membrane potential ($\Delta\psi m$) to either glycolysis or oxidative phosphorylation inhibitors confirmed this relationship. We propose that the mechanism of regeneration of this $\Delta\psi m$, essential for cancer cell proliferation, would involve three major proteins: hexokinase II, ANT2 and the F1 part of the mitochondrial ATP synthase complex. Thus, taking into account the major role of the ANT2 isoform in cell proliferation and its very low expression in differentiated tissues, this protein seemed to us a possible target for an anti cancer strategy.

III-B: Biogenesis of Energy Transducing Systems

III-B p-1. Biogenesis of artificial microperoxidases and *c*-type cytochromes

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Naturally occurring proteins that bind haem are used for electron transfer, enzymatic catalysis, oxygen transport and signalling of gases such as O_2, NO and CO. One class of these proteins are the c-type cytochromes that bind haem covalently to the conserved amino acid sequence Cys–Xaa–Xaa–Cys–His. The two cysteines are cross-linked to the haem vinyl groups, resulting in two thioether bonds per bound haem; histidine serves as an axial ligand of the haem iron. Bacterial c-type cytochromes are synthesized as precursor polypeptides with an N-terminal signal sequence for export of the polypeptide to the periplasm by the general protein secretion system (Sec). The covalent ligation of haem to the c-type cytochromes takes place on the periplasmic side of the cytoplasmic membrane. In *E. coli* eight cytochrome *c* maturation proteins CcmA-H are required specifically for this process.

Our initial aim was to define the minimal sequence requirements within the apo-cytochrome for holo-cytochrome *c* formation by the Ccm system. We expressed small peptides containing a Cys–Xaa–Xaa–Cys–His motif in the periplasm and analysed them for covalent haem attachment. As a result we present a mini-c-type cytochrome of 12 amino acids in length that is produced in vivo and has characteristics similar to commercially available microperoxidases.

This knowledge was further used in a genetic approach that enables specific covalent attachment of haem via a C-terminal peptide tag of 10–16 amino acids to an otherwise nonhaem-binding protein. In addition to the initial enzymatic activity the resulting haem tagged protein is red in colour, has peroxidase activity and is redox active. The haem tag can be used as a tool for the rational design of artificial c-type cytochromes, thereby overcoming previous limitations set by chemical approaches.

III-B p-2. Analysis of human disease-associated mutations introduced into the cytochrome bc_1 complex of *Saccharomyces cerevisiae*

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The high degree of sequence identity between *S. cerevisiae* and human cytochrome *b* validates the use of yeast as a model system to study disease-associated mutations within the mitochondrial cytochrome bc_1 complex. In this study we present data obtained from the physicochemical analysis of six human disease-associated mutations introduced into yeast cytochrome *b*. In man, the mutations G33S, S152P, ?252–259 (an in-frame deletion of eight residues) are associated with exercise intolerance, G167E and G252D with cardiomyopathy and Y279C with exercise intolerance and multisystem disorder. G33S, S152P, G291D and ?252–259 were clearly pathogenic, causing a severe decrease in respiratory function and destabilising the integration of the Rieske iron–sulpur protein (ISP) into the bc1 complex. Y279C was less deleterious, but affected quinol binding at the cytochrome *b* Qo site. G252D had no detectable affect on bc1 complex assembly or activity under the conditions studied, but may confer thermosensitivity to the enzyme at normal body temperature due to weakened interactions with structural lipid. G167E was found to destabilise the bc1 complex and be inhibitory to electron transfer through the high-potential chain, presumably due to decreased mobility of the ISP. These data provide information concerning the molecular basis of respiratory defects due to disease-associated cytochrome *b* mutations in man.

III-B p-3. Effects of haloperidol on energy metabolism, calcium-signalling in human myoblasts, and on permeability transition

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Haloperidol is in use for years as neuroleptic. Some of its extrapyramidal side effects were explained by its known ability to inhibit complex I of the respiratory chain. We investigated the action of this drug on muscle homogenate, muscle mitochondria and intact human myoblasts as a model system for the study of acute energetic depression caused by impaired mitochondria. Inhibition of respiratory chain complexes were measured using standard enzymatic methods. Mitochondrial respiration was measured with high resolution respirometry and cytosolic Ca^{++} was measured fluorimetrically with confocal microscopy. Inhibitor titrations with amytal were performed to estimate flux control coefficients of complex I. Haloperidol inhibits complex I in a concentration dependent manner. The I50 concentration of haloperidol depends on the complexity of the metabolic system and increases from 12 μM (complex I + III) over 24 μM (respiration of muscle homogenate) to 50 μM (respiration of intact cells). The reason for that is the low flux control coefficient of complex I in skinned fibers ($Co = 0.04 \pm 0.04$, $n = 19$) indicating that a partial inhibition of complex I can occur without functional consequences under this conditions. In intact myoblasts the control of complex I on endogenous respiration is larger ($Co = 0.15$) but increased by 50 μM haloperidol ($Co = 0.46$). Also the succinate dependent respiration (which does not include complex I) was decreased by haloperidol due to its new discovered inhibition of F0F1ATPase. After haloperidol addition, cytosolic Ca^{++}-concentration increased immediately, followed by a slow decrease. This haloperidol effect was similar to that caused by addition of rotenone plus oligomycine (inhibitors of complex I and F0F1ATPase). With swellings measurements at liver mitochondria we further found that tendency for Ca^{++} induced pore opening was decreased by haloperidol as it is known from other inhibitors of complex I and F0F1ATPase, too. Our study shows that haloperidol can cause energetic depression due to combined inhibition of complex I and F0F1ATPase.

III-A p-4. Degradation of targeting peptides in mitochondria and chloroplasts: a novel zinc metalloprotease

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Several thousand of the mitochondrial and chloroplast proteins are nuclear encoded and synthesised as precursor proteins containing an N-terminal targeting peptide that is recognised by organellar receptors. Both the mitochondrial and chloroplast targeting peptides show a remarkable similarity in overall properties, yet they maintain a high degree of targeting specificity. After import, the targeting peptides are specifically cleaved off inside the organelles by processing peptidases, MPP in mitochondria and SPP in chloroplasts. The cleaved targeting peptides are toxic to biological membranes, dissipate membrane potential and therefore they should be rapidly degraded or removed. We have identified a novel mitochondrial zinc metalloprotease (Zn-MP) involved in degradation of mitochondrial presequences (J. Biol. Chem. 2002, 277:41931–41939). A combination of biochemical, molecular biological and biophysical approaches such as protein overexpression, site-directed mutagenesis, deletion/truncation mutagenesis, in vitro import as well as Agrobacterium-mediated in vivo transient expression of targeting peptide-GFP constructs have been used in order to characterise properties, intracellular localisation and targeting properties of the Zn-MP. The Zn-MP belongs to the pitrilysin protease family and contains an inverted zinc-binding motif (Plant J. 2003, 36:616–628). It has the ability to degrade both mitochondrial presequences and chloroplastic transit peptides as well as other unstructured peptides, but not folded small proteins. The Zn-MP is dually targeted to both mitochondria and chloroplasts. Targeting peptide of the Zn-MP is organised in domains with different organellar targeting specificity (EMBO Rep. 2003, 4:1073–1078). The domain organisation controlling organellar import efficiency has been also found in the dual targeted glutathione reductase signal peptide (J. Mol. Biol. 2002, 324:577–585). Interestingly, the identified Zn-MP is an organellar homologue of the human Insulin Degrading Enzyme (IDE) that recently has been implicated in Alzheimer's disease by cleaving the amyloid beta-peptide before insoluble amyloid fibrils are formed. Further studies on organellar targeting properties and catalysis are in progress.

III-B p-5. CtaG-copper chaperone and assembly factor for the Cu_B-center of the aa_3-type cytochrome *c* oxidase in *Paracoccus denitrificans?*

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The ctaG-gene codes for a small (20 kD) membrane protein which is conserved throughout many prokaryotic and eukaryotic organisms including yeast, drosophila and humans. A deletion of this gene, located within the cta-operon coding for subunits II and III, leads to a diminished oxidase activity and Cu:heme-ratio in *Paracoccus denitrificans* (Pfitzner 2000). The same effect can be observed for the yeast homologue of CtaG, Cox11, for which the reduced Cu:heme-ratio could be assigned to the absence of CuB by EPR-spectroscopy (Hiser et al., 2000). This observation, together with the fact that the yeast homologue is a copper(I)-binding protein (Carr et al., 2002) suggests that CtaG is a copper chaperone responsible for the biogenesis of the CuB center in the *P. denitrificans* cytochrome *c* oxidase.

The bacterial CtaG has been purified after heterologous expression in *E. coli*. Using CtaG-specific antibodies, which were produced against the heterologously expressed protein, the membrane localization of CtaG in *P. denitrificans* could be verified. TXRF-spectroscopic measurements combined with site-directed mutagenesis of potential metal-binding residues will give insight into the metal-binding properties of CtaG. Further studies on the interaction between CtaG and subunit I of cytochrome *c* oxidase during biogenesis will be carried out.

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III-B p-6. Heme a synthesis in *Bacillus subtilis*: structure and function analysis of CtaA

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Heme A, as prosthetic group, is uniquely found in terminal respiratory oxidases which reduce dioxygen to water. Biosynthesis of heme A from protoheme IX (heme B) occurs with heme O as a stable intermediate (1). The *B. subtilis* CtaA protein is required for the heme O to heme A biosynthetic step; the conversion of the methyl side group on porphyrin ring D of heme O to a formyl group. CtaA might catalyse the entire heme A synthetic step which comprises two monooxygenation reactions (3). Overproduced and isolated CtaA contains heme B and can also harbor heme A (2). The heme B is thought to be a prosthetic group and heme A, when present, is probably the product remaining on the enzyme. A *B. subtilis* ctaA null mutant and gene variants encoding hexahistidyl- or StrepII-tagged CtaA have been constructed. The tagged variants have been shown to be enzymatically active in vivo and allow purification by affinity chromatography of preparative amounts of CtaA from *B. subtilis* solubilised membranes. CtaA orthologues contain four invariant His residues. The functional roles of these residues have been analysed using site-specific mutagenesis. Some mutant variants are inactive in heme A synthesis but can bind both heme B and heme O demonstrating that heme O is a substrate for CtaA.

III-B p-7. Membrane-bound thiol-disulfide oxidoreductases in cytochrome *c* synthesis in *Bacillus subtilis*

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Thiol-disulfide oxidoreductases catalyse the oxidation and reduction of disulfide bonds in cells. These enzymes contain one pair of cysteine residues in the active site. In *Bacillus subtilis*, a model organism for gram-positive bacteria, membrane-bound thiol-disulfide oxidoreductases are involved in development of competence, biosynthesis of cytochrome *c* and in sporulation. The cytochrome *c* machinery in *B. subtilis* is of the so called System II type which is also present in plants. Systems I and III are found in, e.g., *Escherichia coli* and mammalian mitochondria, respectively (1, 2). In cytochrome *c* synthesis in *B. subtilis* the membrane-bound thiol-disulfide oxidoreductase ResA functions on the outside of the cytoplasmic membrane where it reduces the heme-binding site in apocytochrome *c* to allow covalent attachment of the heme-group (3). *B. subtilis* contains four ResA paralogues. The functions of two of these proteins, BdbA and BdbD, have been identified (4, 5). We have now analysed whether the two other paralogues, the YneN and StoA proteins, play any role in cytochrome *c* synthesis. Both YneN- and StoA-deficient strains were found to grow like the parental strain and they had a normal cytochrome *c* content. However the StoA-deficient strains were found to be defective in endospore synthesis. StoA seems to be involved in the formation of the spore cortex which is important for heat resistance of bacterial endospores.

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III-B p-8. The heme-based oxygen sensor, FixL/FixJ system, a paradigm of the two-component signal transducing systems

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Leguminous bacteria fix the atmospheric nitrogen gas into ammonia using ATP during symbiosis in the host root nodules with low oxygen tension. Since their nitrogenases are labile against molecular oxygen, the expression of the structural genes is strictly downregulated under the aerobic conditions.

FixL and FixJ proteins are the oxygen sensing two-component signal transducing system that is responsible for the nitrogenase-gene regulation. FixL is a heme-based sensory histidine kinase, and the oxygen-free form undergoes autophosh-orylation at an invariant histidine residue using ATP at low oxygen tensions. FixJ, in turn, is converted into a transcriptionally active species by receiving the phosphoryl group from FixL. But high oxygen tension leads the inactivation of kinase activity in FixL due to oxygen binding to the heme. The biochemical property that the oxygen-bound and -free forms of FixL are spectroscopically distinguishable has enabled us to determine the oxygen binding affinity of purified FixL, and has endowed the FixL/FixJ system with a paradigm of the two-component systems (Saito et al., Mol. Microbiol. 48, 373–383, 2003; Nakamura et al., Proc. Natl. Acad. Sci. USA. 101, 2742–2746, 2004). In this study, we show that ADP reduces the oxygen binding affinity of the sensor domain in FixL when it is produced from ATP in the kinase reaction.The addition of ADP to a solution of purified wild type FixL resulted in a 4 ~ 5-fold decrease in oxygen binding affinity in the presence of FixJ. In contrast, phosphorylation-deficient mutants, in which the well-conserved ATP-binding catalytic site of the kinase domain is impaired, showed no such allosteric effect. Furthermore, we found that ATP competitively binds to the catalytic site with a higher affinity. This discovery casts a novel light on the significance of homodimerization of two-component histidine kinases; ADP, generated in the phosphorylation reaction in one subunit of the homodimer, enhances the histidine kinase activity of the other by reducing the ligand binding affinity, analogous to a two-cylinder reciprocating engine.

III-B p-9. Acetyl-L-carnitine administration to atrophied rat skeletal muscle increases mitochondrial biogenesis

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Acetyl-L-carnitine (ALCAR) and their derivatives are nutraceuticals used to therapeutically treat problems of energy metabolism. ALCAR pharmacological administration to older animals improves energy productive capabilities leading to higher transcription rate of mitochondrial DNA, improved protein synthesis level and restoring the functional activities of inner membrane bound mithochondrial enzymatic system as well as the cardiolipin content.

Here we studied the impact of ALCAR supplementation on the expression profile of atrophying rat *soleus* muscle as induced by 14 days of hindlimb suspension. ALCAR 1.5% solution was provided *ad libitum* to a group of control and hindlimb suspended animals (n=7) and ALCAR-untreated control and suspended groups were housed under the same condition. ALCAR effects were monitored through a microarray approach using commercially available ATLAS^{TM} Rat 1.2 cDNA filters. Statistical significance was assessed at $p<0.05$.

No difference in *soleus* weight normalized to body mass was noted between ALCAR-treated and untreated groups. Both hindlimb suspended groups underwent a drop in the *soleus* muscle (−21%) compared to the respective control groups. ALCAR was very effective on fatty acids transport and oxidative metabolism. The decrease in mitochondrial muscle carnitine palmitoyltransferase I isoform with the unloading was prevented with ALCAR dietary. As, far as the oxidative phosphorylation metabolism, both nuclear (COX IV, Vb, VII-H) snd mithochondrial (COX I) cytochrome oxidase subunits were upregulated with nutritional intervention. Quantitative Real Time PCR assay was performed to validate the array results and to further investigate the effects of ALCAR on the expression level of mitochondrial genes (ATP6,16S rRNA, ND6) and factors involved in the mitochondrial biogenesis (CaMKII-beta, PGC-1, NRF-1, TFAM). All these genes were found significantly upregulated with ALCAR treatment. The results indicate that nutraceuticals administration biogenesis, as well as to improve the fatty acid import. These findings could suggest a potential role a ALCAR in the therapeutical treatment of muscle disuse conditions as bed rest, immobilization or aging itself.

III-B p-10. Periplasmic redox homeostasis and c-type cytochrome biogenesis

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In Rhodobacter capsulatus, thio-oxidative (DsbA and DsbB) and thio-reductive (CcdA, HelX and Ccl2) branches of the c-type cytochrome (cyt) maturation pathway cooperatively establish a periplasmic redox homeostasis necessary for efficient cyt c biogenesis. Our previous work demonstrated that CcdA-minus mutants, which lack c-type cyts, can be suppressed by mutations inactivating DsbA or DsbB[§]. Currently, we have addressed whether similar relationships also exist between HelX and Ccl2 with DsbA and DsbB. We have isolated photosynthesis competent (Ps^+) suppressors of a Ps^- HelX-deletion mutant that can grow in the presence of thiol-reactive agents. Mapping of these suppressors revealed that they inactivated DsbA. As expected, a $HelX^-DsbA^-$ double mutant was Ps^+, but inactivating DsbB, the physiological partner of DsbA, did not confer a similar Ps phenotype. This observation suggested that, in R. capsulatus an oxidative pathway independent of DsbB might interact with reduced DsbA. In contrast to HelX-DsbA, Ccl2-DsbA or Ccl2-DsbB double mutants remained Ps^- and deficient of all c-type cyts. The fact that the $Ccl2^-$ mutants, unlike the CcdA and HelX mutants, are not suppressed by the absence of the thio-oxidative branch, suggests that Ccl2 may have an additional function along its ability to thio-reduce the apocyts during their maturation.

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[§]Deshmukh, et al., J. Bacteriol. 185 (2003) 3361–3372.

Miscellaneous

Misc p-1. Characterizatin of the mitochondrial cubic membrane

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Cubic membranes are symmetric, periodic structures that occur in numerous cell types from all kingdoms and in virtually any membrane-bound cell organelles. Cubic membranes are based on highly curved surfaces that are mathematically analogous to periodic minimal surfaces used in describing both crystalline and liquid crystalline materials at a variety of length scales. So far three types of cubic membrane morphologies-gyroid (G), double diamond (D) and primitive (P)-have been identified. Through computer simulation of Transmission Electron Microscopy (TEM) and EM tomography, cubic membranes which appear as zigzag patterns in TEM images have been identified.

Our current work is focused on cubic membrane structure. We have studied the cubic membrane structures in two membrane-bound organelles of unicellular microorganisms: the chloroplasts of green algae (Zygnema)[1] and the mitochondria of starved amoeba (*Chaos carolinensis*)[2] and in ischemic Mongrel dogs' myocardial mitochondria. Our recent data obtained with amoeba Chaos strongly suggest that cubic transition of mitochondrial cristae (upon starvation) plays a protective role against reactive oxygen species and may thus be a cellular response to oxidative stress. Transition of the heart mitochondrial crisate into zigzag forms (cubic membrane) in response to acute ischemic insult may represent the earliest myocardial cell adaptation to increased work demand due to acute ischemic stress.

In our presentation we will address our latest result of morphological characterization, bioenergetics and biochemical analysis of the mitochondrial cubic membrane in different living models that are currently undertaken in our laboratory.

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Misc p-2. Transient depletion of ATP induces apoptosis in HeLa cells

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Transient depletion (3 h) and recovery of ATP in HeLa cells leads to the caspase-dependent programmed cell death. To decrease intracellular ATP, cells were incubated in low-glucose medium with inhibitor of glycolysis 2DG (2-deoxy-D-glucose) in the presence of either a mitochondrial inhibitor (rotenone, oligomycin or myxothiozol) or the uncoupler of oxidative phosphorylation, FCCP. This treatment caused a rapid decline of ATP to 35–40% of the initial level during 3 h. If 2DG or inhibitors of oxidative phosphorylation are added separately, lowering of ATP occurs no more then 25%.

The ATP level recovers when cells was transferred to the complete media with high glucose content and without inhibitors. The treatment leads to the programmed cell death that occurred after 18 h, with features typical of apoptosis-ROS generation, caspase activation, cytochrome *c* release into cytosol, nuclear condensation, and DNA fragmentation. Inhibitor of caspases, zVAD-fmk, as well as expressed Bcl-2 protein protected cells from apoptotic death, but not from ATP depletion. Energy deprivation for 3 h alone did not cause immediate changes in cell viability. In this model the apoptotic signal was triggered by the temporarily reduction of the ATP level by about 60%, with the subsequent transfer of the signal and delayed apoptotic death. This project was supported by REBR-NWO and REBR-GFEN grants.

Misc p-3. Analysis of the interaction of adrenodoxin with native and tryptophan-modified NADPH-adrenodoxin reductase

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Introduction: In steroid hydroxylation system of adrenal cortex mitochondria, NADPH-adrenodoxin reductase (AR) and adrenodoxin (Adx) form a short electron-transport chain that transfers electrons from NADPH to cytochromes *P*-450 through FAD in AR and [2Fe−2S] cluster in Adx (NADPH AR Adx P-450) (Arch. Biochem. Biophys. (1966) 117, 660−673). The formation of [AR/Adx] complex is essential for electron transfer mechanism. Early fluorimetric investigations (Biokhimia (russ) (1987) 52, 1258−1262) showed that the chemical modification of AR with *N*-bromosuccinimide (NBS) resulted in Trp residues oxidation, accompanied with inhibition of electron transfer from AR to Adx. The possible ways of electron transfer inhibition due to Trp(s) oxidation may be due to involvement of these amino acid residues in the intracomplex electron transfer, or to AR conformational changes affecting [AR/Adx] complex formation and/or the FAD microenvironment.

In this study a controlled NBS modification of Trps in AR was performed and the affinity of modified protein (AR*) towards Adx was determined by using a resonant mirror biosensor. The most probable NBS-modifiable Trp residues were identified by computational analysis of AR and [AR/Adx] structures.

Results: Chemical modification of tryptophans caused inhibition of electron transport. CD spectroscopy indicated that the NBS-modified AR (AR*) retains the native secondary structure, and the resonant mirror biosensor data indicated that AR* has a lower affinity towards Adx with respect to AR. Activity measurements and fluorescence data indicated that one Trp residue of AR may be involved in the electron transferring activity of the protein. Computational analysis of AR and [AR/Adx] complex structures suggested that Trp193 and Trp420 are the residues with the highest probability to undergo NBS-modification. In particular, the modification of Trp420 hampers the correct reorientation of AR* molecule necessary to form the native [AR/Adx] complex that is catalytically essential for electron transfer from FAD in AR to [2Fe−2S] cluster in Adx.From this study we could conclude that one Trp residue in AR is important for the electron transferring activity of the protein and that the electron transfer inhibition due to oxidation of solvent-exposed Trp(s) is caused by an incorrect assembly of [AR*/Adx] complex.

Misc p-4. CD and NMR studies of transmembrane segments of the mitochondrial oxoglutarate carrier

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The oxoglutarate/malate carrier (OGC), a transport protein of the inner mitochondrial membranes, catalyses the exchange of 2-oxoglutarate for malate and plays a central role in several metabolic processes [Runswich,M.J.,Walker,J.E.,Bisaccia,F., Iacobazzi,V.,and Palmieri,F.(1990) Biochemistry 29, 11033–11040]. In order to understand the mechanism of oxoglutarate translocation through the OGC protein, the elucidation of the carrier structure is essential. The crystallization of transport proteins has been difficult because they are hydrophobic, show a strong tendency to aggregate and undergo the rapid structural changes required for their catalytic cycle. Among the members of the mitochondrial carriers family, only the X-ray crystallographic structure of the carboxyatractyloside-ADP/ATP carrier complex has been determined [Pebay-Peyroula,C.E.,-Dahou-Gonzales,C.,Kahn,R.,Trezeguet,V.,Lauquin,G.J.M.,and Brandolin, G.(2003)Nature 426, 39–43]. We studied the structure of the first two transmembrane segments of the bovine mitochondrial OGC by CD and NMR spectroscopies.

The synthesis of the peptides was performed on a solid-phase automatic peptide synthesiser, Pioneer TMPeptide Synthesis System. The Fmoc strategy was used. The peptides were purified by HPLC and the purity was assessed by mass spectroscopy. Circular dichroism spectra were recorded with a JASCO 600A automatic circular dichrograph.

NMR measurements were carried out on a VARIAN Inova 500 NMR spectrometer. Two-dimensional TOCSY and NOESY spectra were acquired in the phase-sensitive mode.CD data showed that at high concentrations of TFE(>50%) and SDS (>2%) peptides 21–46 and 78–108 assume a-helical structure. NMR data showed a well-defined a-helix in the region K24-V39 of peptide 21–46 and in the region A86-F106 of peptide 78–108. The helix of peptide 21–46 is essentially hydrophobic, whereas that of peptide 78–108 is predominantly hydrophilic.Our helical structures were superimposed on the structure of helices H1 and H2 of the ADP/ATP carrier (PDB accession code: 1okc). The good agreement between the solution structure of our peptides and the crystal structure of the first two transmembrane segments of the ADP/ATP carrier suggests that, in spite of the low sequence homology between them, the structure of OGC may be similar to that of the ADP/ATP carrier. Studies are in progress on the other transmembrane segments and loop regions to test if this conclusion may apply to other regions of the OGC.

Misc p-5. Tellurite reduction by cells of *Rhodobacter capsulatus*: transport, cell viability and oxidative stress

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Phototrophically grown cells of *Rhodobacter capsulatus* are strongly resistant to the toxic metalloid potassium tellurite (K2TeO3, Minimal Inhibitory Concentration, MIC of 1 mM). For comparison, *E. coli* cells show a MIC of 4 M. Tellurite resistance in *Rb. capsulatus* is linked to a consistent cytosolic accumulation of TeO32-, followed by its reduction and precipitation as elemental tellurium crystals (Te0) (Borsetti F et al. 2003, Protoplasma 221:153–161). Recently, we have shown that tellurite uptake is driven by delta pH following a proton symport mechanism which is likely to involve the phosphate transporter (PiT family) (Borsetti F et al. 2003, FEBS Lett 554:315). Here we report that exceeding amounts of tellurite (TeO32-/cell ratio of 200 fgr/cell) affect the cell membrane integrity, as determined by flow cytometry, whereas a significant recovery of the cell viability is seen at much lower TeO32-/cell ratios (4 fgr/cell). To get insights into the mechanism by which the metalloid damages *Rb. capsulatus* cells, we tested the effect of ROS generators (paraquat and/or xantine/xantine oxidase) on cell viability of w.t. (MT1131 strain) and mutant cells of *Rb. capsulatus* (CW5 and CW10 strains) deficient in polypeptides shown to have a role as O2 sensors (CcoG protein) in facultative phototrophs (Oh J and Kaplan S 2004, in: Zannoni D, ed., Respiration in Archaea and Bacteria, vol 15, pp. 287–309, Kluwer Academic Publishers The Netherlands) or ROS protecting agents (Sco1 protein) in Neisseria sp. (Seib KL et al. 2003, FEBS Lett. 20 546:411). In contrast to Neisseria sp., we have observed that the senC polypeptide (homologous of Sco1), has no role in protecting *Rb. capsulatus* against both oxidative stress and tellurite toxicity. On the other hand, cells treated with sublethal amounts of paraquat showed a 10-fold increase in tellurite resistance, this phenomenon being linked to a parallel overproduction of SOD (superoxide dismutase). The latter finding indicates, for the first time, that in *Rb. capsulatus* the tellurite effect on cell viability seems to mimic the cell response to oxidative stress.Work supported by MIUR (PRIN 2003 Bioenergetics).

Misc p-6. Apparent redundancy of electron transfer pathways to oxygen in the extremophilic chemolithautotrophic bacterium acidithiobacillus ferrooxidans

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Acidithiobacillus ferrooxidans is an acidophilic chemolithoautotrophic bacterium that can grow in the presence of either the weak reductant Fe^{2+}, or reducing sulfur compounds that provide more energy for growth than Fe^{2+}. When the cells are grown in iron, we have previously shown that the uphill electron transfer pathway between Fe^{2+} and NAD^{+} involved a bc1 complex that functions only in the reverse direction (A. Elbehti, G. Brasseur and D. Lemesle-Meunier, J. Bacteriol. 182, 2000, 3602–3606); moreover a bioinformatic analysis of the genome of A. ferrooxidans ATCC 23270 strain (TIGER) showed the existence of two different bc1 complexes in this bacterium. (G. Brasseur, P. Bruscella, V. Bonnefoy and D. Lemesle-Meunier, Biochim. Biophys. Acta 1555, 2002, 37–43). In the present work, we demonstrate both the existence of a bc1 complex functioning in the forward direction, expressed when the cells are grown on sulfur, and the presence of two terminal oxidases, a bd and a ba3 type oxidase expressed more in sulfur than in iron grown cells, besides the cytochrome aa3 that was found to be expressed only in iron grown cells. Sulfur grown cells exhibit a branching point for electron flow at the level of the quinol pool leading on the one hand to a bd type oxidase, and on the other hand to a bc1->ba3 pathway. We have also demonstrated the presence in the genome of transcriptionally active genes potentially encoding the subunits of a bo3 type oxidase. A scheme for the electron transfer chains has been established that shows the existence of multiple respiratory routes to a single electron acceptor O2. Possible reasons for these apparently redundant pathways are discussed.

Misc p-7. Use of the fluorescent dye 10-*N*-nonyl acridine orange (NAO) in quantitative and location assays of cardiolipin: a study on different experimental models

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In view of its specific requirement for the functioning of numbers of protein and enzyme complexes, assessment of CL location and concentration within a given subcellular structure is essential to understand its mechanism of action as well as the pathological consequences of its variations. In this regard, relevant is the observation that a temporal link exists between decreased mitochondrial CL, release of cyt.c and initiation of apoptosis (McMillin JB, et Al. BBA 2002;1585:97–107). In the living cell, quantitative analyses of CL have been often performed by using the fluorescent dye NAO (Petit JM, et Al. Eur J Biochem 1992;209:267–7; Garcia Fernandez M.I., et Al. Cell Growth Differ 2002;13:449–455) on the assumption of its high specificity for CL. Yet, there are still some uncertainties concerning the specificity limits of NAO–CL interaction as well as the physical–chemical factors that may quantitatively affect it. For example, there is a relevant disagreement in the literature whether the binding of NAO to the inner mitochondrial membrane depends or not upon the ?Ψ (Jacobson J, et Al. J Neurochem 2002;82:224–233). Moreover, differences may be due to variations in the arrangement of CL within the membrane space limiting the interaction with NAO. The aim of this research was to investigate effects of physical and chemical factors affecting the molecular assembly of CL molecules within the space of a membrane, on the extent of NAO–CL interaction by means of flow cytometry fluorescence variations. The experiments were performed on both artificial membrane models (multilamellar aggregates of CL and CL-containing liposomes) and on isolated mitochondria, studied in different respiratory states. In multilamellar aggregates of CL, the effect on dimerization results proportional only to the surface exposed. When CL is structured within liposomes, NAO dimerization is proportional to the concentration of CL. In isolated mitochondria, it appears that the interaction of NAO with CL is clearly related to the mitochondria respiratory state. These results show that the specific effect of CL is to provide the spatial restriction required for NAO dimerization; this effect is significantly modified by factors that control the geometry of molecular assembly of CL within the space of the membrane under investigation. This finding provides evidence to the proposal that many physiological and pathological states are associated with CL topology in the space of the membrane.

Misc p-8. Functional analyses of P13II, a mitochondrial protein of human T-cell leukemia virus type-1

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HTLV-1 encodes several accessory proteins of incompletely defined function. We recently showed that one of these proteins, named p13II, accumulates in the inner mitochondria membrane and disrupts mitochondrial morphology. Analysis of the effects of p13II on isolated mitochondria showed that a synthetic peptides spanning the active region of the protein induces changes in the permeability of mitochondria to K^+ and modulates their inner membrane potential and Ca^{2+}-retention capacity. These changes are not affected by cyclosporin A, an inhibitor of the permeability transition pore or by ruthenium red, an inhibitor of the calcium uniporter, suggesting that p13II does not act by modulating these mitochondrial channels. These latter features, along with the ion-selectivity of the permeability changes, distinguish the activities of p13II from those described for HIV-1 Vpr, another viral protein targeted to mitochondria.Analysis of the effects of p13II on cell growth revealed that it significantly reduces the incidence and growth rate of tumors in in vivo models of oncogenesis and interferes with cell proliferation in vitro. Preliminary analyses suggest that these effects might result from the ability of p13II to modulate Ca^{2+}-signaling. These findings provide novel clues into the function of p13II as a regulator of cell growth, and underscore a link between mitochondria, Ca^{2+} signaling and tumorigenicity.

Misc p-9. Photosynthetic water oxidation driven back

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Photosystem II of cyanobacteria and plants incorporates the catalytic centre of water oxidation. Powered and clocked by quanta of light the centre accumulates four oxidising equivalents before oxygen is released. The first three oxidizing equivalents are stored on the Mn_4Ca-cluster, raising its formal oxidation state from S_0 to S_3, and the third on YZ, producing S_3YZox. Only thereafter water oxidation, which implies the transfer of four electrons, proceeds in what appears as a single reaction step ($S_3YZox(H_2O)_2 \rightarrow O_2 + 4H^+ + S_0$). Intermediate oxidation products of bound water have not yet been detected. We followed two experimental strategies to track down intermediate reaction products (e.g., bound peroxide): (i) kinetically, by high time resolution of oxygen evolution, and (ii) thermodynamically, by enhanced oxygen pressure.

(i) PS-core particles were centrifuged on a kinetically competent Pt-electrode (rise time 100 μs). The half-rise times of oxygen release were 1.35 ms (20 C, *Synechocystis* WT*) and 13.1 ms (point mutant D1-D61N). The Arrhenius plots were linear between -2 and 32 C. A short lag phase of the polarographic transients(duration at 20 C: 0.45 ms, activation energy: 31 kJ/mol) was absent in the UV-transients attributable to the transition S3-> S_0 of Mn4Ca (at 360 nm). This ''intermediate'' was probably newly formed and transiently still bound oxygen and not of much interest.

(ii) We attempted to block oxygen evolution by a shift of the equilibrium between any putative intermediate and the product state ($O_2 + 4H(+) + S_0YZ$) by increasing the oxygen pressure from 0.21 (i.e., ambient) up to 30 bar. The reduction of the Mn4Ca-cluster after the third flash of light given to dark adapted PSII-cores was photometrically monitored at a wavelength of 360 nm. The extent of the negative absorption transient, which is generally attributed to the reduction of S_3 to S_0, decreased with increasing oxygen pressure. Half-suppression occurred at the rather low oxygen pressure of 2.3 bar, and the maximum was reached at 10 bar. Hydrostatic pressure alone (19 bar N_2) was without any effect. We interpreted these data in terms of a stabilized peroxide intermediate [S_2•(H_2O_2)]. From the titration-data we calculated the standard free-energy profile of the tentative reaction sequence S_3YZox•$(H_2O)_2$-> S_2YZ•(H_2O_2) (deltaG′ = 1.7 kJ/mol), and S_2YZ•(H_2O_2)-> $S_0YZ + O_2 +$ protons (deltaG′ = − 4.7 kJ/mol). This is the first experimental evidence for any intermediate.

Misc p-10. Identification and classification of the yeast transporters

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We propose a nomenclature to characterise nonambiguously all transporter proteins of *Saccharomyces cerevisiae*. This nomenclature is modified from the TC-DB (transporter classification database) created by Milton Saier (http://tcdb.ucsd.edu). Each transporter is characterised by five digits (class, subclass, family, subfamily and cluster) corresponding to functional or phylogenetic criteria. Each family is also characterised by a three letter acronym and an established name (example: The Siderophore-Iron Transporter (SIT) family: 2.A.1.16).All protein sequences were blasted against TC-DB that comprises over 400 established transporters families from bacteria, archaea and eucaryot cells. When the query yeast protein was found to have an homolog in TC-DB that exhibits high similarity as shown by an E-value below $10E-65$, the five digits TC-DB nomenclature was adopted. When the E-values was between 10e-65 and 10e-19, new fourth (Y) digit and/or fifth (Z) digit were created. Among the proteins with E-values higher than $10e-19$, only the sequences with more than two predicted transmembrane spans (TMHMM and HMMTOP) were retained. As they were not mentioned in TC-DB, we classified them in class 9.B corresponding to Putative Uncharacterised Transporters and given a third preliminary digit (9.B.X1, 9.B.X2. . .), waiting future functional characterisation.

We therefore propose a five digit characterisation of all putative or possible yeast transporter even when they are not identified in TC-DB. Our system, termed YETI, allows functional annotation of 726 proteins from *S. cerevisiae* including 34 channels/pores, 221 primary active transporters, 130 electrochemical potential-driven transporters, 80 transporters of unknown mechanisms and 261 putative uncharacterised transporters. This amounts to nearly 20% of the yeast proteins. The annotation of which will be submitted to the major yeast genome databases: SGD, MIPS and GENOLEVURE. Our YETI database is presently being used as reference for the development of a semiautomatic identification procedure that should allow nonambiguous characterisation of all transporters from the numerous yeast species recently sequenced.

Misc p-11. The antiporter-like subunits of respiratory chain complex I show ion translocation ability when expressed in *Bacillus subtilis*

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Complex I (NADH:quinone oxidoreductase) is the least understood enzyme in the respiratory chain. It catalyzes the oxidation of NADH from the Krebs cycle to NAD^+ and the reduction of quinone to quinol in the membrane. The electron transfer from NADH via flavin and several iron–sulfur clusters to quinone is coupled to proton pumping across the membrane, but the mechanism of energy coupling is not known.

Sequence comparison show that the Complex I membrane bound subunits NuoL, M and N are related to one class of Na^+- or K^+/H^+ antiporters, encoded by the gene cluster mrp/sha/pha/mnh in different bacteria. Therefore those subunits are prime candidates for harboring channels for proton translocation. Recently Na^+-transport by bacterial Complex I was demonstrated (Steuber, J., Schmid, C., Rufibach, M. and Dimroth, P. (2000) Mol Microbiol 35: 428–434), but it is currently under debate whether that activity represents a primary or secondary transport (Stolpe, S. and Friedrich, T. (2004) J. Biol. Chem. 278: 26817–26822). In either case, to regard the antiporter-like subunits as mere proton channels is probably a gross oversimplification.

Deletion of the bona fide antiporter encoding genes mrpA and mrpD from the *B. subtilis* chromosome led to distinct Na^+ and pH sensitive growth phenotypes. The ?MrpA strain could survive up to 15 mM Na^+ in the growth media whereas the ?MrpD strain stopped growing already at 5 mM Na^+. This corroborates an earlier prediction that MrpA and MrpD must have distinct functions (Mathiesen, C. and Hägerhäll, C (2002) Biochim. Biophys. acta, 1556: 121–132). In this work the NuoL, NuoM and NuoN polypeptides from *Rhodobacter capsulatus* Complex I were expressed in the deletion strains and the growth properties were analyzed. An increased Na^+ tolerance was seen in most combinations of strains and growth conditions and even reaches wild type levels in some combinations. This demonstrates that the Complex I subunits have ion translocation abilities in vivo.

Misc p-12. Quinone reduction by *Rhodothermus marinus* succinate:menaquinone oxidoreductase

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Di-heme succinate:menaquinone oxidoreductase from *Rhodothermus marinus* (Fernandes et al., 2001) was incorporated in liposomes and an effect of K^+/valinomycin-imposed membrane potential on the enzymatic activity and heme b reducibility by succinate has been studied. Neither quinone reductase activity nor redox equilibration of the low-potential heme b with the succinate/fumarate couple were influenced by the imposed membrane potential. Since the low potential heme has a pH-dependent midpoint redox potential, one possible explanation is that an electron and a proton involved in the reduction of the heme come from the same (negatively charged) side of the membrane, therefore reduction of the heme by succinate is not electrogenic. These results do not support the recently suggested hypothetical models of quinone reduction by di-heme succinate:menaquinone reductases implicating a crucial role of membrane potential in the enzymatic mechanism (Schirawski and Unden, 1998).

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Misc p-13. Role of Fps1 hydrophilic extensions and identification of residues controlling glycerol transport

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The controlled export of solutes is of fundamental importance for cells to survive and adapt to hypotonic conditions. Fps1, a glycerol facilitator from the yeast *Saccharomyces cerevisiae*, is an integral membrane protein belonging to the Major Intrinsic Protein (MIP) family. Fps1 is located in the plasma membrane where it mediates efflux of the compatible solute glycerol in the adaptation to lower osmolarity. Fps1 is an unusual MIP member due to its long hydrophilic extensions at both termini. In addition to crystallisation studies on the full length protein, structural studies on the hydrophilic domains are being carried out separately. The Fps1 N- and C-terminus are produced and purified in both *Escherichia coli* and *Pichia pastoris*. Based on the theory that the regulatory stretches dip into the membrane, we are currently investigating Fps1 variants where the hydrophilic extensions are anchored to the membrane via the closest transmembrane helix. The goal is to achieve reliable 3D models of the hydrophilic extensions, which together with the membrane anchors can reveal the putative interactions between the membrane spanning parts and the regulatory stretches.

In order to learn more about the mechanisms that control Fps1, we have set up a genetic screen for hyperactive Fps1. In this screen we have isolated mutations in 14 distinct residues, all facing the inside of the cell. Our findings provide a framework for further genetic and structural analysis to better understand the mechanism that controls Fps1 function by osmotic changes.

Misc p-14. Structural and functional analysis of the reaction center-light harvesting complex of *Rhodobacter sphaeroides*

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Dimeric photosynthetic reaction center-light harvesting complex (RC-LH1) has been purified from the photosynthetic bacterium *Rhodobacter sphaeroides* grown under semiaerobic conditions. Removal of the detergent in the presence of lipids leads to the formation of two-dimensional crystals. Analysis by cryoelectron mycroscopy at a resolution of 26 Å reveals an ''S''-shaped dimeric complex where the continuity of the LH1 ring that surrounds the RC is interrupted. The higher density of the projection map at the junction between the two monomers of core complex is attributed to a dimer of the PufX peptide (Scheuring et al., 2004, J. Biol. Chem. 279, 3620). These data confirm the structural role of PufX, a single transmembrane protein required for the photosynthetic phenotype (Farchaus et al., 1992, EMBO J., 11, 2779), responsible for the dimerization of the RC-LH1 complex (Francia et al., 1999, Biochemistry 38, 6834).The functionality of the isolated complex, purified from photosynthetically and semiaerobically grown bacteria, was analyzed by time resolved spectroscopy.

Upon excitation of the sample with an actinic laser pulse, the kinetics of charge recombination from the state P + QAQB- to the neutral state PQAQB exibhit a slow phase with an half time of approximately 4 s, at least four times larger than what usually observed in RC complex deprived of the LH1. Stoichiometric determinations of the quinone (Q10) present in the RC-LH1 indicate a Q10/RC-LH1 ratio >10. These quinones are functionally coupled to the RC-LH1 complex, as judged from the extent of cytochrome c_2 rapidly oxidized under continuous illumination. Charge recombination kinetics have been analyzed on the basis of a model proposed by Shinkarev and Wraight (Shinkarev and Wraight, 1993, in ''The photosynthetic reaction center'' Vol. 1, 193) that take into account the binding of quinone at the QB site when a quinone pool is present. The slowing down of the recombination reaction experimentally detected cannot be simply explained by a quinone concentration effect. The model predicts a lower limit of the charge recombination rate constant when the quinone concentration is raised to infinity that is well above the one measured in the RC-LH1, indicating that, even in the presence of saturating quinone conditions the recombination reaction cannot be so slow as experimentally observed. These data suggests that a stabilization of the charge separated state P + QAQB-, leading to a slower recombination reaction, is induced by the LH1 antenna complex.

Misc p-15. Characterization of *Naegleria* pathogenic and nonpathogenic strains by its DNA sequences, several marker proteins of IEF and total proteins profiling of 2D-pages

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The genus Naegleria consists of one pathogenic species, Naegleria fowleri, that cause primary amoebic meningoencephalitis (PAM) in humans and animals. Pathogenic and nonpathogenic Naegleria species must be distinguished during environmental surveys and clinical diagnosis of human cases. For construct the identification and diagnostic methods, and searching for N. fowleri, isolates from the thermal waters and thermally polluted sewage of 14 districts in Japan were investigated in terms of their ribosomal DNA by PCR-RFLP and sequence analyses, and enzyme proteins by IEF. Ribosomal internal transcribed spacers (ITS) analysis, used for the phylogenic characterization at the specific and intraspecific levels and the typing of various organisms, was also employed. N. fowleri have not been identified and ITS sequences of other clones indicated that the isolates belong to the genus Naegleria are N.lovaniensis, N.australiensis, N.italica, N.gruberi or N. spp. The patterns of two diagnostic enzymes, propionyl-esterase and acid phosphatase, after isoelectric focusing, confirmed also their specificity. N. australiensis, which was reported for the first time in Japan, showed two ITS sequences: one was already registered and the other exhibited new clone with TT-insertion(1). Its experimental nasal infection to mouse revealed that these antibodies react with specific amoebae proteins, although no mice were killed. From 2D-PAGE analyses of amoebae total proteins and N-terminal amino acid sequences, four specific proteins of N. fowleri were determined unknown. According to their amino acid sequences of about 20 residues, 11 protein spots were determined to be HSP-70, chaperone protein dnaK, three enzymes of carbohydrate metabolism, pore-forming peptides and others. A 17.7 kDa protein (pI 6.9) was identified as Mp2CL5 membrane protein from N.fowleri(2). The presence of specific actin proteins (42 kDa,pI 5.23,contents;7%) in amoeba total protein was confirmed by Western blotting using polyclonal antibody against actin of Ascaris. When monoclonal antibody of anti-N.fowleri was used for the first antibody, many protein spots labeled by DyeChrome kits indicated the presence of glycoprotein related with N.fowleri pathogenisity.

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Misc p-16. X-ray absorption studies of the local environment of zinc ions bound to the bacterial photosynthetic reaction center

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Binding of transition metal ions to the reaction center (RC) protein of the photosynthetic bacterium *Rhodobacter sphaeroides* slows the light-induced electron and proton transfer to the secondary quinone, QB (Utschig et al., 1998, Biochemistry 37, 8278; Paddock et al., 1999, Proc. Natl. Acad. USA 96, 6183). On the basis of X-ray diffraction (XRD) at 2.5 Å resolution a site has been identified at the protein surface which binds Cd(II) or Zn(II). Both metal ions binds to the same cluster formed by AspH124, HisH126 and HisH128. A water molecule was also proposed to interact with the Zn (Axelrod et al., 2000, Proc. Natl. Acad. Sci. USA 97, 1542). Recent data suggest that inhibition of proton transfer by Cd(II) is predominantly a consequence of competing with protons for binding to HisH126 and HisH128, thus hampering the function of these residues as proton donor/acceptors along the proton pathway to the QB site (Paddock et al., Biochemistry 42,9626, 2003). Determination of the local structure of the bound metal ions is expected to contribute significantly to elucidate the details of the inhibition mechanism. For this reason we performed Zn K-edge X-ray absorption measurements on Zn-doped RCs embedded into polyvinyl alcohol films at both room and liquid nitrogen temperature. Data analysis has been performed using ab-initio simulations and multiparameter fitting; structural contributions up to the fourth coordination shell and multiple scattering paths (involving three atoms) of significant amplitude have been included. Results for complexes characterized by a Zn to RC stoichiometry close to 1 indicate that Zn binds two O and two N atoms in the first coordination shell. The two N atoms come from His, and only one of the two O atoms comes from an amino acid (Asp or Glu); the second O atom belongs to a water molecule. Complexes characterized by approximately two Zn ions per RC show a second structurally distinct binding site, involving three N and one O atom, all coming from nonaromatic residues. The results obtained for the higher affinity site nicely fit the coordination proposed on the basis of XRD data. The second binding site, revealed by our investigation on noncrystalline samples, is most probably located in a more disordered domain of the RC protein and might have a hitherto not appreciated role in charge transfer inhibition.

Misc p-17. Proteoliposomes of negatively charged phospholipids trap long living charge separated state in photosynthetic reaction centers

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Reaction center extracted from the purple photosynthetic bacteria *Rhodobacter sphaeroides* R-26.1 were reconstituted in proteoliposomes formed by the anionic phospholipids Phosphatidylglycerol, Phosphatidylserine and Phosphatidylinositol and with the zwitterionic phospholipid Phosphatidylcholine. The liposomes containing the protein were prepared by size-exclusion chromatography, using the so-called Micelle-to-Vesicle Transition method, in the dark and under illumination. We report the large stabilizing effect induced by the negative charge of the anionic phospholipids on the protein charge separated state which results trapped in a long-living (up to tens of minutes) state with a yield up to 80% when proteoliposomes are formed under illumination. This state is formed regardless the presence or not of the secondary quinone electron acceptor in the QB pocket and shows identical lifetime for both recombination reactions D + QA and D + QB. Furthermore the lifetime and the yield of the long-living state are found to strongly increase when pH decreases. This effect is discussed in terms of a direct interaction between the negative charge of the phosphatidyl group on PG, PS and PI the positively charged residues HISM145 and ARGM267 sitting on the surface of protein scaffolding. We suggest that the long living state can involve a different conformation of the QA surrounding, blocking the RC in some (D + QA) state and that the protonation of residues surrounding QA can be responsible for the strong stabilization of this conformation.

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Misc p-18. A comparative study on membrane potentials of chestnut (*C. sativa mill. cv aveleira*) and spinach thylakoids

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Hitherto, we were not able to find a suitable method in literature that particular deal with the isolation of thylakoid membranes from chestnut leaves. In the course of this study we describe an isolation procedure and compare it with one for spinach thylakoid membranes. The spinach plants were grown in the same field and in close vicinity of the chestnut trees under investigation. Chestnut thylakoid extracts are less enriched in chlorophyll (0.26 vs 1.73 mg ml^{-1}) but with higher Chla/b ratio (3.7 vs 3.1), suggesting that they are more heliophilic than spinach thylakoids. As expressed by energy-dependent fluorescence quenching of ACMA, chestnut thylakoids incubated in a high salt-derived media, 200mM Sorbitol, 2mM Tricine-NaOH (pH 8.4), 150mM KCl and 4mM MgCl2 delivering 90% of reduction, which is dissipated by 168mM Nigericin (939mM Nigericin in spinach) or 25mM NH4Cl (50mM in spinach). Oxygen evolution in chestnut thylakoids was evaluated as 75% of the observed in spinach. According to the temperature studies, a transition point at 21 °C was found in chestnut (26 °C in spinach). Nevertheless, thylakoid fatty acid composition indicates an unsaturation index of chestnut (158) lower than that from spinach (234).

Misc p-19. Azidoquinone labeling of *Bacillus subtilis* succinate:quinone oxidoreductase

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X-ray crystallography, photolabeling and mutagenesis studies of succinate:quinone reductase (SQR or respiratory chain Complex II) and quinol:fumarate reductase (QFR) have shown that many of these enzymes contain two different quinone binding sites in the membrane spanning domain, one proximal (Qp) and one distal (Qd) to the flavoprotein and iron–sulfur protein subunits. The *Escherischia coli* QFR has been crystallized with quinones present at both sites whereas the structure of *E. coli* SQR revealed one quinone binding site, Qp. However, in *Bos taurus* SQR, that structurally and functionally resemble the latter enzyme, both a Qp and a Qd has been identified, using a radioactively labeled azidoquinone. Another QFR, that of Wolinella succinogenes, contained no quinones in the solved structure, although the enzyme is undisputedly a QFR. The *Bacillus subtilis* SQR enzyme contains two proposed quinone binding sites but only the presence of Qd, that interacts with the inhibitor HQNO, has been directly demonstrated. To understand the functional mechanisms of SQR and QFR it is important to understand why two quinone binding sites can be present and under which conditions they are functional.

In this study we have used two different azidoquinones (AzQ) that function as substrates for *B. subtilis* SQR, showing the same kinetic characteristics as the corresponding quinones without the azido group. The AzQs inactivated the enzyme upon long wavelength UV-light illumination, in a light and exposure time dependent fashion. The labeling was performed using the enzyme in the oxidized, succinate reduced and fully reduced state, in the presence and absence of HQNO.

The advantage of this method is that the quinone is only modified in one position (the azido group) and the labeled peptide is subsequently identified with mass spectrometry. The method is applicable to all enzymes interacting with quinone, for example Complex I. By synthesizing more quinones with the azido-group located at different positions it is also possible to map the binding site geometries in greater detail.

Misc p-20. Characterization and expression of the flagellar genes from the thermophilic PS3

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Many species of bacteria swim by means of flagellar rotation, its energy 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. P and L ring, assembled into peptidoglycan layer and outer membrane, located around the rod. Bacillus PS3 does not have an outer membrane and therefore, there are no bearing structures. 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 assembly termed 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, until now rotation mechanism is not understood, since complexity of motor proteins etc. Flagellum reconstitution and crystallization could be clarified these mechanism and functions.

In this work the characterization and expression of thermophilic bacterium B.PS3 flagellum motor gene cluster (fla/che operon >26kbp) were carried out. B.PS3 consists of thermostable protein and so, comparison of these primary structures with mesophilic bacterium is available to search not only these functions but also the factors of thermostability.

We determined 21 genes of fla/che operon. These deduced amino acid sequences correspond to switch complex (FliM,FliN,FliG), MS ring (FliF), rod (FlgB,FlgC), hook (FlgE), hook basal body (FliE), export apparatus (FliI,FliH,FlhA,FlhB,FliR), chemotaxis (CheY), and, flagellar biosynthesis protein (FliJ,FliK,FliL,FliZ,FliP,FliQ,FlhF) were compared with that of another microorganisms. High homology was observed in C-terminal of FliE. FlgC was longer about 20 residues than that of *Escherichia coli*. FliI (except for N and C terminal region) has homologous region to other bacterial ATPase F1βs.

PS3 flagellin overexpressed in *E. coli* as fused protein with maltose binding protein (MBP) was cleaved with enterokinase, but it could not completely cleave. We assumed it cause by interaction of flagellin with MBP-flagellin. We also overexpressed FliF. It has two transmembrane regions, so these fused protein expressed in insoluble fraction.

Misc p-21. Carnitine acylcarnitine carrier deficiency: novel mutations, phenotype–genotype correlation and dietetic therapy

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The carnitine/acylcarnitine carrier (CAC), which catalyzes the electroneutral exchange of cytosolic acylcarnitine for mitochondrial carnitine, allows the import of fatty acyl moieties into mitochondria, where they are oxidized by the enzymes of the b-oxidation pathway. The CAC deficiency is a life-threatening recessively inherited disorder of lipid b-oxidation which manifests in early infancy with hypoketotic hypoglycemia, cardiomyopathy, liver failure and muscle weakness (Stanley et al N. Engl.Med. 327, 19–23, 1992). We have identified seven new CAC-deficient patients from different countries who exhibited significant clinical heterogeneity (V. Iacobazzi, M. Pasquali, R. Singh. D. Matern, P. Rinaldo, C. Amat di San Filippo, F. Palmieri and N. Longo Am. J. Med. Genet 126A, 150–155, 2004; V. Iacobazzi, F. Invernizzi, S. Baratta, R. Pons, W. Chung, B. Garavaglia, C. Dionisi-Vici, A. Ribes, R. Parini, M. D. Huertas, S. Roldan, G. Lauria, F. Palmieri and F. Taroni Human mutation, in press). Two patients deceased within 6 months from onset, while the remaining five are still alive, ranging from 1.5 to 8 years old. Sequence analysis of the CAC gene (SLC25A20) disclosed six novel mutations and three previously reported mutations. Four patients were homozygous for the identified mutations. Two of the novel mutations (c.718 + 1G>C and c.843 + 4_843 + 50del) altered the donor splice site of introns 7 and 8, respectively. The 47-nt deletion in intron 8 caused either skipping of exon 8 alone or skipping of exons 6–8. Five mutations ([p.Gly54Trp; p.Thr55Ala], p.Arg133Trp, p.Asp231His, p.Ala281Val and p.Gln238Arg) resulted in amino acid substitutions affecting evolutionarily conserved regions of the protein. One of these exonic mutations (p.Ala281Val) was associated with a splicing defect characterized by skipping of exons 6–8. Combined analysis of clinical, biochemical and molecular data failed to indicate a correlation between the phenotype and the genotype. Patients were treated with a low-fat/high-carbohydrate diet, frequent feedings, medium chain triglycerides (MCT) supplementation and carnitine. After few weeks, a reduction of long-chain acylcarnitines and reversion of cardiac symptoms and hypoglycemia were observed.

Misc p-22. Ligand binding dynamics in heme-based oxygen sensor FixL studied by ultrafast spectroscopy

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Heme-based oxygen sensors are part of ligand specific two-component regulatory systems. FixL from *Bradyrhizobium japonicum* is an example of such a sensor. In this protein the binding of oxygen to the heme in the receptor domain causes changes in an associated enzymatic domain that eventually regulates transcription factors. We used femtosecond absorption spectroscopy to investigate the binding dynamics of O_2, CO and NO and the characteristics of the first signaling intermediate upon oxygen release with wild type and mutant forms of FixL. In wild-type specifically, the oxygen binding kinetics in FixL are extremely fast and efficient. Within 20 ps, 90% of oxygen is already recombined in a geminate way and only 10% of the dissociated oxygen leaves the heme pocket, showing that the heme pocket acts as an oxygen trap [1]. The heme spectrum after photolysis of oxygen is perturbed compared to the steady state oxygen-binding spectrum, which shows the residual interaction with the dissociated ligand. Arginine 220 is located in the heme pocket and is proposed to stabilize the bound ligand and participate in the signal transduction [2]. Substitution of R220 with isoleucine, glutamic acid and glutamate all had similar effects: It strongly decreased the part of fast oxygen geminate recombination pointing at an important role of this residue in oxygen trapping. Also the residual interaction with dissociated oxygen is lost as witnessed by the less perturbed dissociation spectrum. The steady-state heme conformation and ligand characteristics are similar in FixL, the oxygen sensor, and myoglobin, the oxygen storage protein. In the heme pocket of Mb a histidine can form a hydrogen bond to the ligand. We prepared the R220H mutant of FixL in which the oxygen binding kinetics became biphasic. In addition to the fast phase of oxygen binding appeared a slower, nanosecond component. The amplitude of each phase was about 50% of the total amplitude. The binding of CO also was dramatically affected. In the R220H mutant about 50% of carbon monoxide rebinds geminately with a time constant 2.4 ns, which is observed nor in WT FixL nor in Mb, where only bimolecular binding of CO occurs. The ensemble of our data indicate that R220 plays a key role in transmission of the signal within the heme domain. Preliminary MD simulations support this view.

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Misc p-23. AlPcS4-Photosensitised photodamage to cells and mitochondria

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ROS generation by photosensitisers and light underlies photodynamic therapy (PDT), a promising modality for treatment of cancer and other diseases. The efficacy of the photosensitiser depends on lipophilicity determined intracellular localisation.

Here, we studied the progressive responses of human epidermoid carcinoma cells A431 in vitro to photodynamic treatment mediated by lysosomaly localised hydrophilic photosensitiser aluminum (III) phthalocyanine tetrasulphonate (AlPcS4). Decrease in the activity of a lysosomal enzyme β-*N*-acetylglucosaminidase and the cellular ATP level was an early response to AlPcS4 photosensitisation. The initial decrease in mitochondrial membrane potential $\Delta\psi_m$ was followed by the subsequent temporal increase exceeding the potential in control cells. The increase in the activity of caspase-3, the apoptosis executing enzyme activated by cytochrome *c* following its translocation from mitochondria to cytosol, reached the maximum at 6 h postexposure. Based on these preliminary observations, it was presumed that cellular photodamage mediated by AlPcS4, which was primarily localised to the lysosomes, iniated the reactions proceeding not only in lysosomes, but also in cytosol and, supposedly, mitochondria.

Therefore, the effect of AlPcS4-mediated photodynamic treatment on isolated rat liver mitochondria was investigated. We showed that AlPcS4-PDT had increased permeability of the inner mitochondrial membrane due to lipid peroxidation. On the other hand, a damage to electron transport chain was induced. Fluorescence microscopy confirmed the leakage of AlPcS4 to cytosol, thus substantiating the possibility of lysosomaly localised photosensitiser to mediate photodamage to mitochondria.

Misc p-24. VDAC1 and the mitochondrial permeability transition

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The mitochondrial permeability transition pore (PTP) plays an important role in Ca^{2+} homeostasis, bioenergetics and cell death. However, the molecular composition of the PTP is still under investigation. In search for chemical inhibitors of the PTP, a novel compound was identified in functional assays. This compound, Ro 68-3400, blocked the PTP with high affinity (nM) apparently through covalent modification of isoform 1 of the mammalian voltage-dependent anion channel, VDAC [Cesura et al., (2003) J. Biol. Chem. 278, 49812–49818]. Surprisingly, in functional assays Ro 68-3400 had also an inhibitory effect on Ca^{2+}-induced PTP opening in mitochondria isolated from VDAC1 $-/-$ mice compared to wild-type CD1 mice. Well-known PTP-inhibitors such as cyclosporin A and ubiquinone were also able to inhibit Ca^{2+}-induced pore opening to similar degrees in both CD1 and VDAC1 $-/-$ mouse mitochondria. Western Blot analysis demonstrated that in mitochondria isolated from CD1 mice all three isoforms of VDAC were expressed. However as expected, in mitochondria isolated from VDAC1 $-/-$ mice, only VDAC isoforms 2 and 3 were expressed. Labeling of mitochondria isolated from CD1 and VDAC1 $-/-$ mice with 3H-Ro 68-3400 demonstrated that proteins of $\sim$ 32 kDa—the expected molecular weight of VDAC isoforms—were specifically labeled in both preparations. The proteins labeled in CD1 and VDAC1 $-/-$ mouse mitochondria will now be purified and identified by MALDI-MS and nano ESI-MS/MS analysis. These results suggest that in isolated mitochondria the PTP may work effectively even in the absence of a functional isoform 1 of VDAC. Yet, experiments using VDAC1 $-/-$ mice suggest that VDAC1 may nevertheless play a role in hepatic apoptosis in vivo. Experiments aimed at defining the basis for these intriguing findings are under way in our laboratories.

Misc p-25. Mitochondrial functional heterogeneity in normal and pathological cells revealed by confocal imaging

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Various mitochondrial subpopulations can be present in the cells, that may be differently involved in physiological and pathological processes demonstrating intracellular heterogeneity of mitochondrial function. Heterogeneous mitochondrial damage is suggested in various pathologies including ischemia–reperfusion injury and apoptosis. However, the origin and role of mitochondrial heterogeneity under physiological and, in particular, pathophysiological conditions remain to be elucidated.

Modern confocal and two-photon fluorescent microscopy was used for the direct imaging of mitochondrial heterogeneity. The analysis of autofluorescence of mitochondrial flavoproteins and NADH in hepatocytes and carcinoma cells demonstrated significant heterogeneity of mitochondrial redox state. Importantly, static heterogeneity of mitochondria can be distinguished from the dynamic heterogeneity where distinct time responses of mitochondria can be observed. Dynamic and static heterogeneity of mitochondrial flavoproteins were observed in experiments with intact hepatocytes. Addition of substrate dihydroxyacetone to these cells resulted in flavoproteins reduction with strong decline of flavoprotein fluorescence of almost all mitochondria. However, some small mitochondrial cluster or single mitochondrion remained at almost initial fluorescence signal, clearly demonstrating their different response to substrate addition. Confocal imaging of in situ mitochondria in the heart demonstrated clear colocalization of mitochondrial flavoproteins and NADH autofluorescence as redox indicators, and fluorescence of TMRE and Rhod-2 as specific probes for mitochondrial membrane potential and calcium, demonstrating also comparative homogeneity of these parameters. In contrast, ischemia–reperfusion resulted in a highly heterogeneous mitochondrial functional state (redox state, mitochondrial calcium content and inner membrane potential), pointing to heterogeneous mitochondrial damage and local permeability transitions, possibly due to region-specific ROS production.

Summarizing, functional heterogeneity of mitochondria was apparent in several ways, with respects to their energetic and redox status. Importantly, the extent of mitochondrial functional heterogeneity may significantly increase under pathological conditions, leading to metabolic instability and organ failure. Direct imaging of mitochondria in situ provides, therefore, new insights into the mechanisms of mitochondrial function.

Misc p-26. Electrogenic proton transfer in the photosystem II core complexes

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An electrometric technique was used to investigate the reaction related to functioning of plastoquinone acceptor(s) in the spinach photosytem II core complexes incorporated into phospholipid vesicles. Beside a rapid phase of transmembrane potential generation corresponding to charge separation between redox-active tyrosine YZ and the primary quinone acceptor QA, an additional electrogenic phase with a characteristic time of ca. 850 mks at pH 7.3 was observed after the second laser flash in the presence of decyl-plastioquinone. The magnitude o this phase was ca. 10% of that related to the YZoxQA- formation. The sensitivity of this additional phase to DCMU, the flash-number dependence of it amplitude as well as the amplitude and the rate constant pH-dependences indicate that it is due to the dismutation of QA- and QB- and to subsequent protonation of a doubly reduced plastoquinone QB2-.

The data obtained allow us to suggest that in contrast to bacterial reaction center complexes, the distance between QB binding site and membrane surface is much smaller in photosystem II complexes.

Misc p-27. Subclinical hypothyroidism in lithium treated psychiatric outpatients at reference laboratory of Iran–Tehran

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Introduction: Lithium carbonate widely used in the treatment of psychiatric disorders, is well known to associate with thyroid hypofunction.In most of the cases lithium induced hypothyroidism is subclinical(10–20%)(1.Ozpoyraz N, Tamam L, Kulan E. Thyroid abnormalities in lithum-treated patients. Adv Ther: Jul–Aug, (2002), 19(4):176–184). Furthermore, studies have reported higher incidence of thyroid antibodies in patients treated with lithium (24%) compared with those not taking lithium (12%) (2. Lazarus JH. Endocrine and metabolic effects of lithium. New York: Plenum, (1986), 99–124). In addition,it has been reported that conversion of subclinical hypothyroidism to overt hypothyroidism in the presence of circulating antibodies is high. Therefore,routine screening of patients receiving lithium has been advocated. (3. Bensenor I. Screening for thyroid disorders in asymptomatic adults from Brazilian populations. Sao Paulo Med J, Sep 2, (2002), 120(5): 146–151). Methods: A total of 46 psychiatric patients participated in the study. From each patient a questionnaire regarding a previous thyroid gland problem,time of on set, and duration of lithium treatment was filled out. Blood samples were drawn 8–10 h after oral dose/s of lithium. The Lithium level was determined using flame atomic absorption spectroscopy (4. Analytical Methods(F-AAS). (1989). Varian Australia Pty Ltd. Publication # 85-10000900). Then sera were sent to the RIA lab for the determination of TSH, (T3), and (T4) levels, as well as anti-TPO and anti-Tg. Results: eight cases (17%), displayed overt hypothyroidism. Subclinical hypothyroidism was observed in 16 cases (35%), and euthyroidism was observed in 22 case (48%). Normal TSH levels for adult are 0.5–3.0 mU/l. We carried out the pearson product–moment correlation between Li induced subclinical hypothyroidism and lithium concentration, duration of Li use, age,and sex. The significant differences between two factors were patient‘s age ($n = 16$, $r = 0.71$, $P < 0.01$) and duration of lithium use ($n = 16$, $r = 0.76$, $P < 0.001$). Sex as well as Li concentration were not significant ($P > 0.05$). Thyroid antibodies were present in 27% of euthyroid group and 31% cases of subclinical group. Conclusion: Our data indicated that 16 (35%) asymptomatic patients displayed subclinical hypothyroidism after starting Li treatment. Moreover,duration of lithium use and age ($X = 50$) regardless of sex are important risk factors in monitoring these patients. TSH appears to be efficient than positive antibody presence.

Misc p-28. Light-induced pH changes in reaction centres solution monitored by piranine

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The photosynthetic reaction center (RC) of purple bacteria is an integral membrane enzyme that, upon light absorption and in the presence of exogenous electron donors, undergoes a photocycle resulting in the double reduction and double protonation of the loosely bound lypophilic electron carrier ubiquinone-10. The light-generated ubiquinol leaves the RC and reaches a second redox enzyme (i.e., ubiquinol:cytochrome oxido-reductase) where is oxidised and releases the protons, generating the transmembrane proton-gradient required for the metabolic activities of the cell. In isolated RCs, the photocycle can be restored in the presence of an external electron donor (i.e., cytochrome *c* or ferrocene) and a pool of quinone molecules that exchange with the quinone bound to the QB-binding site of the protein.

Being UQ10 a highly hydrophobic molecule, upon illumination protons will be taken up from the solution to form the quinol, ending up sequestrated into the micellar phase. In solution with low buffer capacity the increase of the pH accompanying the photocycle can be easily followed by pyranine, a pH-sensitive fluorescent dye. The catalytic reaction has been studied by using three different secondary electron donors: ferrocene, cytochrome *c* and cytochrome c_2.

A quantitative agreement between the moles of quinones added to the solution and the moles of proton uptaken up to [UQ10] 40 M with the electron donors tested was observed. The useful range of pH for this techniques spans from 6.8 to 9 for pyranine. The investigation has also been preliminarily performed in the case of reaction center reconstituted in proteoliposomes of phosphatidyl choline, concentrating on the use of cytochromes as electron donors.

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Misc p-29. Cytochrome *c* exhibits pool behavior in mitochondria of a cardiolipin-lacking mutant of *Saccharomyces cerevisiae*

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Using blue native polyacrylamide gel electrophoresis (BN-PAGE) for the study of *Saccharomyces cerevisiae* mutants lacking CL (cardiolipin), we previously demonstrated that CL plays a specific role in supporting the association of complexes III (bc1 complex) and IV (cytochrome *c* oxidase) into a supercomplex in the inner mitochondrial membrane (Zhang et al. (2002) J. Biol. Chem. 277: 43553–43556). In the current study we compared dependence of kinetics of NADH oxidation via complexes III, IV and cytochrome *c* on concentration of the complex III specific inhibitor antimycin in CL-containing (wild type) and CL-lacking (mutant) mitochondria. Under physiological conditions, a linear relationship between respiratory activity and antimycin concentration was observed for wild type mitochondria consistent with a single functional unit model of the electron transport chain of *S. cerevisiae* (Boumans et al. (1989) J. Biol. Chem. 273: 4872–4877). Under the same conditions, CL-lacking mitochondria displayed a hyperbolic relationship indicating that cytochrome *c* interacts with complexes III and IV as a freely diffusible carrier. Identical cytochrome *c* pool behavior kinetics was observed for both wild type and CL-lacking mitochondria in the presence of chaotropic agents which disrupt the interaction between the respiratory complexes. Mild detergent extracts of wild type and CL-lacking mitochondria subjected to BN- and colorless native-PAGE in the presence of 0.01% digitonin retained supercomplex formation in the former case but displayed individual homodimers of complex III and complex IV in the latter case. Our data indicate destabilization of the interaction of complexes III and IV and cytochrome *c* in the absence of CL in vivo.

Misc p-30. NAO as a probe for cardiolipin in mitochondria: study of changes in fluorescence emission spectra

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Nonyl acridine orange (NAO) is a fluorescent molecule displaying high affinity for the mitochondrial anionic phospholipid cardiolipin (CL). Binding of the dye to CL was reported to cause a shift in fluorescence emission from green to red. In this work, change in the emission spectra of NAO induced by the interaction with liposomes of different lipid composition was investigated. Free NAO in aqueous medium was titrated with CL liposomes. Quenching of green fluorescence after addition of liposomes was detected for 0.5–60 μM NAO; red fluorescence was noticeable only for values of NAO concentration higher than 2 μM and for a ratio NAO:CL of 2:1. In the case of the CL analogue, deoxycardiolipin (dCL), higher concentrations of NAO were necessary to detect the red fluorescence, but still in the ratio of NAO:dCL of 2:1. Under the same conditions addition of phosphatidylglycerol (PG) liposomes induced quenching of NAO green fluorescence that was not accompanied by a red shift. We suggest that NAO binding to PG involves interaction with the negatively charged headgroup of the lipid but does not result in the stacking of NAO. In the case of CL and dCL, stacking of aromatic rings of NAO results from the hydrophobic interaction of nonyl residues of at least two NAO molecules with the four acyl chains of the CL molecule (Mileykovskaya et al. 2001, FEBS Lett. 507, 187). Mixing of NAO with the cationic phospholipid phosphatidylcholine (PC) resulted in little green fluorescence quenching, while emission in the red spectral region was not detectable. The data are consistent with the low affinity of NAO for PC. Titration of 4 μM NAO with CL/PC mixed liposomes (2 μM CL and a variable PC percentage), did not show any red fluorescence up to ratio of CL:PC equal to 1:1, suggesting that the red fluorescence is specific for NAO binding to CL-patches rather than to individual CL molecules. 10 μM NAO was incubated with mitochondria isolated from wild type, crd1 mutant (lacking CL), and pgs1 mutant (lacking CL and PG) of *Saccharomyces cerevisiae*. At the ratio of NAO to mitochondrial CL of 2:1, the red emission intensity was related to the amount of CL; the same results were achieved with mitochondria enriched in CL by fusion with CL liposomes. Treatment of wild type mitochondria with ADP, malate, pyruvate and phosphate resulted in a noticeable increase in the NAO red fluorescence. We suggest that under these conditions rearrangement of CL-patches in the membrane might take place.

Misc p-31. Effect of proapoptotic proteins on VDAC channels

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During apoptosis, cytochrome *c* is released from mitochondria into the cytosol where it participates in caspase activation. Various and often conflicting mechanisms have been proposed to account for the increased permeability of the mitochondrial outer membrane that is responsible for this process. The voltage-dependent channel, VDAC, is the major permeability pathway for metabolites in the mitochondrial outer membrane and, therefore, is a very attractive candidate for cytochrome *c* translocation. Our experiments suggest that Bax does not form a new large pore with VDAC. Moreover, a detailed analysis of the characteristic properties of VDAC channels such as voltage gating, ion selectivity, and single channel conductance, did not show any change after Bax addition regardless of lipid composition, medium pH, or ionic content. We conclude that there is no functionally detectable interaction between VDAC channels, isolated from mammalian mitochondria, and either monomeric or oligomeric forms of Bax (from two separate labs, R.Y. and B.A.). This is consistent with the failure to detect Bax-VDAC interaction by immunoprecipitation or cross-linking (Mikhailov et al., 2001) and with the insensitivity of the effect of Bax, expressed in yeast cells, to the knock-out of VDAC (Gross et al., 2000). We conclude that Bax does not induce cytochrome *c* release by acting on VDAC.

In contrast to Bax, another proapoptotic protein, Bid, proteolytically cleaved with caspase 8, affects the voltage gating of VDAC. We have shown that cut Bid induces closure of VDAC channels both on single and multichannel membranes in a dose-dependent manner (Rostovtseva et al., 2004). Our data support a model where Bcl-2 family proteins control MOM permeabilization by regulating the opening or closure of VDAC channel (Vander Heiden et al., 2001). By reducing the permeability of VDAC channels, the proapoptotic protein Bid might interfere with metabolite exchange between mitochondria and the cytosol, with the subsequent loss of outer membrane integrity.

Misc p-32. Modelling the interaction and electron transfer in the complex between type I and type II cytochromes c_3

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Protein electron transfer is a very important phenomenon that in many cases requires the formation of a complex between the redox proteins. Analysis of redox protein complexes is generally very difficult through experimental techniques given their transient occurrence. Here (Teixeira, VH, Baptista, AM, Soares, CM, Biophys.J., in press) we take advantage of theoretical computational methodologies to analyse the complex formed by these two interacting redox proteins, type I and type II cytochromes c_3, from the periplasm of sulphate reducing bacteria, where they interact in the molecular hydrogen metabolism.

Given that no X-ray structure was available, comparative modelling techniques were used to build the three-dimensional structure of the type II cytochrome c_3. Various possible complexes for the molecular interaction between type I and type II cytochromes c_3 were generated by rigid body docking methodologies, followed by molecular dynamics simulations in explicit solvent to optimize them. The relative stability of the different complexes was evaluated by a hybrid method that combines molecular mechanics and continuum solvent methods (MM-PBSA). The thermodynamics of reduction and protonation were studied by continuum electrostatic techniques and Monte Carlo methods developed in our lab accounting for the joint binding equilibrium of protons and electrons.The results evidence interesting interaction solutions that help to explain the electron transfer process between these two cytochromes c_3. Thermodynamic analysis shows that complex formation induces redox potential shifts in both cytochromes. These redox potential shifts are larger on type I cytochrome c_3. The largest redox potential change upon complex formation is observed in haem IV from type I cytochrome c_3, showing approximately 80 mV. Further analysis of the redox potentials in both redox proteins before and after complex formation shows possible mechanisms in the control of electron transfer processes at the molecular level.

Misc p-33. Oxygen-dependent interrelations between photosynthetic and respiratory electron transport chains in *Synechocystis* SP. PCC 6803

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Cyanobacterium *Synechocystis* sp. PCC 6803 represents an appropriate model for the study of oxygen-dependent interrelations between photosynthetic and respiratory electron transport chains. In order to elucidate the mechanisms of regulation of electron transport in intact cells, we studied the influence of preillumination conditions and oxygen concentration on the light-induced redox transients of the PS 1 primary donor P700, respiration, and generation of a proton gradient across the thylakoid membranes. Redox transients of P700 were monitored by the EPR method. Kinetics of oxygen consumption/production was measured with oxygen sensitive spin probes. The generation of the transmembrane proton gradient was determined pH-sensitive spin probes. To discriminate between the factors controlling the rates of electron flow in photosynthetic and respiratory electron transport chains, we compared the behaviour of P700 and oxygen exchange processes in wild type cells and different mutants (a gift from Dr.W.Vermaas) with impaired PS 1, PS 2, NDH-2 (ndbABC), SDH, and terminal oxidases (Cta1, CydAB, CtaDEII). Cells were placed either into a gas permeable plastic tube (aerated cells) or into a quartz cuvette where the concentration of oxygen was modulated in darkness or during illumination of cells. It has been shown that the rates of electron flow through both chains, photosynthetic and respiratory chains, are under the strong control of the membrane potential and oxygen concentration in a cell suspension. Oxygen deprivation due to terminal oxidases after sufficiently long adaptation of cell to the dark caused the retardation of electron efflux from P700, whereas electron donation from plastoquinol to $P700^+$ (via the cytochrome *b*/*f*-complex and cytochrome c_6 or/and plastocyanin) was supported at high level due to overreduction of plastoquinone pool. In addition to the linear photosynthetic electron transport, cyanobacteria were capable of maintaining cyclic electron transfer around PS 1 and respiratory chain which involved the NDH-1 complex. We conclude that photosynthetic electron transport in cyanobacteria is controlled by two main mechanisms: (i) oxygen-dependent acceleration of electron transfer from PS 1 to $NADP^+$, and (ii) retardation of electron flow between PS 2 and PS 1 governed by the transmembrane pH difference across the thylakoid membrane.This work was supported by grants from RFBR (03-04-48981, 03-04-49332), INTAS (99-1086, 01-483), and ISTC (2296).

Misc p-34. Protein/lipid interaction in bacterial photosynthetic reaction center: the role of phosphatidylcholine and phosphatidylglycerol in charge stabilization

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The role of characteristic phospholipids of native membranes, phosphatidylcholine, PC, phosphatidylglycerol, PG, and cardiolipin, CL, was studied in the energetics of the acceptor quinone side in photosynthetic reaction centers of *Rhodobacter sphaeroides*. The rates of the first, kAB(1), and the second, kAB(2), electron transfer and that of the charge recombination, kBP, the free energy levels of QA−QB and QAQB− states, and the changes of charge compensating protein relaxation were determined in RCs incorporated into artificial lipid bilayer membranes. In RCs embedded in PC vesicle, kAB(1) and kAB(2) increased (3125−4132 and 740 s^{-1} to 3300 s^{-1}, respectively), kBP decreased (0.77−0.39 s^{-1}) compared to that measured in detergent at pH 7. In PG, kAB(1) and kBP decreased (to values of 710 s^{-1}, and 0.26 s^{-1} respectively), while kAB(2) increased to 1506 s^{-1} at pH 7. The free energy between the QA−QB and QAQB− states decreased in PC and PG (deltaG0(QA-QB-> QAQB-)= −76.9 and −88.5 meV, respectively) compared to that of measured in detergent (−61.8 meV). The changes of the QA/QA- redoxpotential measured by delayed luminescence showed: (1) differential effect of lipids whether incorporated in micelles or vesicules; (2) altered binding interaction between anionic lipids and RC; (3) direct influence of PC and PG on the free energy levels of the primary and secondary quinones probably through the intraprotein hydrogen bonding network; (4) larger increase of the QA/QA− free energy in PG than in PC both in detergent micelles or in single component vesicles. Based on recent structural data, implications of the binding properties of phospholipids to RC and possible interactions between lipids and electron transfer components will be discussed.

Misc p-35. Influence of a transmembrane pH gradient on protonation probabilities of bacteriorhodopsin

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Bacteriorhodopsin pumps protons across a membrane using the energy of light. The proton pumping is inhibited when the transmembrane proton gradient that the protein generates becomes larger than 4 pH units. This phenomenon is known as the back-pressure effect. In this paper, we investigate the structural basis of this effectby predicting the influence of a transmembrane pH gradient on the titration behavior of bacteriorhodopsin. For this purpose we introduce a method that accounts for a pH gradient in protonation probability calculations. The method considers that in a transmembrane protein, which is exposed to two different aqueous phases, each titratable residue is accessible for protons from one side of the membrane depending on its hydrogen-bond pattern. This method is applied to several ground-state structures of bacteriorhodopsin, which residues already present complicated titration behaviors in the absence of a proton gradient. Our calculations show that a pH gradient across the membrane influences in a nontrivial manner the protonation probabilities of six titratable residueswhich are known to participate in the proton transfer: D85, D96, D115, E194, E204, and the Schiff base. The residues connected to one side of the membrane are influenced by the pH on the other side because of their long-range electrostaticinteractions within the protein. In particular, D115 senses the pH at the cytoplasmic side of the membrane and transmits this information to D85 and the Schiff base. We propose that the strong electrostatic interactions found between D85, D115, and the Schiff base as well as the interplay of their respective protonation states under the influence of a transmembrane pH gradient are responsible for the back-pressure effect on bacteriorhodopsin.

Misc p-36. Electrons compete to enter the respiratory chain: physiological consequences

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In the yeast Saccharomyces cerevisiae, the two most important systems or conveying excess cytosolic NADH to the mitochondrial respiratory chain are external NADH dehydrogenases (Nde1p and Nde2p) and the glycerol3phosphate dehydrogenase shuttle. In this last system, NADH is oxidised to NAD+ and dihydroxyacetone phospate is reduced to glycerol 3 phospate by the glycerol-3-phospate dehydrogenase (Gut2p). Both Nde1p/Nde2p and Gut2p are located in the inner mitochondrial membrane with catalytic sites facing the intermembranal space.

We previously showed that the activation of NADH dehydrogenases inhibited the Gut2p in such a manner that, at a saturating concentration of NADH, glycerol 3-phospate is not used as respiratory substrate. This effect was not a consequence of a direct action of NADH on Gut2p activity because both NADH dehydrogenase and its substrate were needed for Gut2p inhibition. This is not due to an inhibition of Gut2p by Nde1p/Nde2p. Studies on the functionally isolated enzyme showed that neither Nde1p nor Nde2p directly inhibit Gut2p. Thus, this could be due to a competition for the entrance of the electrons into the respiratory chain. Studies realized with singke deletion mutants of either NADH dehydrogenase showed that glycerol-3-phosphate oxidation via Gut2p is fully inhibited when NADH is oxidized via Nde1p whereas only 50% of glycerol oxidation is inhibited when Nde2p is functioning. by comparing respiratory rates with distinct respiratory substrates, we show that (1) electrons from Nde1p/Nde2p are favored from electrons coming from Ndip (internal NADH dehydrogenase) (2) when electrons are coming from both Nde1p/Nde2p and succinodehydrogenase, their use by the respiratory chain is shared up to a comparable extend. This points out top a very specific competition for electrons into the respiratory chain which may be due to the supramolecular organization of the respiratory chain.

This regulation might have a psysiological function in situ. Indeed, since yeast has both internal and external NADH dehydrogenases it has always be assumed that redox potentials in both compartments (cytosol and mitochondria) where similar. However in this study, we show a full priority of electrons coming from external NADH dehydrogenase which enable the cell to establish distinct redox in mitochondria versus cytosol.