View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by UniCA Eprints

Rendiconti Seminario Facoltà Scienze Università Cagliari Vol. 73 Fasc. 1 (2003)

COMUNICAZIONE SULL'ATTIVITÀ SVOLTA DA ALCUNI RICERCATORI DELLA FACOLTÀ

XVI CONGRESSO SOCIETÀ ITALIANA DI BIOCHIMICA SEZIONE SARDEGNA

ORGANYZED BY

GIOVANNI FLORIS, ROSARIA MEDDA, ALESSANDRA PADIGLIA, SILVIA LONGU

PURIFICATION AND FUNCTIONAL CHARACTERIZATION OF THE HEMOCYANIN FROM *SCYLLARIDES LATUS*

BARBARA MANCONI[1], SILVIA PUXEDDU[1], BARBARA SALIS[1], MARIAGIUSEPPINA PELLEGRINI[1], SUSANNA SALVADORI[2], ALESSANDRA OLIANAS[1]

[1] Dipartimento di Scienze Applicate ai Biosistemi, Università di Cagliari
[2] Dipartimento di Biologia Animale ed Ecologia, Università di Cagliari

The hemocyanin of *Scyllarides latus* is present in the hemolymph by an hexameric aggregation state, as deduced by its molecular mass (440 kDa), calculated by a Superose-6 gel chromatography. Purification of Hc has been performed by an ultracentrifugation and a DEAE-Sepharose CL6B chromatography.

The effect of pH on the oxygen binding properties was measured in the pH range 7.0-8.4 and a low O_2 affinity and a little pH effect was observed. Addition of L-lactate at physiological hemolymph concentrations did not affect the oxygen binding parameters; but addition of 0.5 mM urate, which corresponds to the physiological hemolymph concentration, caused a large increase of O_2 affinity. The Bohr coefficient was very small both in the absence and presence of urate, and cooperativity of O_2 binding was almost constant in both these conditions (n_{50} = 3.0 ± 0.5). The large effect of urate on haemocyanin oxygen-affinity was further studied by a titration curve, and the quantitative value of this effect, expressed as $\Delta \log P_{50}/\Delta \log[\text{urate}]$, was in agreement with those reported in the case of other Crustacean Hcs. The effect of temperature on the oxygen affinity in the absence and presence of urate was also measured and the ΔH values are in accordance with the exothermic contribution of the urate binding to the oxygenated Hc.

STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF THE PHOSPHATE-BINDING SITE OF THE CATHODIC HEMOGLOBIN FROM ANGUILLIFORMES

Daniela Masia[1], Luigi Sollai[1], Laura Serra[1], Maria Teresa Sanna[1],
Anna Maria Deiana[2], Bruno Giardina[3]

[1]Dipartimento di Scienze Applicate ai Biosistemi, Università di Cagliari
[2]Dipartimento di Biologia Animale ed Ecologia, Università di Cagliari
[3]Istituto di Biochimica e Biochimica Clinica, Università Cattolica del Sacro Cuore, Roma

The hemoglobin system of Anguilliformes have two types of Hb which differ markedly in their structural and functional properties. The cathodic Hb components show high intrinsic O_2 affinities and lack wide normal Bohr and Root effects.

Our study revealed that saturating amounts of GTP drastically reduced the O_2 affinity of both *C. conger* and *A. anguilla* cathodic Hb ($\Delta \log P_{50} = 1.25$ at pH 7.0), increased the cooperativity and converted the reverse Bohr effect into a small normal Bohr effect. A further investigation of the GTP effect on oxygen affinity, carried out by fitting the titration curve of both *C. conger* and *A. anguilla* cathodic Hb, evidenced the presence of two independent binding sites. The computer modelling study suggests that *C. conger* cathodic Hb may bind organic phosphates at two distinct binding sites located along the central cavity of the tetramer by hydrogen bonds and/or electrostatic interactions with amino acid residues of both chains which have been identified; among these residues, the two Lys-α(G6) seem to have a key role in the GTP movement from the external binding region to the internal central cavity of the tetrameric molecule.

EXPERIMENTAL EVIDENCES OF AN ADDITIONAL PHOSPHATE-BINDING SITE IN THE CATHODIC HEMOGLOBIN OF *CONGER CONGER*

Luigi Sollai[1], Daniela Masia[1], Barbara Salis[1], Alessandra Olianas[1], Maria Teresa Sanna[1],
Anna Maria Deiana[2], Susanna Salvadori[2], Mariagiuseppina Pellegrini[1]

[1]Dipartimento di Scienze Applicate ai Biosistemi, Università di Cagliari
[2]Dipartimento di Biologia Animale ed Ecologia, Università di Cagliari

The 20% of the hemolysate of *C. conger* consisted of a cathodic Hb which showed a reverse Bohr effect, high O_2 affinity and slightly low cooperativity in the absence of any effector. The addition of chloride ions only produced a small decrease in O_2 affinity, while

saturating amounts of GTP drastically reduced the O_2 affinity, increased the cooperativity and converted the reverse Bohr effect into a small normal Bohr effect.

The titration curve obtained with increasing concentration of GTP evidenced the presence of two independent binding sites. Since there is a very close similarity between the titration curves of *C. conger* cathodic Hb and those of *C. maccormicki* Hbs, and also between the amino acid residues, which have been suggested to form the additional binding site in the case of both *C. maccormicki* Hbs, and those present at the same positions in *C. conger* cathodic Hb, we suppose that the computer modelling study, which is in course, should confirm the presence of an additional binding site also in the case of *C. conger* cathodic Hb. It might have a low-affinity binding role, which would enhance the ability of Hb to capture phosphate from the solution and readily transfer it to the main binding site by means of a site-site migratory mechanism, thus acting as an entry-leaving site.

COMPARATIVE STUDY ON FUNCTIONAL AND STRUCTURAL PROPERTIES OF THE BARBARY SHEEP AND EUROPEAN MOUFLON HAEMOGLOBINS

D. Murtas[1], A. Fais[1], B. Era[1], El Said El Sherbini[2], El Sayed GR.[2] and M. Corda[1]

[1]Department of Sciences Applied to Biosystems, University of Cagliari (Italy)
[2]Faculty of Veterinary Medicine, Mansoura University

The Barbary sheep (*Ammotragus lervia*) is widely distributed in North Africa and classified as being closely related to either the genus Ovis or to the genus Capra, since it is known from previous studies that genus Ammotragus has its b-globin chains structurally similar to those of genus Ovis and the α-globin chains to the genus Capra.

In order to give a phylogenetic position to the species *Ammotragus lervia*, we started our studies by comparing its functional and structural properties to another species' one: *Ovis musimon*.

Electrophoretic analyses show two different haemoglobins, one of which is present in a higher rate than the other, and the presence of only a β- globin chain and two α-globin chains named $^{I}\alpha$ and $^{II}\alpha$.

The oxygen-binding properties of the Barbary sheep haemoglobin have been investigated in the pH range 6.5-8.0 both in the absence and the presence of chloride anions and 2,3-DPG. The Barbary sheep haemoglobin has a lower oxygen affinity than that of Mouflon Hb B phenotype. Both Barbary sheep and Mouflon are modulated, in a different way, by Cl^- and 5 mM DPG.

These results indicate that, in physiological conditions, the chloride anion is the main allosteric modulator of oxygen affinity.

On the contrary the different functional response to 5 mM 2,3-DPG could be explained by a molecular adaptation to particular environmental conditions, in which an increase of 2,3-DPG biosynthesis could occur and facilitate the oxygen unloading.

HEMOGLOBIN BELFAST (β15, Trp → Arg) DEFINITION OF THE CLINICAL AND HEMATOLOGICAL PHENOTYPE

B. Puddu[1], A. Fais[1], L. Perseu[2], S. Barella[3], S. Satta[3,4], R.Galanello[3,4]

[1] Dipartimento di Scienze Applicate ai Biosistemi, Università degli Studi di Cagliari
[2] Istituto Neurogenetica e Neurofarmacologia CNR
[3] Ospedale Regionale Microcitemie A.S.L. 8 Cagliari
[4] Dipartimento di Scienze Biomediche e Biotecnologie, Università degli Studi di Cagliari

We report the third occurrence of hemoglobin Belfast, a mild unstable b-chain variant, in a large family with 9 subjects affected. DNA analysis showed a T → A mutation at codon 15 of the β globin gene, predicting a Trp → Arg aminoacid substitution. The clinical phenotype is silent or very mild the only clinical finding being an intermittent moderate jaundice.

Hemoglobin (Hb) Belfast (β15, Trp → Arg) is a hemoglobin described for the first time in a Irish patient and subsequently in a French patient. In both families only a single patient was reported and the associated severe polycistic kidney disease and metastatic carcinoma, do not allow to have exact information on the clinical and hematological phenotype of this variant.

Functional studies indicate that the oxygen affinity of the hemolysate from adult carriers of Hb Belfast is identical to that of normal hemolysate, whereas it is higher of about 30% than that of total hemolysate in the purified Hb Belfast at all experimental conditions examined.

These data are different to those described in a previous papers which describe a small increase in oxygen affinity both for hemolysate and purified Hb Belfast.

XENON BINDING SITES ON HORSE AND PIG METMYOGLOBINS INVESTIGATION BY ^{129}XE NMR SPECTROSCOPY

R. Melis[1], M. Corda[1], B. Era[1], A.Schirru[1], C. Piras[1], M. Casu[2]

[1] Dipartimento Scienze Applicate ai Biosistemi, Università di Cagliari
[2] Dipartimento di Scienze Chimiche, Università di Cagliari

In this study, the interaction of xenon with horse and pig metmyoglobin in aqueous solution is investigated by ^{129}Xe NMR spectroscopy. Horse and pig myoglobin differ by

15 aminoacids. Among these residues only one (H19) is at internal position in heme contact. Chemical shifts of both proteins (1mM) are measured as a function of xenon concentration. In these systems, xenon is in fast exchange between all possible environments.

The experimental data for horse and pig metmyoglobin were interpreted using a thermodynamic model which supposes that xenon forms a 1:1 complex with the protein and exchanges between a cavity in the proteins and all the other environments. At low xenon concentration (1 atm) horse metmyoglobin shows an upfield shift for the xenon resonance relative to the xenon resonance in the solvent, while in pig metmyoglobin a downfield shift is observed. As the xenon concentration increases, the xenon resonance shifts downfield in horse and upfield in pig metmyoglobin. The two proteins are characterized by ^{129}Xe spin lattice relaxation time (0.3 s in horse and 0.7 s in pig metmyoglobin) much shorter than that measured in water (~ 500 s). These observed differences in the direction and magnitude of the paramagnetic shift and in the magnitude of the spin lattice relaxation time can be attributed to differences in the relative orientation and proximity of Xenon to the unpaired electron in these proteins.

FUNCTIONAL STUDIES REVEAL THAT CYTOSOLIC 5'-NUCLEOTIDASE ACTIVE SITE HAS A STRUCTURE SIMILAR TO THAT OF HALOACID DEHALOGENASE SUPERFAMILY

Allegrini S.*, Careddu M.G.*, Cuccu G.**, Pesi R.**, Tozzi M.G.**

*Dipartimento Scienze del Farmaco, Sassari
**Dipartimento Fisiologia e Biochimica, Pisa

Cytosolic 5'-nucleotidase is an ubiquitous mononucleotide phosphatase, acting also as a phosphotransferase. Although its physiological role is not yet fully understood, it is generally believed that the enzyme participates with other six 5'-nucleotidases in the nucleotide pool regulation. In particular, cytosolic 5'-nucleotidase seems to play a major role in regulating intracellular IMP concentration. This enzyme is also involved in the metabolism of purine prodrugs. 5'-Nucleotidase hyperactivity has been related to paediatric neurological syndromes, including Lesch-Nyhan syndrome. 5'-Nucleotidase acts through the formation of a phosphoenzyme intermediate. The phosphorylation occurs on Asp52, which belongs to a conserved motif – DXDX(T/V) – shared by enzymes of the wide haloacid dehalogenase superfamily, including also some phosphatase/ phosphotranspherase enzymes. This motif is present in at least four other 5'-nucleotidases. Sequence alignments of 5'-nucleotidase with other members of haloacid dehalogenase superfamily, as well as comparison of the active site structure of two other members of the family, phosphoserine phosphatase and mitochondrial deoxyribonucleotidase, allowed us to identify five residues as possibly involved in the cytosolic 5'-nucleotidase active

site. Both conservative and non-conservative point mutants of these amino acids were prepared. Changes in the kinetic parameters of 5'-nucleotidase caused by these substitutions are in good agreement with those described for other enzymes of this family, suggesting a similar role in the catalytic mechanism.

CLONING AND EXPRESSION IN *ESCHERICHIA COLI* OF ADENOSINE PHOSPHORYLASE FROM *BACILLUS CEREUS*

LUCIANO FRASSETTO, PAOLA DESSANTI, FRANCESCO SGARRELLA

Dipartimento di Scienze del Farmaco, Università di Sassari

Adenosine phosphorylase catalyses the reversible phosphorolytic cleavage of (deoxy)adenosine to adenine and (deoxy)ribose-1-phosphate. In *Bacillus cereus* adenosine phosphorylase appears to be involved in the mechanism of induction of enzymes catabolizing exogenous nucleosides. In order to clone the adenosine phosphorylase gene, integral chromosomal DNA purified from *B. cereus* was used as a template for PCR. The PCR product was purified from 1% agarose gel, digested with NdeI and EcoRI and inserted into pET5b plasmid. The recombinant plasmid was cloned in *Escherichia coli* DH5α, purified and used to transform *E. coli* BL21 (DE3 lysogen) in order to express adenosine phosphorylase. Transformants were grown in the presence of 50 μg/ml ampicillin and recombinant enzyme induced by 1mM isopropyl-β-D-thiogalacto-pyranoside. Adenosine phosphorylase was purified by ammonium sulphate fractionation, gel filtration on Sephacryl S300 HR and affinity chromatography on adenosine-agarose. The enzyme efficiently cleaves adenine nucleosides, while it does not catalyse phosphorolysis of hypoxanthine or guanine nucleosides. A molecular weight of 29 kDa was determined by 16% SDS-PAGE, a value close to that calculated from the amino acid sequence (25,657 Da). Moreover, a molecular weight of 180 kDa was determined by gel filtration on Sephacryl S-300 HR, indicating a hexameric structure for the native enzyme. Therefore, adenosine phosphorylase may be considered as a member of the hexameric subfamily of type I nucleoside phosphorylases.

DEGRADATION OF JUGLONE BY *PLEUROTUS SAJOR CAJU*

NICOLETTA CURRELI, ANTONIO RESCIGNO, BRUNA PISU, FRANCESCA SOLLAI, AUGUSTO RINALDI and ENRICO SANJUST

Cattedra di Chimica Biologica, Università di Cagliari

Pleurotus sajor caju is able to degrade and decolorise a wide range of toxic compounds such as plant polyphenols, naphthoquinones and anthraquinones. Many of

these compounds are substrates for none of the degradative enzymes produced by *Pleurotus*, but they can be oxidised by low-molecular-weight species which are reaction products of laccase, Mn-dependent-peroxidase and aryl-alcohol-oxidase. One of the involved mechanisms of juglone degradation is the induction and excretion of aryl-alcohol-oxidase (AAO), which oxidises an aryl alcohol to the correspondent aldehyde, concomitantly reducing O_2 to H_2O_2, the actual oxidising species. The aldehyde produced by AAO is constantly recycled by a membrane bound NAD(P)H-dependent-reductase.

It seemed interesting to verify whether the reductase (as it reduces compounds ranging from aryl acids to quinones) was able to react with juglone. Infact reduced juglone (namely 1,4,5-trihydroxy-naphthalene, THN) is unstable even in acidic medium, and readily auto-oxidises to give juglone, polymerisation compounds and ROS. THN-mediated ROS production by the mycelium was demonstrated by using peroxidase and syringaldazine; the recovery of juglone after the cycle of reduction-reoxidation was about 1%. In conclusion *Pleurotus sajor caju* performs another mechanism for ROS production other than AAO. This mycelium bound system is constitutive and obviously faster in comparison of the inductive mechanism of production of AAO. Further work is needed to assess the relative importance of the two different ROS producing systems.

MOLECULAR ANALYSIS AND PRIMARY STRUCTURE OF CALMODULIN GENE FROM *EUPHORBIA CHARACIAS*

ANNA MURA, ANTONELLA TRAVAGLIONE, SIMONE SATTA, PAOLO BOTTA, GIORGIO PISTIS and ALESSANDRA PADIGLIA

Department of Applied Sciences in Biosystems, University of Cagliari (Italy)

Calmodulin is a protein highly conserved across all lines of eukaryotic phylogeny. In plant cells calmodulin seems to be the primary sensor for the regulation of intracellular free Ca^{++} levels, and the combination of Ca^{++} with calmodulin leads to the activation of number of target proteins initiating the physiological response. Full-length cDNA encoding *Euphorbia characias* calmodulin has been cloned using polymerase chain reaction (PCR) with primers synthesized using consensus degenerate hybrid oligonucleotide primer (CODEHOP) design strategy, combined with a BLAST analysis of a large number of plant calmodulin genes. The primers, corresponding to the conserved Ca^{++}-binding domains EF-hand I and EF-hand IV, were used in a RT-PCR experiments. A band of 360 bp was amplified and sequenced after Southern blot analysis. Using 5'/3' rapid amplification of cDNA ends (RACE) strategy with specific primers, a full length cDNA was obtained. A single open reading frame of 447 bp encodes a protein of 148 amino acid with a molecular mass of 16.9 KDa and a predicted pI of 4.05. The protein contains four conserved calcium-binding domains. Alignments of *Euphorbia* calmodulin

nucleotide sequence with other higher-plant calmodulins reveal a 80-95% homology. An interesting relationship between *Euphorbia* calmodulin and *Euphorbia* peroxidase, an enzyme that shows a catalytic efficiency enhanced by three orders of magnitude in the presence of calcium ions, may be proposed.

INFLUENCE OF DIVALENT CATIONS ON *EUPHORBIA CHARACIAS* PEROXIDASE

SILVIA LONGU, ALESSANDRA PADIGLIA, GIOVANNI FLORIS, MARTINA CABIDDU, ROSARIA MEDDA

Department of Applied Sciences in Biosystems, University of Cagliari, Cagliari (Italy)

Peroxidases (E.C. 1.11.1.7, donor: hydrogen peroxide oxidoreductase) are widely distributed and have been isolated from many higher plants, animal tissues, yeasts and microorganisms. We previously purified a peroxidase from the latex of *Euphorbia characias* (ELP) and some of its physicochemical characteristics have been reported. Interesting the specific activity of this enzyme is very slow comparing with that reported for other peroxidases. Based on this fact, we decide to investigate the effect of divalent cations on the activity of ELP.

When native ELP was incubated in the presence of Ca^{2+} ions, an activation was observed with a notable increase of k_c and a decrease of K_m for hydrogen peroxide. The k_c/K_m value of 18900 was calculated with an increase of 485 folds on respect to the native enzyme. The binding of Ca^{2+} ions is strongly pH-dependent and after dialysis or filtration through a G-25 column chromatography the activating effect was lost. Other divalent cations such as Sr^{2+} and Ba^{2+} gave rise to minor activation of the enzyme (20 and 10 times respectively), whereas Mg^{2+} and Mn^{2+} had no effect on ELP activity.

These results may be indicative that the free enzyme and the enzyme-Ca^{2+} complex (E-M) have different affinities for the substrate. Thus, in the presence of Ca^{2+} ions the *Euphorbia* peroxidase is completely converted into the active form E-M.

STUDY OF THE *EUPHORBIA CHARACIAS* PEROXIDASE ACTIVATION BY CA^{2+} IONS

PAOLA MACCIONI, SILVIA LONGU, MARTINA CABIDDU, ANNA ONNIS, SILVIA PINNA, ANTONELLA CONTINI, ROSARIA MEDDA

Department of Applied Sciences in Biosystems, University of Cagliari, Cagliari (Italy)

The superfamily of heme peroxidases is a group of enzymes that utilize hydrogen

peroxide to oxidize a second reducing substrate. We have previously isolated and characterized a peroxidase from the latex of *Euphorbia characias* and we have shown that the catalytic efficiency of the enzyme was enhanced in the presence of calcium ions. A possible explanation of the mechanism of activation is reported after investigation by a wide range of spectroscopic and analytic techniques including formation of the cyanide derivative, stopped–flow determinations, laser photolysis experiments, intrinsic fluorescence, CD and EPR spectroscopy.

The results indicate that the combination of enzyme with substrate (or cyanide) and with calcium are not independent so that the enzyme activity is strictly regulated by the presence of Ca^{2+} ions whose main effect is to favour the oxidation of the ferric enzyme by H_2O_2 to form a Compound I, whereas the other steps of the catalytic cycle seem to be affected to a lesser extent. Only minor changes in the secondary or tertiary structure of the protein are detected by fluorescence or CD measurements in the presence of Ca^{2+} ions, except for a significant perturbation of the Fe^{3+} inner sphere geometry, as detected by EPR measurements.

THERMODYNAMICS OF METAL ION-PEPTIDE INTERACTION: A CAPILLARYELECTROPHORETIC APPROACH

Tiziana Cabras[1], Andrea Mastinu[1], Alberto Vitali[2], Raffaele Petruzzelli[3], Diana Valeria Rossetti[4], Cecilia Zuppi[4], Maria Benedetta Fadda[1]

[1]Dipartimento Scienze Applicate Biosistemi, Università Cagliari
[2]Istituto Chimica Riconoscimento Molecolare, CNR
[3]Dipartimento Bioscienze Mediche, Università Chieti
[4]Istituto Biochimica Biochimica Clinica, Università Cattolica, Roma

Metal ion binding is often essential to exploit peptide biological activity. Many examples revealed the existence of specific peptide binding motives for each metal ion of biological interest. Capillary electrophoresis (CE) may offer a significant contribute to investigate thermodynamics of peptide-ion interaction. Ion binding usually generates modifications of peptide structure and charge, that reflect in measurable modifications of electrophoretic mobility as a function of ligand activity. Easy modification of background electrophoretic solution, connected to fast analysis time and minimal sample requirement are distinctive advantages of CE. Thus, CE was utilized to investigate binding of Zn(II) to bacitracin A_1 (Bac). Measurement of Bac mobility at different pH values allowed determining the five protonic dissociation constants and the Stokes radii of the different Bac protonated forms. Provided this knowledge, measurement of Bac mobility at several fixed pH and increasing Zn(II) concentration allowed determining the association constants of Bac for Zn(II), the Stokes radii and the acidic constants of Bac-metal ion

complex. Experiments in water-trifluoroethanol solutions, that mimic membrane apolar environment, evidenced that under this conditions bacitracin assumes a conformation with higher affinity toward Zn(II).

MIUR and «Ricerca Educazione Sanitaria» founds are acknowledged.

POST-TRANSALATIONAL MODIFICATIONS OF SEVERAL RELEVANTHUMAN SALIVARY PROTEINS

Massimo Castagnola[a,b], Giuseppina Onnis[c], Rosanna Inzitari[a], Vincenzo Piras[D], Gloria Denotti[d], Alessandro Lupi[b], Irene Messana[c]

[a]Istituto Biochimica Biochimica Clinica, Università Cattolica
[b]Istituto Chimica Riconoscimento Molecolare, CNR, Roma
[c]Dipartimento Scienze Applicate ai Biosistemi,
[d]Dipartimento di Odontostomatologia, Università di Cagliari, Cagliari.

Protein composition of human saliva is very complex. Some salivary proteins are of plasmatic origin, like albumin and immunoglobulin, but many others are peculiar of the oral cavity. They include histatins, statherin, proline-rich proteins (PRP) and salivary cystatins, which range from peptides of few amino acids to high-dimension proteins, with different acidic properties. Protein concentration varies largely due to physiological rhythms and stimuli, age and habits. Moreover, saliva is characterized by a great qualitative variability due to different genetic patterns and to many post-translational modification products. Comprehension of the specific role exerted by each protein is thus a huge task. The availability of apparatus that take profit from the connection between the high resolution power of liquid chromatography (HPLC) and the analytical potential of electrospray-ion trap mass spectrometry (ESI-MS) represents an improvement to reach this aim. By this technique we determined the percentage of different phosphorylated cystatin S, new phosphorylation patterns, several dimers not yet characterized and modified forms of acidic PRP. A correlation between these modifications and the healthy status of the donor might give relevant information to detect new markers of pathologies either systemic or pertaining to oral cavity.

MIUR and «Ricerca Educazione Sanitaria» founds are acknowledged.

ANALYSIS OF THE CYSTATIN COMPLEX BY COUPLING HPLC TO ION TRAP MASS SPECTROMETRY

I. Podda[1], M. Castagnola[2,4], I. Messana[1], G. Denotti[3], A. Lupi[2]
A. Vitali[2], V. Piras[3], T. Cabras[1], M.B. Fadda[1]

[1]Dip. Scienze Applicate Biosistemi
[2] Ist. Chimica Riconoscimento Molecolare, CNR
[3] Dip. Scienze Odontostomatologiche, Univ. Cagliari
[4] Ist. Biochimica Biochimica Clinica, Univ. Cattolica, Roma.

Human salivary cystatins belong to family 2 of cysteine protease inhibitors. Five major cystatins, cystatin S, S1, S2, SA, SN, and two minors, cystatin C and D, were already detected in human saliva. Results of the analysis of human saliva by coupling HPLC with an ion-trap electrospray mass spectrometer are here reported. Acidic extract of saliva from healthy subjects was analyzed by a Vydac C8 column (150 x 2,1 mm, 5 μm) and analytes were revealed by an ESIT-LCQ Thermo Finnigan apparatus. Cystatins were eluted in the order D, SN, S, (S1, S2), SA. All the known salivary cystatins were identified, with the exception of cystatin C. However, a peak corresponding to a mass probably pertaining to a modified form of cystatin C was detected. Several peaks were tentatively attributed to peptides originated by oxidative modifications of cystatins on the basis of mass values. These derivatives are probably connected to donor habits, such as smoke and feeding. On the basis of the present results, we can conclude that RP-HPLC ESIT-MS coupling is a reliable and sensitive method for the separation and characterization of cystatins.

MIUR and «Ricerca Educazione Sanitaria» founds are acknowledged.

DETECTION OF PROLIN-RICH PROTEINS, HISTATINS AND STATERIN BY HPLC COUPLED TO ION TRAP MASS SPECTROMETRY

T. Cabras[1], I. Podda[1], M.Castagnola[2,4], M.B. Fadda[1]
A. Lupi[2], G.Gambarini[3], M. Cordaro[3], I. Messana[1]

[1]Dip. Scienze Applicate Biosistemi, Univ. Cagliari
[2] Istituto Chimica Riconoscimento Molecolare, CNR
[3]Clinica Odontoiatrica, «La Sapienza»,Roma
[4] Ist. Biochimica Biochimica Clinica, Univ. Cattolica

Human saliva peptides and small proteins, like statherin, histatins and acidic and basic prolin-rich proteins (PRP), were investigated in this study. Whole saliva was treated with 0.2% aqueous trifluoroacetic acid (1:1,v/v), precipitate was discharged and acidic

solution submitted to RP-HPLC-ion trap mass spectrometry, equipped with electrospray injection. HPLC separations were performed either with Hypersil BDS-C18 or with Vydac C8 columns. Mass spectrometer was a Thermoquest MDQ apparatus. The three major histatins (His 5, His 3, His 1) were detected, whereas concentration of the minors (H-2, H-4, H-6-12) was probably under method detection limit. Quite all the known basic PRP were detected, except IB-6 peptide, that is known to produce two peptides by proteolytic fragmentation during secretion. Only one of the two fragments, peptide PH, was detected. Masses pertaining to acidic PRP were revealed, together with other masses probably attributable to post-translational modifications of PRPs: phosphorylation, N-terminus pyroglutamic acid formation and glycosylation. HPLC peak of statherin was well resolved and easily detected. In the HPLC profile many other peaks, with masses not corresponding to any known salivary peptide, were detected. Thus, many salivary proteins are still pending for identification.

MIUR and «Ricerca Educazione Sanitaria» founds are acknowledged.

ANTIOXIDANT PROPERTIES OF DIFFERENT ROASTED COFFEE ON LDL

V. Pasciu#, F. Lumbau*, C. Carru*, M.C. Nicoli§, F. Gambella°, G. Pes*, L. Deiana*

#Department of Biomedical Sciences, Division of Biochemistry
*Chair of Clinical Biochemistry
°Di.S.A.A.B.A, University of Sassari
§ Department of Food Sciences, University of Udine

Beverages containing natural (polyphenols) or heat-induced antioxidant have been reported to possess cardiovascular health benefits. In the present study we have compared the antioxidant activities of coffee having different roasting degree, light, medium and dark (CTN 110, 85 60), using the *in vitro* low density lipoprotein model. Addition of coffee increased LDL resistance to *in vitro* oxidation. Three coffee under test showed different antioxidant activity; in particular the increase in coffee roasted level from CTN 110 to CTN 60, reduced coffee antioxidant activity.

Coffee antioxidant activities were also evaluated by measuring its ability to reduce DPPH°. Light roasted coffee, having an EC_{50} value lower than the other coffee, had antiradical ability greater than the other roasting degree ones.

The ability of the three differently roasted coffee to reduce copper ion was also evaluated. The amount of differently roasted coffee that completely reduce the same copper concentration (250 μM) were: 45, 75, 80 μg/ml for light, medium and dark roasted

coffee respectively. Our observed results suggest that the major anti-oxidant power of light roasting coffee (CTN 110), found in our *in vitro* system of LDL oxidation, may be associated to one greater ability to scavenging radicals and reducing copper ions.

APOPTOSIS AND CARDIAC DIFFERENTIATION IN GT-R1 ES CELLS

Marina Fadda, Bruna Tadolini, Francesco Usai, Carlo Ventura

Department of Biomedical Sciences, section of Biochemistry, Sassari.

Early stages in cardiogenesis can be studied in vitro by using pluripotent embryonic stem (ES) cells, which have a developmental potential similar to murine blastocyst pluripotent cells and are able to spontaneously differentiate into all three primary layers when allowed to form embryoid bodies.

The aim of this study is to assess whether apoptosis and particularly caspase-8 signaling may have a role during the cardiac differentiation GT-R1 ES cells.

The current experimental data show that GT-R1 cells have a spontaneous high apoptosis level, increasing along the differentiation process. Caspase-8 mRNA is constantly overexpressed throughout each day of differentiation, but the corresponding protein is hardly detectable through classical procedures such as western blotting.

As a result of cell treatment with a specific caspase-8 inhibitor, a significant decrement of mRNA levels of a number of cardiac-specific genes has been found. The MTT test for cell viability has reinforced such a role of caspase-8 inhibition in reducing GT-R1 cell orientation towards the cardiogenic lineage. Preliminary data suggest a possible re-orientation of non-differentiated pluripotent cells into different specialized cells, i.e. towards the neuronal lineage, and a strong linkage between caspase-8 and many PKC isoforms. The last results are very promising, since they are consistent with our recently acquired data (Ventura C. et al. (2003) Circ. Res. 92 (6), 617-622), showing a fundamental role of PKC signaling in GT-R1 cardiac differentiation.

EFFECTS OF RESVERATROL, A NATURAL POLYPHENOL, ON THE PROLIFERATION OF HUMAN ENDOTHELIAL CELLS

Anna Maria Posadino, Gianfranco Pintus, Ilania Ruiu, Annalisa Cossu, Gian Gavino Pinna

Department of Biomedical Sciences, Biochemistry, University of Sassari

The resveratrol is a natural polyphenol mainly located in the grapevine (Arichi H. et

al. (1982) Chem Pharm Bull 30: 1766-70). This molecule has interesting pharmacological actions on human health. In fact recent studies show resveratrol anticancer proprieties (Jang M et al (1997) Science, 275: 218-220) and a number of cardioprotective effects, among which an increase in high-density lipoprotein (HDL) cholesterol (Gaziano JM et al (1993) New Engl. J. Med 329, 1829-1834), and the inhibition of low-density lipoprotein (LDL) oxidation (Frankel EN et al (1993) The Lancet 341, 1103-4).

The purpose of our work is the analysis of resveratrol effects on the growth and the proliferation of human endothelial cells extracted from the umbilical vein cord, since its effects on not transformed cells are still unclear.

Our results show that resveratrol produces a biphasic effect on cell proliferation, because low concentrations increase cellular growth, whereas high concentrations induce the opposite effect. Such results have been evalueted by measuring cellular viability through MTT test, and by measuring DNA synthesis and fragmentation through radioisotopic techniques. The analysis of resveratrol high concentrations effects, would suggest an apoptosis process activation: such results have been confirmed by RT-PCR of some genes having a key role in programmed cell death regulation (Bax and Bcl-2).

CHARACTERIZATION OF A HUMAN HYBRID -GAγT- GLOBIN GENE

PIRASTRU M., DESOLE G., MULTINEDDU C., MANCA L.

Dipartimento di Scienze Fisiologiche, Biochimiche e Cellulari – Università di Sassari

In humans at birth, fetal hemoglobin is a mixture of tetramers containing approximately 70% of Gγ and 30% of Aγ chains. Though, Gγ or Aγ chains may be present in abnormal amounts, suggesting the presence of anomalous γ-globin gene arrangements. These γ-globin gene arrangements may be due to different crossing-over events between the Gγ-Aγ- normal arrangements and have been so far identified by means of quantitation of the Gγ/ Aγ ratio combined with the gene mapping analyses. Low Gγ globin chain values (35 to 45%) have been observed in 0.3% of Sardinian newborn.

In the present study a newborn, heterozygous for the mutated AγT chain, displayed an abnormal Gγ/Aγ ratio with decreased Gγ value (42.6%) and a high AγT level (37.6%), providing evidence for the existence of a γ-globin gene anomaly.

DNA sequencing revealed the presence of a hybrid GAγT-gene. This *locus* has been completely sequenced as well as the normal Gγ-and AγT-genes located on the other chromosome. The hybrid gene resulted from an unequal crossover between Gγ and AγT gene. Its encoded protein is structurally identical to the AγT chain, though produced at a rate characteristic for the Gγ chain.

Similarities in sequence of the Gγ and Aγ *loci* have made it impossible to determine the 5' and 3' endpoints of the deletion.

This work was supported by grants from: Fondazione Banco di Sardegna, Regione Autonoma della Sardegna, target project «Programma Operativo di Interventi in Materia di Prevenzione ed Educazione Sanitaria», and MIUR-PRIN 2002.

THE POLYMORPHISM OF α-GLOBIN GENES IN TWO CAPRINE SPECIES

SPEZIGA S.M.[1], PALICI DI SUNI M.[1], MEREU P.[1], MASALA B.

[1]Dipartimento di Scienze Fisiologiche, Biochimiche e Cellulari
Centro Interdisciplinare per lo Sviluppo della Ricerca Biotecnologica e per lo Studio della Biodiversità della Sardegna e dell'Area Mediterranea
Università di Sassari

The production of structurally different α- and β-globin chains is responsible for the hemoglobin polymorphism detected in ruminants. Concerning caprines, heterogeneity at the level of α-chains has been observed in sheep, goat and aoudad (*Ammotragus lervia*), whereas nothing is described in the case of mouflon (*Ovis gmelini musimon*).

As in most of mammals, the α-globin *locus* is duplicated in caprines and are designed as the $^{I}\alpha$ and $^{II}\alpha$. In sheep and goat different alleles are present at the $^{I}\alpha$ and $^{II}\alpha$ *loci*. None of the genes of aoudad and mouflon have been characterized at the level of nucleotide sequence.

Here, nucleotide sequencing was performed in order to compare the α-genes of sheep and goat to those of the corresponding *loci* of wild aoudad and mouflon. Result shows that in mouflon the two α *loci* encode a polypeptide that is identical to that in sheep. Data at coding regions level shows substitutions mostly resulting in synonymous codons. With respect to the goat $^{I}\alpha$ chain, the aoudad and the mouflon/sheep $^{I}\alpha$ globins differ by one residue: 74, Asp or Glu, and 104 Ser or Thr, respectively, while aoudad and mouflon/sheep $^{I}\alpha$ globins differ for two residues: 74, Glu or Asp and 104 Ser or Thr. The $^{II}\alpha$ chains of goat and in aoudad differ by one residue: 74, Asp or Glu, respectively. Four or five residues are different when the structure of mouflon/sheep $^{II}\alpha$ chain is compared with the corresponding protein sequences of goat and aoudad, respectively.

This study was supported by: RAS (L.R. 19/1996 «Cooperazione allo Sviluppo»), and Structural Funds by EU (PIC INTERREG III and Cofinanziamento Dottorati di Ricerca).

EFFECTS OF CRYOPRESERVATION ON GLYCOSAMINOGLYCAN CONTENT OF PORCINE AORTIC VALVE

CRISTINA TEDDE, ANGELA MARIA LEONE, MIRIAM FARINA, GIAN MARIO CHERCHI, *LUCA DAINESE, *GIANLUCA POLVANI, MARILENA FORMATO

Dipartimento Scienze Fisiologiche, Biochimiche e Cellulari, Università di Sassari
*Cattedra di Cardiochirurgia, Centro Cardiologico «F. Monzino» IRCCS, Università di Milano

Mechanical properties of extracellular matrix are critically important for allograft long term durability. The effect of cryopreservation on glycosaminoglycan (GAG) content and distribution was studied on cryopreserved aortic leaflet in relation to its position (closed or open).

Sixteen aortic porcine valves were cryopreserved in closed and open position. GAGs were evaluated from aortic wall, commissural zone and leaflets. The methodology used for isolation and characterization of GAGs includes delipidation, proteolytic treatment, anion-exchange chromatography, ethanol precipitation, acetate cellulose electrophoresis and capillary electrophoresis of GAG enzymatic depolimerization products. The three areas were significantly different in their total GAG content and composition. The qualitative analysis of GAGs showed very high relative percentages of hyaluronan and undersulphated chondroitinsulphate in valve tissue. The differences in GAG composition between leaflet areas and aortic wall regard not only the relative levels of each type of GAG, but also, as in the case of CS, their sulphation degree, suggesting peculiar biochemical pathways for some of these extracellular components in valve selected areas. Moreover, valve cryopreservation in closed position produces a significant generalized loss of GAGs at level of commissure. On the basis of our data, the leaflet position during cryopreservation appears to be a critical determinant in valve commissure GAG maintenance.

SULFATED POLYSACCHARIDE CONTENT AND DISTRIBUTION IN SOME MARINE SPONGE SPECIES

ANGELA MARIA LEONE, MARILENA FORMATO, §RENATA MANCONI, CRISTINA TEDDE PIERINA DEMURO, °ROBERTO PRONZATO, GIAN MARIO CHERCHI

Dipartimento Scienze Fisiologiche, Biochimiche e Cellulari,
§ Dipartimento Zoologia ed Antropologia Biologica, Università di Sassari
°Dipartimento Studio Territorio e sue Risorse, Università di Genova

The structural features and biological role of glycosaminoglycans (GAGs) in sponge extracellular matrix (ECM) are still unknown and their potentialities as taxonomical diagnostic traits of the phylum Porifera remain a matter of debate.

Therefore, we studied the distribution of sponge ECM sulfated glycans in relation to their morpho-functional roles. To this extent we investigated GAG composition in selected areas of sponge body, such as digitations in *Aplysina* spp., «cortex» and «medulla» in *Tethya aurantium*, and basal plate in *Chondrilla nucula*.

GAGs were extracted from specimens preserved in absolute ethanol. After exhaustive proteolytic treatment, free GAGs were purified by ion-exchange chromatography, quantified and analyzed by discontinuous acetate cellulose electrophoresis.

Total GAG content in the extracellular matrix of different species showed a wide variability. Moreover, sponge GAGs do not fit the standards from vertebrates and are not suitable substrata for specific eliminases. These findings strongly suggest a lower sulfation degree associated with a different sugar composition, substitution and sequential arrangement compared to mammalian GAGs. Some taxa displayed peculiar GAG patterns, however they failed to be diagnostic for a taxonomic discrimination. Finally, the morpho-functional analysis performed in specialized areas suggests a key role for GAGs in modulating morphogenetic processes typical of sponges.