2. METALLOPROTEINS

KEYNOTE LECTURES

KL08
Electrocatalytic hydrogen cycling by hydrogenases in the presence of O2: functional challenges for technology
Fraser Armstrong1, Kylie A. Vincent1, James A. Cracknell1, Annemarie Wait1, Gabrielle Goldet1, Baeidel Friedrich1, Oliver Lenz2, Marcus Ludwig3, 1University of Oxford, Department of Chemistry, Oxford, United Kingdom; 2Institut für Biologie/Mikrobiologie, Humboldt-Universität zu Berlin, Germany; 3Michigan State University, Biochemistry and Molecular Biology, East Lansing, MI, USA; 2Michigan State University, Chemistry, East Lansing, MI, USA; 3Institut für Biologie/Mikrobiologie, Humboldt-Universität zu Berlin, Germany.

Hydrogenases are usually inactivated under oxidizing conditions [1]. Yet many aerobic microbes obtain energy using [NiFe]-hydrogenases that can oxidize traces of H2 in air [2,3]. Enzymes from Ralstonia have such high selectivity for H2 against O2 that they can be used as electrocatalysts in the simplest of fuel cells - lacking a membrane or even just an anode and cathode separated by an electrolyte film [4,5]. Using a ‘blue’ Cu oxidase, laccase as cathode catalyst, sufficient power is produced from 3% H2 in air to power a wristwatch - not a high demand, but a visible and demanding test of a hydrogenase’s ability to function in air. This lecture addresses how these hydrogenases function in low levels of H2.

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KL09
Fe(II)/2-oxoglutarate-dependent hydroxylases
Robert P. Hausinger1, Piotr K. Grzyzka1, Meng Li2, Jana M. Simmons1, Tina A. Müller1, 1Michigan State University, Microbiology and Molecular Genetics, East Lansing, MI, USA; 2Michigan State University, Chemistry, East Lansing, MI, USA; 3Michigan State University, Biochemistry and Molecular Biology, East Lansing, MI, USA.

2-oxoglutarate-dependent hydroxylases are mononuclear non-heme Fe(II) enzymes that couple the oxidative decarboxylation of an oxoacid to the transformation of a primary substrate. The archetype representative of this enzyme family is TauD, an Escherichia coli enzyme that converts taurine (2-aminooxosulfonate) to sulfite and aminoacetaldehyde. Recent spectroscopic findings related to TauD and its variants will be presented and corresponding insights into the enzyme mechanism will be described. To illustrate the versatility of this group of catalysts, three representative enzymes from eucaayotic sources will be discussed. Aspergillus nidulans contains XaA which was demonstrated to be a xanthine hydroxylase; this enzyme utilizes 2-oxoglutarate-dependent chemistry rather than the better known molybdopterin system for producing uric acid. Trypanosoma brucei, the protozoan responsible for African sleeping sickness, is known to modify specific thymine bases in its DNA to create the J base; evidence is presented related to the potential use of a 2-oxoglutarate-dependent thymine hydroxylase catalyzing this activity. Finally, humans possess eight homologues to AlkB, an enzyme that repairs alklation-damaged DNA; the properties of two of these enzymes (hABH1 and hABH5) are described.

KL10
Towards the Mechanism of N2 Reduction by Nitrogenase
Brian M. Hoffman, Northwestern University, Evanston, IL, USA.

Contact e-mail: bmh@northwestern.edu

A major obstacle to understanding the reduction of N2 to NH3 by nitrogenase has been the impossibility of synchronizing electron delivery to the MoFe protein so that intermediates along the N2 reduction pathway can be accumulated for characterization. Recently, however, in collaboration with the groups of Dennis Dean and Lance Seefeldt, a number of intermediates have been trapped by freeze-quenching, and ENDOR spectroscopy has proven to be the method of choice for characterizing substrate-derived species bound to FeMo-co of trapped enzymatic intermediates. This presentation will describe current understanding of the structure of trapped intermediates and of the mechanistic sequence of hydrogen addition to N2.

Even when an intermediate is trapped, the process of electron delivery requires that this occur without synchronous electron delivery, and as a result the number of electrons (and protons), n, that has been accumulated during its formation is unknown. Consequently, the intermediate is untethered from Lowe-Thornely (LT) kinetic schemes for reduction, which are indexed by n. We have shown that a trapped intermediate itself provides a ‘synchronously prepared’ initial state whose relaxation to the resting state under conditions that prevent electron delivery to the MoFe protein can be analyzed to disclose n and the nature of its relaxation reactions. We will describe the relaxation protocol and outline current understanding of the connections of trapped nitrogenous intermediates to the LT kinetic scheme for N2 reduction, as well as the mechanisms by which intermediates relax.

KL11
Copper Prion Interactions. Specificity from mammalians to Fishes
Henryk Kozlowski1, Anna Janicka1, Pawel Stańczak2, Daniela Valensin3, Gianni Valensin3, 1Faculty of Chemistry, University of Wroclaw, Wroclaw, Poland; 2Department of Chemistry, University of Siena, Siena, Italy.

Contact e-mail: henrykkoz@wchuwr.pl

Prion diseases are fatal neurodegenerative disorders including spongiform encephalopathies in cattle and sheep and Creutzfeldt-Jacob syndrome in humans. Although biological functioning of prion protein (PrP) is still unknown it seems to be generally accepted that it may play a critical role in copper homeostasis and copper based enzymatic activity.

Mammalian protein possesses two specific binding sites in the unstructured domain: i) octarepeat region and ii) neurotoxic peptide fragment. In all cases histidine residues play a basic role in the metal protein interactions. Avian proteins contain the hexapeptide repeats having also one His residue critical for Cu(II) binding. Surprisingly the fish proteins e.g. that of Japanese pufferfish may be even more potent Cu(II) binder than those of mammalians and avian. In the latter case the repeat domain is very different than those in human or chicken, but containing multi-histidine region is very effective in the interactions with copper.

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To date, acetylene is the only hydrocarbon known to be metabolized in the absence and presence of molecular oxygen. The novel W,Fe-S enzyme acetylene hydratase (AH) from the strictly anaerobic bacterium \textit{P. acetylenicus} stands out from its class in that it catalyzes a non-redox reaction, the addition of water to the C=C triple bond to form acetaldehyde. However, Ti(III)-citrate or dithionite, is required for activity. AH belongs to the dimethylsulfoxide reductase family, and it contains a bis-MGD-ligated W and a [4Fe:4S] cluster, with a redox potential of $-410 \pm 20$ mV [1]. The 3D structure (1.26 Å) reveals a water molecule at the W site that gets activated by an aspartate to attack acetylene bound in a hydrophobic pocket. A strong shift in $pK_a$ of the aspartate residue is required, caused by the [4Fe:4S] cluster. To access this novel W site, AH evolved a substrate channel distant from where it is found in related Mo and W enzymes [2]. \textit{P. acetylenicus} can also insert Mo (but not V) into the bis-MGD cofactor of AH, but the specific activity of the Mo isoenzyme was significantly lower.

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**KL13**

**Discriminating Heavy Metal Binding to De Novo designed peptides using spectroscopy and x-ray crystallography**

Vincent L. Pecoraro1,2, Olga Iranzo1, Debra Touw1, Christopher Cabello1, Kyung-Hoon Lee3, Saumen Chakravorty1,1
1University of Michigan, Chemistry, Ann Arbor, MI, USA; 2University of Michigan, Biophysics Research Division, Ann Arbor, MI, USA.

Contact e-mail: vlpec@umich.edu

The ability to control the coordination properties of metals (number of ligands, geometry, etc.) in proteins is an essential feature for the design of metalloenzymes. We will discuss the factors that allow us to differentiate the relative binding affinities of the same metal for different sites. We will discuss the factors that allow us to differentiate the relative binding affinities of the same metal for different sites. We will also discuss the factors that allow us to differentiate the relative binding affinities of the same metal for different sites.

Using the designed α-helical 3-stranded coiled coil peptides known as the TRI peptides (sequence G-(LKALEEK)$_n$G in which one or two of the leucines (L) is substituted by cysteine (C)) we will describe the protein chemistry of toxic heavy metals such as Pb(II), As(III), Hg(II) and Cd(II) with thiolate rich protein sequences. We will show that Cd(II) can be controlled to bind as either a three or four coordinate structure in aqueous solution and we will show that individual peptides can be made that contain two 4-coordinate, two 3-coordinate or a mixed 3 and 4 coordinate peptides. We will also discuss selective metal binding where Cd(II) can be forced to one site in this protein to the exclusion of a second identical binding site and when simultaneously challenged with both Pb(II) and Cd(II), one can selectively sequester metals into binding sites that again appear identical. This selectivity will be discussed using crystallographic studies of an As(III) peptide.

**KL14**

**The molybdenum cofactor: Biosynthesis, function and deficiency**

Guenter Schwarz, Institute of Biochemistry at the University of Cologne, Cologne, Germany.

Contact e-mail: gschwarz@uni-koeln.de

The molybdenum cofactor (Moco) forms the active site of all molybdenum enzymes, except nitorgenase. Molybdenum enzymes catalyze important redox reactions in global metabolic cycles. Moco consists of molybdenum covalently bound to one or two dithiolates attached to a unique tricyclic pterin moiety commonly referred to as molybdopterin (MPT). Moco is synthesized by an ancient and conserved biosynthetic pathway that can be divided into four major steps, according to the biosynthetic intermediates precursor Z (cyclic pyranopterin monophosphate), MPT, and adenylylated MPT. Before molybdenum is inserted, a Cu(I) atom is bound via the MPT dithiolene sulfurs. In a fifth step modifications such as attachment of nucleotides, sulfuration, or bond formation between Molybdenum and the protein result in different catalytic centers formed by Moco. Upon completion of cofactor biosynthesis Moco can be bound to a carrier protein. A defect in any of the steps of Moco biosynthesis results in the pleitropic loss of all molybdenum enzyme activities, causing the death of the organism. Human Moco deficiency is a hereditary metabolic disorder characterized by severe neurodegeneration resulting in early childhood death. Latest developments in the field of bacterial and eukaryotic Moco biosynthesis will be presented. Finally, novel insights into the biosynthesis of related tungsten-containing pterin cofactors will be discussed.

**KL15**

**Methyl-coenzyme M reductase and the anaerobic oxidation of methane**

Rudolf K. Thauer, Max-Planck-Institut für terrestrische Mikrobiologie, Marburg/Lahn, Germany.

Contact e-mail: thauer@mpi-marburg.mpg.de

Methane has long been known to be used as carbon and energy source by some aerobic alpha- and delta-proteobacteria. In these organisms the metabolism of methane starts with its oxidation with O2 to methanol, a reaction catalyzed by a monoxygenase and therefore restricted to the aerobic world. Methane has recently been shown to also fuel the growth of anaerobic microorganisms. The oxidation of methane with sulfate and with nitrate have been reported, but the mechanisms of anaerobic methane oxidation still remains elusive. Sulfate-dependent methane oxidation is catalyzed by methanotrophic archaea, which are related to the Methanosarcinales and which grow in close association with sulfate.
reducing delta-proteobacteria. There is evidence that anaerobic methane oxidation with sulfate proceeds at least in part via reversed methanogenesis involving the nickel enzyme methyl-coenzyme M reductase for methane activation.

Krüger, M., Meyerdierks, A., GlocKner, F. O., Amann, R., Widder, F., Kubé, M., Reinhard, R., Kahnt, J., Boecher, R., Thauer, R. K., & Shima, S. (2003) A conspicuous nickel protein in microbial mats that oxidize methane anaerobically. Nature 426, 878-881.

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SESSION LECTURES

KL16
Zinc and copper in the brain: Unraveling the role of metallothionein-3
Milan Vašák, Department of Biochemistry, University of Zürich, Zürich, Switzerland.
Contact e-mail: mvasak@bioc.unizh.ch

Zinc and copper homeostasis plays a crucial role in brain physiology and in neurodegenerative diseases. Metallothionein-3 (Zn-MT-3) is mainly expressed in the brain and was found downregulated in Alzheimer’s disease (AD). The protein occurs intra- and extracellularly in comparable amounts. Intracellular Zn-MT-3 is expressed in glutamatergic neurons that release zinc from their synaptic terminals. We could demonstrate a specific binding of Zn-MT-3 to the small GTPase Rab3A-GDP. This interaction indicates that Zn-MT-3 is not merely a cellular zinc buffer, but actively participates in synaptic vesicle trafficking upstream of vesicle fusion. Involvement of aberrant metal-protein interactions and oxidative stress play a key role in AD. The production of reactive oxygen species (ROS) and neuronal toxicity are linked to the binding of redox-active metals like copper to the amyloid-beta peptide (Aβ). The protective effect of extracellular Zn-MT-3 from Aβ toxicity in neuronal cell cultures has been demonstrated, but its origin is not understood. By using several complementary spectroscopic, biochemical, and cell biological techniques we could show that Zn-MT-3 not only scavenges free Cu(II) ions but also removes Cu(II) bound to Aβ. A metal swapping between Zn-MT-3 and the soluble and aggregated Aβ-Cu(II) complexes, accompanied by copper reduction, abolishes the ROS production and profoundly reduces the cellular toxicity. The generated Cu(I)/Zn-MT-3 contains an oxygen stable Cu(I) thiolate cluster and two disulfide bonds. The results provide insights into the protective role of Zn-MT-3 from Aβ-Cu(II) toxicity in AD.

KL17
Construction of organometalloproteins: A new approach for bioorganic chemistry
Yoshihito Watanabe, Nagoya University, Grad. Sch. Sci. Dept. Chemistry, Nagoya, Japan.
Contact e-mail: yoshi@mucc.cc.nagoya-u.ac.jp

Myoglobin (Mb) is a small (17 kDa), well characterized heme protein that is often used as a model system for other heme proteins and the reactions they catalyze. Very recently, we have designed a myoglobin mutant, F43W/H64D Mb, in which an aromatic substrate locates immediate above the heme as a model for a substrate bound form of cytochrome P450 and site specific aromatic hydroxylation was found to proceed by a stoichiometric amount of H2O2 in a few seconds. As an extension of our efforts for the construction of organometalloproteins, we have replaced the heme prosthetic group with a series of M(salophen) complexes. We have also applied this strategy to heme oxygenase (HO) which is known to catalyze the conversion of heme to biliverdin. In this particular case, we have introduced Fe(salophen-schiff-base) bearing a carboxylate group, instead of heme, located at the active site of HO. The crystal structure of an Fe(salophenaldehyde-schiff-base) HO composite shows a hydrogen bond between propionic acid and Arg177 of HO. Due to the hydrogen bonding formation, the ET rate from NADPH to the composite is 3.5-fold faster than that of Fe(salophenaldehyde) HO, though a redox potential of Fe(salophenaldehyde-schiff-base)/HO (-79 mV vs. NHE) is lower than that of Fe(salophenaldehyde)/HO (+15 mV vs. NHE). Instead of these small protein cavities, we have also employed a protein having a large cavity, i.e., ferritin (Fr). We have prepared a zero-valent palladium cluster by chemical reduction of palladium ions in the apo- ferritin cage and examined its catalytic activity.

SL030
New fluorescent probes for Cytochrome P450 3A4 (CYP3A4)
Antoinette Chougnet, University of Basel, Department of Chemistry, Basel, Switzerland.
Contact e-mail: antoinette.chougnet@unibas.ch

Steroid derivatives were synthesized carrying fluorescent groups such as anthracene, dansyl, deazaflavin, and pyrene attached to C-6, e.g. see 1. These compounds are unique inhibitors of Cytochrome P450 3A4 displaying similar IC50 values in the low micro-M range for the 3A4 substrates midazolam, testosterone and nifedipine. On binding to 3A4 the fluorescence of the dansyl, deazaflavin or pyrene probes is quenched due to photophysical interaction of the fluorophore with the heme. Addition of drug candidates with nM-µM binding constants causes displacement of the probes from the active site and hence leads to restoration of the fluorescence. Accordingly relative affinities of drug candidates to CYP3A4 can be easily and accurately determined by fluorescence measurements.

The mechanism of fluorescence quenching was investigated with the synthetic model compound 2 in which deazaflavin is strapped over the distal side of an iron(III)-porphyrin to mimic the envisaged enzyme-inhibitor interaction within the active site of 3A4, see 1. Femtosecond pump-probe and fluorescence spectroscopies were used to study the photophysical processes of 2. Accordingly, rapid intramolecular energy transfer and enhanced intersystem crossing processes induced by the high-spin Fe(III) are responsible for the complete suppression of deazaflavin fluorescence.

SL031
Protein Evolution and Geochemical Changes: From Cytochrome c6 to Plastocyanin
Miguel A. De la Rosa, Instituto de Bioquímica Vegetal y Fotossíntesis, Sevilla, Spain.
Contact e-mail: maros@us.es

Plastocyanin and cytochrome c6 are two proteins with different structures - the former is a cupredoxin, the latter is a heme protein - but they can act in a similar way to transfer electrons from the cytochrome b6-f complex to photosystem I. They are thus an excellent model system for the analysis of biological convergent evolution at the molecular level. It seems that cytochrome c6 was
first synthesized by the most primitive oxygen-evolving photosynthetic organisms in the early Earth's life, when iron was much more available than copper because of the reducing character of the atmosphere. Later on, plastocyanin and other copper proteins appeared as the atmospheric oxygen concentration increased so as to make the copper element available to living cells. The transition from cytochrome c6 to plastocyanin may have indeed favored the evolution of the reaction mechanism with their two membrane partners, and electrostatics gained weight over hydrophobic forces in the binding interactions. The whole reaction mechanism consists of several steps, including long-range attractive movements, molecular rotations and orientation at short distances, formation of an encounter complex with a well-defined interaction area and an electron transfer pathway, and electron transfer itself. So such a functional evolution affected the way these small electron carriers recognize and bind their membrane partners, changed the step becoming the kinetic bottleneck of the reactions, and provided an increase in both specificity and efficiency.

**SL032**

Mechanism(s) of the re-oxidation reactions of substrate-reduced amine oxidases with dioxygen

Eric M. Shepherd, Justine Roth, David M. Dooley, Montana State University, Chemistry and Biochemistry, Bozeman, MT, USA; Department of Chemistry, Johns Hopkins University, Baltimore, MD, USA.

Contact e-mail: dmdooley@montana.edu

Although the catalytic mechanism of copper-containing amine oxidases is reasonably well understood, a critical issue remains to be definitively resolved. This is the mechanism by which substrate-reduced enzyme (containing the aminooxidin form of the TPQ cofactor) is oxidized by molecular oxygen. We have investigated the reaction of O2 with substrate-reduced amine oxidases by stopped-flow kinetics, focusing on the amine oxidase from *Arthrobacter globiformis* and the measurement of 18O isotope effects. The effects of temperature, dioxygen concentration, and solvent (D2O versus H2O) have been examined. These data provide additional insight into the reaction of O2 with the [Cu(II) - aminooxidin] and the [Cu(I) - semiquinone] states of the substrate-reduced amine oxidases examined.

**SL033**

Biosynthesis of Iron-Sulfur Clusters: an essential iron-sulfur protein for respiratory metabolism in *Escherichia coli*

Marc Fontecave, Laboratoire de Chimie et Biologie des Métaux, UMR 5249 Université Joseph Fourier, CNRS ; CEA ; DSV/iRTSIV, Grenoble, France.

Contact e-mail: Marc.Fontecave@cea.fr

Several multiprotein complexes assisting Fe-S cluster assembly have been identified in both procaryotes and eucaryotes (1). In *Escherichia coli* contains two biosynthetic machineries named SUF and ISC (1). Both contain homologous proteins, SufA and IscA respectively, which have been proposed to function as scaffold proteins, even though this is not firmly established. Scaffold proteins provide sites for binding iron and sulphur atoms and transient assembly of clusters which can be subsequently transferred for maturation of Fe-S proteins. We have identified in *Escherichia coli* a new A-type scaffold, named ErpA, which was found essential for *E. coli* to grow in the presence of oxygen or alternative electron acceptors. Defect in isoprenoid biosynthesis is largely unknown so far. A spectroscopic investigation of the most peculiar metal ion binding abilities of a number of plant metallothioneins will be presented, also considering the redox state of the environment. In addition, I will elaborate shortly on our possibly most peculiar finding so far, the existence of a two-metal ion cluster, called the γ-domain, which was unreported of in the MT superfamily so far (1).

Financial support for this project comes from the Swiss National Science Foundation (200020-113728/1 to EF).

[1] Porozau EA, E. Freisinger E, J. Biol. Inorg. Chem. 2007, DOI 10.1007/s00775-006-0195-5.

**SL034**

Plant MTs - long neglected members of the metallothionein superfamily

Eva Freisinger, Institute of Inorganic Chemistry, University of Zurich, Zurich, Switzerland.

Contact e-mail: freisinger@aci.uzh.ch

Metallothioneins (MTs) are a class of small, cysteine-rich proteins ubiquitous across all the different kingdoms of life. Characteristic is also the pronounced affinity for metal ions with d10 configuration, which are bound in form of metal-thiolate clusters using the deprotonated thiolate groups of the cysteine residues as ligands. Although the discovery of the first plant metallothionein reaches back more than 20 years, their characterization was paid little attention. This is even more surprising considering that the amino acid sequences and cysteine distribution patterns of plant MTs differ distinctively from the isoforms of other organisms. Plant MTs are sub-grouped into four families, whose members show a tremendously broader sequence variety than those of the mammalian subfamilies. Most striking are perhaps the differences in cysteine content ranging from 10-17 residues per protein, or 15-20% of total amino acids. The reason why plants display such sequence diversity is largely unknown so far.

A spectroscopic investigation of the most peculiar metal ion binding abilities of a number of plant metallothioneins will be presented, also considering the redox state of the environment. In addition, I will elaborate shortly on our possibly most peculiar finding so far, the existence of a two-metal ion cluster, called the γ-domain, which was unreported of in the MT superfamily so far (1). Financial support for this project comes from the Swiss National Science Foundation (200020-113728/1 to EF).

[1] Porozau EA, E. Freisinger E, J. Biol. Inorg. Chem. 2007, DOI 10.1007/s00775-006-0195-5.

**SL035**

Structure and function of the Na+ -translocating NADH dehydrogenase from *Vibrio cholerae*, a flavo-FeS redox pump

Julia Fritz-Steuber, University of Zurich, Zurich, Switzerland.

Contact e-mail: steuber@bioc.uzh.ch

The Na+ -translocating NADH dehydrogenase (Na+-NQR) is a multisubunit respiratory complex containing one 2Fe-2S cluster and several flavins that couples the oxidation of NADH to the transport of Na+ across the inner bacterial membrane. The enzyme from *Vibrio cholerae* contributes to extracellular superoxide production which might augment the pathogenicity of the bacterium in the human host (Lin et al, 2007). In our effort to assign electron transfer reactions to individual cofactors in the complex, we studied the redox properties of the individual NqrF subunit which contains one FAD and one [2Fe-2S] cluster. The FAD in subunit NqrF represents the flavin cofactor with the most negative midpoint potential in the Na+-NQR, according to its function as hydride acceptor from NADH and electron entry site into the complex. From there, electrons are transferred to the FeS cluster in subunit NqrF which represents the first example for a vertebrate-type [2Fe-2S] cluster in a respiratory complex (Lin et al., 2005). The Na+-NQR complex was overproduced in *Vibrio cholerae* and purified by affinity chromatography. Electron microscopic analysis of the Na+-NQR complex stained with uranyl formate revealed particles with a larger diameter of 3-4 nm.

Lin P.-C., Puhar, A., Türk, K., Piligkos, S., Bill, E., Neese, F., Steuber, J. (2007) J. Biol. Chem., 282, 22560 - 22563

Lin P.-C., Türk, K., Häse, C.C., Fritz, G., Steuber, J. (2007) J. Bacteriol., in press
Molybdenum and tungsten are the only 4d (Mo) and 5d (W) transition metals that are required for the normal metabolism of biological systems. Mo enzymes are present in bacteria, plants, animals, and humans; their W counterparts occur less frequently. Virtually all the reactions catalysed by these enzymes involve the net transfer of an oxygen atom either to or from the substrate. The structures of several Mo- and W-enzymes have been determined by crystallographic study has shown that the pyran ring can be closed remarkably conserved in the Mo and W enzymes, but one DMSO reductase has been examined. The structure of the MPT is D145, a conserved active site residue of Rhodobacter sphaeroides these equivalent Mo and W centres will be presented. The role of DMS to DMSO. Reasons for the significantly different activity of DMSOR shows (virtually) no ability to catalyse the oxidation of DMSO to DMS; however, unlike Mo-DMSOR, W-DMSOR is active with either Mo or W as the catalytic centre. This W-DMSO cofactor(s).

We have shown that Rhodobacter capsulatus DMSO reductase is active with either Mo or W as the catalytic centre. This W-DMSO reductase is the only isoenzyme for which structural, spectroscopic, electrochemical, and kinetic characterisation have been achieved thus allowing for direct comparisons with its Mo counterpart. In respect of catalytic activity, W-DMSOR >> Mo-DMSOR for the reduction of DMS to DMS; however, unlike Mo-DMSOR, W-DMSOR shows (virtually) no ability to catalyse the oxidation of DMS to DMSO. Reasons for the significantly different activity of these equivalent Mo and W centres will be presented. The role of D145, a conserved active site residue of Rhodobacter phaeorhabdus DMSO reductase has been examined. The structure of the MPT is remarkably conserved in the Mo and W enzymes, but one crystallographic study has shown that the pyran ring can be closed or open. Possible roles for MPT in the catalysis accomplished by the Mo and W enzymes will be discussed.

### The EPR spectra of the Enzyme-Substrate (Lys-Ala-Naphthylamide) Complex in Cu(II)-Dipeptidyl Peptidase III

Junzo Hirose1, Haiyuki Iwamoto2, Kayoko Fukasawa3, 1Fukuyama University, Fukuyama, Japan; 2Matsumoto Dental University, Shiojiri, Japan. Contact e-mail: hirose@fubuc.fukuyama-u.ac.jp

Dipeptidyl peptidase III (DPP III), which has a HELLLGHE...E (residues 450-455, 508) motif as the zinc-binding site, is classified as a zinc-aminopeptidase, but the zinc-binding motif (HEExxxH (x: appropriate amino acid)) of DPP III is different from a common zinc-binding motif (HEExxH) such as that of thermolysin(1). The Cu2+ derivatives of thermolysin and carboxypeptidase A do not have the appropriate amino acid)) of DPP III is different from that of thermolysin(1). The Cu2+ derivative of thermolysin has the high enzyme activity and we showed that the motif part of DPP III directly influences the expression of the enzyme activity in the copper derivative of DPP III. For Arg-Arg-Ala-Naphthylamide, the value of kcal/Km of Cu(II)-DPP III was 1/3 that of Zn(II)-DPP III. Lys-Ala-naphthylamide was also the good substrate for Cu(II)-DPP III but slowly digested at low temperature by Cu(II)-DPP III. The EPR spectra of Cu(II)-DPP III mixed with substrate(1-10 mM), Lys-Ala-naphthylamide, at low temperature and immediately frozen in liquid nitrogen were measured and were different from that of Cu(II)-DPP III. The EPR spectra of the substrate-Cu(II)-DPP III complexes gradually change to original EPR spectrum of Cu(II)-DPP III when the substrate-Cu(II)-DPP III complexes were incubated at 10 °C. These results clearly indicated that the substrate-Cu(II)-DPP III complex could be measured as EPR spectrum.

### Molecular Design of Converting an Electron Transfer Metalloprotein into a Biocatalyst

Zhong X. Huang, Fudan University, Chemistry, Shanghai, China. Contact e-mail: zhuang@fudan.edu.cn

Various classes of natural hemoproteins perform a wide variety of biological activities, such as oxygen carrier, electron transfer, biochemical reaction catalyses, and signal transduction, although they share the same protoheme IX as prosthetic group. The artificial design of metalloproteins with desired or novel properties and reactivity is one of the challenges in chemical biology research. Hemoproteins are the excellent natural models and starting-molecules. In order further to convert an electron transfer hemoprotein into a catalytic metalloprotein molecular design based on cytochrome c has been employed. Compared to the heme active site of peroxidases a series single-, double- and triple-site mutant proteins of yeast iso-1-cytochrome c were generated to eliminate the axial ligand and to introduce a distal histidine and arginine in the heme pocket at residue position Met80, Tyr67, Pro71. The obtained three variants, M80V, Y67H, and Y67H/M80V showed much higher peroxidase activity than the wild-type cytochrome protein. More interestingly, the Cyt c Y67H variant showed the kcat/Km value towards classical guaiacol catalytic reaction not only the highest among the cytochrome c variants but also higher than that of native horseradish peroxidase under the same condition. A wide range of spectroscopic studies and biocatalytic activity assay provide important insight into the structure-property-reactivity-function relationship of hemoproteins. This project is supported by the National Science Foundation of China
Weak non-covalent interactions in biological molecules including proteins, nucleic acids, and lipids play important roles for expressing the specific functions and structures in many varieties of biological systems.

Pseudoazurin (PAz) is a blue copper protein, which functions as the electron carrier in several microorganisms. The aromatic acid substituents of PAz, Met16Phe, Met16Tyr, and Met16Trp were studied to elucidate the effect of weak indirect interactions involving p-i-pi interaction on the active site structures and properties.

Electronic absorption spectra of Met16X variants indicate the changes in the intensity of the two main LMCT transitions in the visible region. EPR spectra of Met16X variants at 77 K demonstrate that the copper site has two different conformers, characterized by rhombic and axial EPR signals. The EPR spectra indicate that the active structure is sufficiently flexible to be modulated by weak interactions between a ligand histidine imidazole ring and the newly introduced aromatic rings of Met16Phe, Met16Tyr, and Met16Trp. The spectroscopic and electrochemical results suggest the second shell weak interaction may have a role to modulate the structure and reactivity of blue copper protein active site.

A part of this work is supported by Research Promotion Bureau, Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan to TK, under the contract No. 17-214 and a Grant-in-Aid for Scientific Research from JSPS (No. 18550147), Japan to TK.

Contact e-mail: kohzuma@mx.ibaraki.ac.jp

Contact e-mail: ortwin.meyer@uni-bayreuth.de

Weak Interaction Regulates the Active Site Structure of a Blue Copper Protein, Pseudoazurin

Takamitsu Kohzuma, Ibaraki University, Institute of Applied Beam Science, Mito, Japan.

Contact e-mail: kohzuma@mx.ibaraki.ac.jp

"Four-dimensional" protein structures: examples from metalloproteins

Claudio Luchinat, CERM (Centre for Magnetic Resonance), University of Florence, Sesto Fiorentino (Florence), Italy.

Contact e-mail: luchinat@cern.unifi.it

The fact that an object, for example, a protein, possesses a three-dimensional structure seems an obvious concept. However, if the object is flexible, the concept is less obvious. Proteins are indeed not rigid, but may sample more or less wide ranges of different conformations. It has been recently proposed to call the range of sampled conformations the "fourth dimension" of a protein structure. A particular, but quite common, case of conformational freedom is constituted by multidomain proteins, where two or more well structured and relatively rigid protein domains are tethered by linkers that are more or less flexible. In most cases, the function itself depends on flexibility. Static structural information from, e.g., X-ray, does not provide information on flexibility, and may even be misleading. Nuclear magnetic resonance is a better technique to detect dynamics and assess conformational heterogeneity. We have recently found that the presence of a paramagnetic metal in a metalloprotein permits the use of NMR in a novel way to describe the conformational freedom of protein domains (i.e. their "four-dimensional" structure). A formally well defined way to define the reciprocal arrangements of the domains in terms of their Maximum Allowed Probability (MAP), is being proposed.

SL040
Weak Interaction Regulates the Active Site Structure of a Blue Copper Protein, Pseudoazurin

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3Bertini I, Gupta Y, Luchinat C, Parigi G, Peana L, Wang S, submitted

SL042
Conformational stability of the CuA center of cytochrome oxidase

Nusrat J. M. Sanghamitra, Shyamalava Mazumdar, Tata Institute of Fundamental Research, Department of Chemical Sciences, Mumbai, India.

Contact e-mail: shyamal@iisr.res.in

Cytochrome oxidase forms the terminal step in the respiratory electron transfer chain. The dinuclear copper centre (CuA) at the subunit II of this enzyme is the electron entry site of the enzyme. The CuA site has been shown to exist in a charge-delocalised form or in a valence trapped form, and both of these two forms have been proposed to be important for the function of the enzyme. Studies on the CuA site from various sources have indicated that these two forms exist in a pH coupled equilibrium.

The present talk will describe some of our recent studies on the stability of the CuA site and the role of proton transfer on the stability of the active conformation of the metal center. The results will be discussed in the light of understanding the possible role of these two forms on the biological electron transfer mediated by the CuA center.

SL043
Post-translational assembly of the molybdenum-sulphur active site cluster of CO dehydrogenase

Astrid M. Pelzmann1, Marion Ferren1, Holger Dobbeck1, Manuel Grund1, Wolfram Meyer-Klaue2, Ortwin O. Meyer1, 3University of Bayreuth, Bayreuth, Germany; 2European Molecular Biology Laboratory (EMBL), Outstation Hamburg at DESY, Hamburg, Germany.

Contact e-mail: ortwin.meyer@uni-bayreuth.de

CO dehydrogenase of Oligotropha carboxidovorans oxidises CO at a unique binuclear [CuSMoO 2] cluster in its active site (1). Post-translational cluster assembly involves the gene functions coxDEFG. Insertional mutagenesis of coxD had the following consequences: (i) The resulting mutant OM5D::km was unable to utilize CO under chemolithoautotrophic conditions, but (ii) retained the ability to utilize H 2; (iii) When grown with H2 and induced with CO, OM5D::km synthesized non-functional apo-CO dehydrogenase. (iv) EPR, EXAFS and crystallography revealed a [Mo(OH) 3] centre in the active site of the apo-enzyme, which (v) could be reconstituted to a functional [CuSMoO 2] cluster employing sulphide and Cu(I)-thiourea.

CoxD is a MoxR-like AAA+ ATPase, enzymes involved in ATP dependent processes of protein refolding and degradation (2) and a member of the APE2220 phylogenetic subfamily of MoxR-proteins (3). The coxD gene is adjacent to coxE which contains a Von Willebrand Factor Type A (VWA) domain, and both genes are part of a cox gene cluster (4). These features separate the APE2220 subfamily from the PA2707 subfamily of MoxR. We present evidence that CoxD operates as a molecular chaperone assisting CoxEFG and other essential functions to mediate the post-translational insertion of sulphur and copper into the CO dehydrogenase active site and the subsequent assembly to a catalytically competent [CuSMoO2] cluster.

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SL044
Probing the mechanism of Formate Dehydrogenases using EPR Mo(V) signals
José J. G. Moura, REQUIMTE, Departamento de Quimica, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Caparica, Portugal.
Contact e-mail: jose.moura@dq.fct.unl.pt

Formate dehydrogenase (Fdh) catalyzes the oxidation of formate to carbon dioxide. The crystal structures of three Fdh's have been reported: E.coli Mo-Fdh-H and E.coli Mo-Fdh-N and D. gigao W-Fdh. The catalytic subunit contains the active site and one [4Fe-4S] cluster. The oxidized active site is coordinated to 2xMGD, one Se atom from a selenocysteine, and a sixth ligand X, identified as a hydroxyl in Ec Fdh-N, Ec Fdh-H, and D. desulfuricans Fdh, but the Dg Fdh X-ray data suggest the presence of a S atom. A reevaluation of the crystallographic data of the Ec Fdh-H reduced by formate in the presence of azide proposed that this ligand is better fitted as a sulfur than an oxygenic ligand (Raaijmakers and Romão, JBIC (2006)). We report detailed EPR studies on as-prepared, formate reduced and azide inhibited samples of Dg Fdh, in order to compare with reduced Ec Fdh-H in the presence of azide. The re-evaluation of the 3D structure of the formate reduced Ec Fdh-H in the presence of azide shows that the SeCys ligand is not coordinated to Mo. Based on this observation it was suggested a completely different mechanism. In the presence of formate and absence of inhibitors the EPR signals assigned to competent catalytic species are different from those obtained in presence of azide, we will focus on the clarification of mechanistic aspects of Fdh. Relevant references will be given at the presentation.

Acknowledgments: MG Rivas, P González, C Brondino, I Moura, MJ Romão. FCT-MCTES, SEPCYT, CAI+D-UNL.

SL045
Impact of heme to protein linkages in peroxidases on redox chemistry and catalysis
Christian Obinger1, Jutta Vlastis2, Marcia Belli3, Gianantonio Battistuzzi2, Paul G. Furtmüller1,1, BOKU - University of Natural Resources and Applied Life Sciences, Department of Chemistry, Vienna, Austria; 2University of Modena and Reggio Emilia, Department of Chemistry, Modena, Italy.
Contact e-mail: christian.obinger@boku.ac.at

The mammalian peroxidases myeloperoxidase (MPO), eosinophil peroxidase, lactoperoxidase (LPO) and thyroid peroxidase participate in host defence against infection, hormone synthesis and pathogenesis. The most striking feature of these heme enzymes is the existence of two covalent ester bonds between the prosthetic group and the protein in the functional, mature proteins. Myeloperoxidase is unique in having an additional vinyl-sulfonium bond. Based on the crystal structures of human MPO and its complexes with cyanide, bromide and thiocyanide as well as on the characterization of proteins and their interactions with other molecules. Solution structures have been determined for several paramagnetic systems by using 1H NMR-based experiments, and in several cases paramagnetic effects have been exploited to derive structural information useful for refinement of a structure.1

The magnitude of paramagnetic effects on nuclear relaxation rates depends on the nature of the metal ion and its electronic relaxation rate. Depending on the metal-nucleus distance, electron-nucleus coupling effects may prevent the observation of the proton signals of residues close to the metal site, due to induced broadening of the NMR lines. This is the case for type II copper(II)-containing proteins, where protons close to the paramagnetic copper center are characterized by relaxation rates too large to be studied with a conventional NMR approach.2

Carbon-13 direct-detection NMR offers advantages for the study of paramagnetic proteins because paramagnetic dipolar contributions to nuclear relaxation depend on the square of the gyromagnetic ratio of the observed nucleus; thus, a switch from 1H to 13C detection guarantees a sizable decrease in relaxation rates.3,4 This is demonstrated in Cu,Zn superoxide dismutase, where 13C direct-detection NMR experiments enable detection of resonances as close

SL046
Maturation of thiocyanate hydrolase is promoted by its activator protein, P15K
Masafumi Odaka1, Takatoshi Arakawa2, Shota Horii2, Hiroyuki Mino2, Yoko Katayama3, Hiroshi Nakayama2, Naoshi Dohmae4, Masafumi Yoda4,1, Department of Biotechnology and Life Science, Toky University of Agriculture and Technology, Tokyo, Japan; 2Division of Material Science (Physics), Graduate School of Science, Nagoya University, Nagoya, Japan; 3Department of Environment and Natural Resource Science, Tokyo University of Agriculture and Technology, Tokyo, Japan; 4Biomolecular characterization team, RIKEN, Saitama, Japan.
Contact e-mail: modaka@cc.tuat.ac.jp

Nitrile hydratase (NiHase) family proteins are Co- or Fe-containing enzymes having two post-translationally modified cysteine ligands, cysteine-sulfenic acid (Cys-SOH) and cysteine-sulfenic acid (Cys-SOH). NiHase family proteins require their specific activator proteins for the functional expression. Here, we show the function of the activator protein, P15K, for thiocyanate hydrolase (SCNase), a Co-type NiHase family protein. When P15K was co-expressed with α, β or γ subunit of SCNase in Escherichia coli, only the γ subunit which contained all ligand residues formed a stable complex (γP15K) with a stoichiometry of γ: P15K = 1: 1. It is of interest that the γ subunit was expressed as inclusion bodies when it was expressed solely. Then, we studied the effect of Co ion on the expression of γP15K. When γP15K was expressed in the Co-enriched medium, the isolated γ subunit as well as γP15K was obtained. Both the isolated γ subunit and γP15K incorporated significant amount of Co ions and possessed the Cys-SOH modification like native SCNase. Considering that P15K has significant amino acid sequence similarity with the β subunit and that the metal-binding site is located at the interface between β and γ subunits, the function of P15K is to associate with the γ subunit and to assist the Co-incorporation as well as the generation of the cysteine modifications. We have been trying to reconstitute SCNase from γP15K and the γ subunit. Based on the results obtained, we will discuss the maturation mechanism of the Co-type NiHase family proteins.

SL047
Towards a protocol for solution structure determination of copper(II) proteins through protonless 13C direct-detection NMR
Roberta Pierattelli, University of Florence, Dept of Chemistry and CERM, Sesto Fiorentino, Italy.
Contact e-mail: pierattelli@cerm.unifi.it

Nuclear magnetic resonance spectroscopy plays a prominent role in the characterization of proteins and their interactions with other molecules. Solution structures have been determined for several paramagnetic systems by using 1H NMR-based experiments, and in several cases paramagnetic effects have been exploited to derive structural information useful for refinement of a structure.1

The magnitude of paramagnetic effects on nuclear relaxation rates depends on the nature of the metal ion and its electronic relaxation rate. Depending on the metal-nucleus distance, electron-nucleus coupling effects may prevent the observation of the proton signals of residues close to the metal site, due to induced broadening of the NMR lines. This is the case for type II copper(II)-containing proteins, where protons close to the paramagnetic copper center are characterized by relaxation rates too large to be studied with a conventional NMR approach.2

Carbon-13 direct-detection NMR offers advantages for the study of paramagnetic proteins because paramagnetic dipolar contributions to nuclear relaxation depend on the square of the gyromagnetic ratio of the observed nucleus; thus, a switch from 1H to 13C detection guarantees a sizable decrease in relaxation rates.3,4 This is demonstrated in Cu,Zn superoxide dismutase, where 13C direct-detection NMR experiments enable detection of resonances as close
as 6 Å from the metal ion, and allow determination of structural
restraints necessary for structure refinement. Solid-state NMR, that is developed on $^{13}$C direct-detection, provides
an additional tool for the NMR investigation of Cu(II)-containing
proteins.

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SL048
The axial ligand and associated hydrogen bonding
network in the distal site of heme oxygenase control the
dynamical freedom of the enzyme during catalysis
Mario Rivera1, Juan C. Rodriguez2, 1University of Kansas, R. N.
Adams Institute for Bioanalytical Chemistry and Department of
Chemistry, Lawrence, KS, USA; 2University of Kansas, R. N.
Adams Institute for Bioanalytical Chemistry and Department of
Chemistry, Lawrence, Kansas, KS, USA.
Contact e-mail: mrivera@ku.edu

NMR experiments have been used to investigate $\mu$-$\mu$-ms motions in
heme oxygenase from Pseudomonas aeruginosa (pa-HO) in its
ferrous state, inhibited by CN$^-$ (pa-HO-CN) and $N_3^-$ (pa-HO-$N_3$),
and in its ferrous state, inhibited by CO (pa-HO-CO). Comparative
analysis of the data indicates that the distal ligand affects the
conformational freedom of the polypeptide in regions removed from
the heme. Interpretation of the dynamical information in the context
of the structure of pa-HO shows that secondary structure harboring
residues involved in the network of structural H-bonded waters
undergo $\mu$-$\mu$-ms motions in pa-HO-CN, which was studied as a model
of the resting state form. In comparison, similar motions are
suppressed in the pa-HO-CO and pa-HO-$N_3$ complexes, which
were studied as mimics of the oxyferrous and ferric hydroperoxide
intermediates. These findings suggest that in addition to proton
delivery to the nascent Fe$^{3+}$OO$, the H-bonding network serves
two additional roles: (i) propagate the electronic state in each of the
catalytic cycle steps to key but remote sections of the polypeptide
via small rearrangements and (ii) modulate the conformational
freedom of the enzyme, thus allowing it to adapt to the demanding
changes in axial coordination and substrate transformations taking
place during a catalytic cycle. This idea was probed by disrupting the
H-bonding network by replacing R$^80$ for L. 

SL049
Metal binding site in the macrocyclic peptide Kalata
Lars Skjeldal, Norwegian University of Life Sciences (UMB),
Dept. of Chemistry, Biotechnology and Food Science, Aas,
Norway.
Contact e-mail: lars.skjeldal@umb.no

Metal binding site in the macrocyclic peptide Kalata.
The African medical plant Oldenlandia affinis, was used as a
decoy by the natives during child-birth to support labour. The
delivery time was remarkable short, and the uterine contractions
extremely strong. The active compound in the plant is the cyclic
peptide Kalata B1, which contains three disulfide linkages. The
disulfide connectivities (5-13, 17-29 and 22-27) and the three
dimensional structure were elucidated by high field NMR [Skjeldal
et al (2002)Arch.Biochem.Biophys., 399, 142-148]. The peptide
binds to the micelle surface with relatively high affinity [Shenkarov
et al (2006) FEBS J.273, 2658-2672]. We have also found that the peptide is able to bind manganese in a
specific metal binding site, which could be a binding site for
calcium. The structure is deposited in PDB as IJZJ.

SL050
Redox properties of the c-type heme centers in the novel
PQQ-dependent alcohol dehydrogenase from nitrogen
fixing Gluconacetobacter diazotrophicus
Martha E. Sosa-Torres1, Sáül Gómez-Manzo2, Edgardo Escamilla-Marván2, Peter M. H. Kromeck3, 1Facultad de Quimica,
Universidad Nacional Autónoma de México, México D.F., Mexico;
2Instituto de Fisiología Celular, Universidad Nacional Autónoma de
México, México D.F., Mexico; 3Fachbereich Biologie, Universität
Konstanz, Konstanz, Germany.
Contact e-mail: mst@servidor.unam.mx

Multi-heme proteins and enzymes are important constituents of
bacterial electron transfer chains and are involved in many
respiratory processes. In sulfate-reducing bacteria the tetraheme
protein cytochrome $c_5$ and the nonaheme cytochrome $c$ play
important roles in transferring electrons between the [Ni,Fe]-
hydrogenase and membrane-bound respiratory complexes [1]. In
nitrogen-fixing bacteria, such as Gluconacetobacter diazotrophicus,
a multi-heme PQQ-dependent alcohol dehydrogenase (PQQ-ADH)
may also serve as a protectant for the oxygen-labile nitrogensenase [2].
The PQQ-ADH of G. diazotrophicus is a heterodimer composed of
SU1 (PQQ cofactor and one c-type cytochrome) and SU II (three c-
type cytochromes). This latter PQQ-ADH is rather unique in the
sense that the heme centers remain in the reduced Fe(II) state even
when isolated in the presence of dioxygen. We will describe the
redox properties of the four different c-type cytochromes in the
ADH from G. diazotrophicus by applying UV/Vis- and EPR
spectroscopy-monitored redox titrations. Preliminary
 electrochemistry measurements are also included. An
intramolecular electron transfer (IET) pathway between the c-
type heme centers within subunits I and II, and to the ubiquinone is
proposed.

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SL051
Histidine-rich protein, Hpn and metallothionein: are they
similar?
Hongzhe Sun1, Ruiguang Guo1, Yibo Zeng1, Hui Wang1, Jiandong
Huang1, 1Department of Chemistry, The University of Hong Kong,
Hong Kong, China; 2Department of Biochemistry, The University of
Hong Kong, Hong Kong, China.
Contact e-mail: hsun@hkusua.hku.hk

Helicobacter pylori (H. pylori), a Gram-negative, and spiral-shaped
bacterium, has been known to be the causative agent of chronic
gastritis and peptic ulcers. The organism produces a large amount of
a nickel-containing enzyme urease, which is believed to neutralize
gastric acid by producing ammonia for the survival of the
bacteria [1,2]. A constant supply of nickel is therefore essential for the
survival of the organism, a nickel-containing enzyme urease, which is believed to neutralize
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survival of the organism, a nickel-containing enzyme urease, which is believed to neutralize
gastric acid by producing ammonia for the survival of the
bacteria [1,2].
species in solution and binds to five Ni\textsuperscript{2+} and four Bi\textsuperscript{3+} per monomer moderately ($K_d$ of 7.1 and 11.1 µM respectively)\textsuperscript{[3]}

Although \textit{in vitro}, it binds to Cu\textsuperscript{2+} stronger than Ni\textsuperscript{2+} and Bi\textsuperscript{3+}, the \textit{in vivo} protection by the protein is in the order of Ni\textsuperscript{2+}> Bi\textsuperscript{3+}> Cu\textsuperscript{2+}\textsuperscript{[4]}

Hpn may therefore serve to buffer intracellular Ni\textsuperscript{2+} in much the same way as that the small and cysteine-rich protein, metallothionein interacts with Zn\textsuperscript{2+}/Cu\textsuperscript{2+}\textsuperscript{[5,6]}

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SL052
The Structure of the Hyponitrite Species in the Heme Fe-CuB Center of ba3 Oxidase from Thermus thermophilus 
Costas Varotsis\textsuperscript{1,2}, Takahiro Ohta\textsuperscript{3,4}, Teizo Kitagawa\textsuperscript{2}, Tewfik Souliman\textsuperscript{5}, Effychia Pinakoulaki\textsuperscript{6}, \textsuperscript{1}University of Crete, Department of Chemistry, Heraklion, Greece; \textsuperscript{2}Okazaki Institute for Integrative Bioscience, National Institutes of Natural Sciences, Okazaki, Japan; \textsuperscript{3}Stanford University, Department of Chemistry, Stanford, CA, USA; \textsuperscript{4}University of Limerick, College of Science, Limerick, Ireland; \textsuperscript{5}University of Cyprus, Department of Chemistry, Nicosia, Cyprus.

Contact e-mail: varotsis@chemistry.uoc.gr

The formation of the hyponitrite species from two NO molecules is the key intermediate in the reduction of NO to N\textsubscript{2}O in denitrification. The reaction is used by bacteria as an alternative to oxygen-based respiration. The bacterial Nitric oxide reductase (Nor) and the ba\textsubscript{3}-oxidoreductase from \textit{Thermus thermophilus} catalyze the reduction of NO to N\textsubscript{2}O. Here we report on the formation of the hyponitrite species in the heme a\textsubscript{1}/Cu\textsubscript{b} binuclear center of ba\textsubscript{3}-oxidoreductase from \textit{T. thermophilus}. The N-N bond ($\nu_{\text{N-N}}$= 1334 cm\textsuperscript{-1}) of the hyponitrite-bound to the heme Fe is detected only under 244 nm Raman excitation, and the bonding vibration of heme Fe-N\textsubscript{2}O at 624 cm\textsuperscript{-1} under 428 nm Raman excitation. The $\nu_{\text{N-N}}$ is sensitive to pH and pD exchanges indicating the interaction of the hyponitrite species with a H\textsubscript{2}O molecule. We propose a mechanism for the formation of the hyponitrite species that is initiated by the reduction of Cu\textsubscript{b} by NO. With the identification of the hyponitrite species the hypothesis of a common phylogeny of aerobic respiration and bacterial denitrification is fully supported and the mechanism for the 2e\textsuperscript{-}/2H\textsuperscript{+} reduction of NO to N\textsubscript{2}O can be described with more certainty.

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ORAL PAPERS
0025
Resonance Raman Spectroscopic Studies on the Alkaline Transition of a Blue Copper Protein, Pseudoazurin and Its M16X Variants
Rehab F. Abdelhamid\textsuperscript{1}, Takamitsu Khozuma\textsuperscript{2}, \textsuperscript{1}Institute of Applied Beam Science, Ibaraki University, Mito, Japan; \textsuperscript{2}Institute of Applied Beam Science, Ibaraki University, , 2-1-1 Bunkyo, Mito, Ibaraki 310-8512, Japan.
Contact e-mail: rehab.abdelhamid@gmail.com

Several blue copper proteins involving pseudoazurin (Paz) shows a structural transition at alkaline pH. The spectroscopic and electrochemical studies of the alkaline transition of Paz were reported [1, 2]. Very recently the effect of the second sphere interaction in the vicinity of the active site of Paz M16X variants, M16Y, M16F, M16W, and M16V has been reported [3]. Resonance Raman (RR) spectroscopic studies of wild-type and the M16X variants were performed to know the details of the effect of second sphere interaction on the alkaline transition of pseudoazurin. RR spectra of wild-type and M16X mutants at pH 7.2 and 10.2 were measured in the frequency region of 200-550 cm\textsuperscript{-1}. The RR spectra of the wild-type and the M16X spectra at pH 7.2 showed five intense Raman bands at 418, 398, 367, and 355 cm\textsuperscript{-1} and shoulder bands at 335, 439, and 454 cm\textsuperscript{-1} except M16Val. The RR spectra of wild-type and M16X variants indicated the disappearance of several Raman bands at pH10.2

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0026
Crystallographic and spectroscopical studies of peroxide-derived myoglobin intermediates
Hans Petter Hersleth\textsuperscript{1}, Asmund K. Rohr\textsuperscript{2}, Carl Henrik Görbitz\textsuperscript{1}, Karl Kristoffer Andersson\textsuperscript{3}, \textsuperscript{1}University of Oslo, Chemistry, Oslo, Norway; \textsuperscript{2}University of Oslo, IMBV, Oslo, Norway. Contact e-mail: k.k.andersson@imbv.uio.no.

High resolution crystal structure of myoglobin in the pH range 5.2-8.7 [1-4] have been used as models for the peroxide-derived compound II intermediates in haem peroxidases and oxygenases. The observed Fe-O bond length (1.86-1.90 Å) is consistent with a single bond. This finding is supported by observation of a new low energy $^{16}$O-peroxide-sensitive resonance Raman Fe-O mode at 687 cm\textsuperscript{-1} [4]. We observe some radiation-induced changes in both compound II (forming a novel semi-stable intermediate H) and in the resting ferric state of myoglobin by single-crystal microspectrophotometry [4]. Our data suggest for myoglobin the compound II intermediate consists of a Fe\textsuperscript{IV}=O species with a single bond. The presence of Fe\textsuperscript{IV}=O is indicated by Mössbauer spectroscopy [4], and quantum refinements and DFT-calculations suggest that this species is protonated [2,3]. The presence of a Fe\textsuperscript{IV}=O state seems to be general for compound II intermediates. Additionally, an isoelectronic form of the compound 0 intermediate has been generated by radiation-induced changes (to be submitted J. Am. Chem. Soc).

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These data provide valuable insight into the structural selectivity is achieved.

The accessory protein, HypB, is necessary for Ni(II) insertion into the NiFe cluster of hydrogenase in E. coli. By using XAS data and mutagenic studies we have been able to form a structural picture of the high and low-affinity metal sites present in the HypB protein (2). We have determined the amino acid ligands in addition to the Ni(II) ion in both the high-affinity and low-affinity sites present in the HypB protein (2). We have established a method for refining anomalous scattering contributions for individual atoms within a crystal structure for multiple data sets collected along an absorption edge [1], yielding information on redox state and electron distribution at each atom. This allows for the detailed analysis of redox events and the tracing of electron flow within protein systems. The test case of an antiferromagnetically coupled [2Fe:2S] cluster is presented, where a mixed-valence reduced state with localized charges confirms the reliability of the approach, and implications for highly complex metal clusters are discussed. [1] Einsle, O., Andrade, S.L.A., Dobbeck, Jacques Meyer, D.C. (2007) J. Am. Chem. Soc. 129, 2210-2211.

O029

Biogenesis of iron-sulfur clusters and intracellular iron metabolism under oxidative stress conditions

Huangen Ding, Juanjuan Yang, Liana C. Coleman, Louisiana State University, Baton Rouge, LA, USA.

Contact e-mail: hding@lsu.edu

Iron is an essential element for living organisms. Ironically, iron is highly toxic to cells since free iron can promote the production of hydroxyl free radicals via the Fenton Reaction. Here we report the interplay of the iron binding between the iron-sulfur cluster assembly protein IscA and the Friedreich’s ataxia protein frataxin, under physiological and oxidative stress conditions. Previous studies indicated that both IscA and frataxin are able to bind iron. However, their iron binding mechanisms are very different. In IscA, the variant three cysteine residues are required for the iron binding. On the other hand, the carboxyl groups of the highly conserved aspartate and glutamate residues in frataxin are essential for the iron binding. In this study, we have shown that in the presence of the thiol reductase system which emulates intracellular redox potential, frataxin fails to bind any iron even at a 10-fold excess of iron. IscA, on the other hand, can efficiently recruit iron and transfers the iron for the iron-sulfur cluster assembly in IscU. In the presence of hydrogen peroxide, however, IscA completely loses its iron binding activity due to the oxidation of the thiol groups, whereas frataxin becomes a competent iron-binding protein and attenuates the iron-mediated production of hydroxyl free radicals. The results suggest that IscA is capable of recruiting intracellular iron for the iron-sulfur cluster assembly under normal physiological conditions, whereas frataxin may serve as an iron chaperon to sequester redox active free iron and alleviate cellular oxidative damage under oxidative stress conditions.

O030

Assignment of individual metal redox states in metalloproteins by crystallographic refinement at multiple X-ray wavelengths

Oliver Einsle1, Susana L. A. Andrade1, Holger Dobbeck2, Jacques Meyer1, Douglas C. Rees4, 1Institute for Microbiology and Genetics, Göttingen, Germany; 2Laboratorium Proteinkristallographie, Bayreuth, Germany; 3CEA-Grenoble, Grenoble, France; 4California Institute of Technology, Pasadena, CA, USA.

Contact e-mail: oeinsle@uni-goettingen.de

In recent years X-ray crystallography revealed the existence and fine structure of numerous multi-metal and multi-center metal clusters in proteins. X-ray data are routinely collected at or around absorption edge in order to exploit anomalous scattering for phase determination, but the additional information contained in these edges - as used in X-ray absorption spectroscopy - is commonly not utilized in crystallography. We have established a method for refining anomalous scattering contributions for individual atoms within a crystal structure for multiple data sets collected along an absorption edge [1], yielding information on redox state and electron distribution at each atom. This allows for the detailed analysis of redox events and the tracing of electron flow within protein systems. The test case of an antiferromagnetically coupled [2Fe:2S] cluster is presented, where a mixed-valence reduced state with localized charges confirms the reliability of the approach, and implications for highly complex metal clusters are discussed. [1] Einsle, O., Andrade, S.L.A., Dobbeck, H., Meyer, J. & Rees, D.C. (2007) J. Am. Chem. Soc. 129, 2210-2211.

O027

Redox thermodynamics of the Fe3+/Fe2+ couple in wild-type and mutated heme peroxidases

Gianantonio Battistuzzi1, Marzia Bellei2, Christa Jakopitsch2, Jutta Vlasits2, Paul G. Furtmüller2, Marco Sola1, Christian Obinger2, 1University of Modena and Reggio Emilia, Modena, Italy; 2BOKU – University of Natural Resources and Applied Life Sciences, Wien, Austria.

Contact e-mail: battistuzzi.gianantonio@unimore.it

The thermodynamics of the one-electron reduction of the ferric heme in wild-type and mutated heme Synechocystis catalase-peroxidase and human myeloperoxidase were determined through spectro-electrochemical experiments [1, 2]. The data are interpreted in terms of ligand binding features, electrostatic effects and solvation properties of the heme environment. Reduction-induced solvent reorganization effects turn out to be the major effectors of reduction entropy and influence reduction enthalpy, which mainly depends on the anionic character of the proximal histidine and the polarity of the heme environment.

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S62 J Biol Inorg Chem (2007) 12 (Suppl 1):S53–S98
O031
Investigation of the coordination structures of the molybdenum(V) sites of sulfite oxidizing enzymes by pulsed EPR spectroscopy: Direct detection of coordinated sulfate using $^{35}$S labeling
John H. Enemark1, Andrei V. Astashkin1, Arnold M. Raitsimring1, Kasyunta Johnson-Winters1, Eric L. Klein2, Robert S. Byrn3, Russ Hille2, Heather L. Wilson1, K. V. Rajagopalan1, 1University of Arizona, Tucson, AZ, USA; 2The Ohio State University, Columbus, OH, USA; 3Duke University Medical Center, Durham, NC, USA. Contact e-mail: jenemark@u.arizona.edu

Sulfite oxidizing enzymes (SOEs) are physiologically vital and occur in all forms of life. During the catalytic cycle the five-coordinate square-pyramidal oxo-molybdenum active site passes through the Mo(V) state, and intimate details of the structure can be obtained from pulsed EPR spectroscopy through the hyperfine interactions (hfi) and nuclear quadrupole interactions (nqi) of nearby magnetic nuclei (e.g., $^1$H, $^1$H, $^{15}$O, $^{13}$P, $^{183}$W). By employing spectrometer operational frequencies ranging from ~4 to ~32 GHz, it is possible to make the nuclear Zeeman interaction significantly greater than the hfi and nqi, and thereby simplify the interpretations of the spectra. The SOEs exhibit three general types of Mo(V) structures which differ in the number of nearby exchangeable protons (one, two or zero). The CW-EPR and electron spin echo envelope modulation (ESEEM) data for sulfite oxidase from several SOEs exhibit three general types of Mo(V) = 6 with sulfite labeled with $^{33}$S ($\omega_{\text{nq}} = 4.8$ MHz) demonstrates the presence of coordinated sulfate, a postulated intermediate in the catalytic cycle of sulfite oxidizing enzymes. The fatal R160Q mutant of human sulfite oxidase also shows $^{35}$S couplings from bound sulfate. These are the first examples of direct detection of coordinated sulfate in molybdenum enzymes and only the second case of pulsed EPR detection of a $^{35}$S-metals interaction in metalloenzymes.

O032
Role of highly conserved three-histidines ligand environment of type-2 copper site in copper containing nitrite reductase
Hiroshi Fujii, Masato Kujime, National Institutes of Natural Sciences, Okazaki, Japan. Contact e-mail: hiro@nims.ac.jp

Denitritation is an important component of the global nitrogen cycle that is responsible for regulating the amount of fixed nitrogen available for plant growth. In denitritation process, the reduction of NO2- to NO is catalyzed by nitrite reductase, containing either heme c1 or copper. The x-ray crystal structures of copper-containing nitrite reductases (Cu-NiRs) show a common reaction center, containing a pair of copper ions, type-1 Cu and type-2 Cu. The type-1 Cu site has (His)3-Cys-Met donor set, which is typical for electron-transfer copper proteins. On the other hand, the type-2 Cu site has three histidine ligands, (His)3, and a water as the fourth ligand in the resting state. The type-2 Cu site has been suggested to be a reaction center where nitrate is reduced to NO with an electron transferred from the type-1 Cu site. The (His)3 coordination environment is highly conserved in all Cu-NiRs. In order to investigate the functional role of the (His)3 coordination environment in the type-2 Cu site, we set out to prepare copper(I)-nitrite complexes from stericly hindered tris(4-imidazolyl)carbinol (TIC) and tris(pyr azolyl)methane (TPM) ligands to mimic the (His)3 coordination environment in the type-2 Cu site of CuNiR. Particularly, the copper(I) nitrite complex having sterically hindered tris(4-imidazolyl)carbinol is expected to be the best model complex for the copper(I) nitrite complex in CuNiR. We report here synthesis, structural features, spectroscopic properties, and reactivity of copper(I) nitrite complexes with sterically hindered TIC and TPM ligands.

O033
Unnatural Amino Acid Containing Mutants of KatG Exhibit Enhanced Peroxidase Activity
Rania Damianis, Reza A. Ghiladi1, North Carolina State University, Raleigh, NC, USA. Contact e-mail: Reza_Ghiladi@NCSU.edu

Catalase-peroxidases (KatG) are bifunctional hemoproteins possessing both catalase and peroxidase activities. The crystal structures of different KatGs each revealed the presence of a novel active site modification comprised of two covalent bonds between three amino acid side chains: Trp107, Tyr229, and Met255 (Mycobacterium tuberculosis numbering). Absent from these peroxidases, this Met-Tyr-Trp ‘crosslink’ has been suggested to impart catalase activity to the KatG, although how it carries out this function has been the subject of recent debate. To better understand the role which this crosslink plays in enzyme catalysis, we have expressed several KatG(Tyr229AA) mutants, where UAA represents the following unnatural amino acids: p-Ac, p-NH2, p-N, p-Br, p-I, and p-OMe derivatives of phenylalanine. Notably, these mutants exhibit up to a 300-fold increase in peroxidase catalytic efficiency ($k_{\text{cat}}/K_m$) compared to the WT enzyme (see Table). Specifically, $k_{\text{cat}}$ was enhanced by ~20-60 fold, whereas $K_m$ was improved slightly (~1-8 fold), indicating that the gain in catalytic efficiency is mainly from increasing the rate of the peroxidase reaction, with only a minor contribution from improving substrate binding. While these KatG mutants were unable to form Met-UAA-Trp crosslinks, resulting in loss of catalase activity, they nevertheless represent one of the first examples in hemoprotein engineering where an enzymatic activity has been enhanced through the use of UAAAs. Details of these and our complementary stopped-flow UV-visible spectroscopic studies will be presented.

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\begin{array}{cccc}
\text{Peroxidase Activity of WT KatG and Selected Mutants} \\
\hline
\text{WT} & 0.062 \pm 0.001 & 8.44 \pm 0.45 & 7.3 \pm 0.4 & 1 \\
\text{p-Br-F} & 1.27 \pm 0.08 & 1.50 \pm 0.21 & 849 \pm 99 & 116 \\
\text{p-Ac-F} & 2.37 \pm 0.01 & 2.60 \pm 0.04 & 910 \pm 12 & 124 \\
\text{p-I-F} & 1.70 \pm 0.02 & 1.57 \pm 0.08 & 1083 \pm 55 & 148 \\
\text{p-NH2-F} & 3.46 \pm 0.23 & 2.37 \pm 0.23 & 1461 \pm 141 & 200 \\
\text{p-OMe-F} & 2.17 \pm 0.07 & 1.13 \pm 0.07 & 1920 \pm 120 & 263 \\
\text{p-N3-F} & 2.36 \pm 0.03 & 1.09 \pm 0.06 & 2155 \pm 117 & 295 \\
\hline
\end{array}
\]

O034
Design, synthesis, and structures of tethered substrates as probes of the active sites of cytochrome P450cam, cytochrome c peroxidase, and nitric oxide synthase.
David B. Goodin1, Edith Glazer1, Richard Wilson1, Andrew Annalora1, Stefan Vetter2, Harry B. Gray3, 1The Scripps Research Institute, La Jolla, CA, USA; 2Florida Atlantic University, Boca Raton, FL, USA; 3California Institute of Technology, Pasadena, CA, USA. Contact e-mail: dbg@scripps.edu

We are exploring the use of synthetic molecular wires to probe the active site and function of heme enzymes including P450cam, cytochrome c peroxidase (CcP), and nitric oxide synthase (NOS). These wires, consisting of ligand analogs tethered to a reporter or substrate, are designed to bind specifically to the active site channel of a given enzyme. Adamantane analogs linked through hydrocarbon or polyether linkers bind in the active site channel of P450cam, inducing a large range of conformational changes in the F and G helix regions. Benzimidazole analogs attached to peptide linkers bind to an engineered channel in place of the electron transfer pathway of CeP, allowing the electron transfer to be probed through an electrode interface. Finally, analogs of 6(R)-tetrahydro-L-biotin (I4H) linked to Ru based photosensitzers bind to perin free iNOS. We will report on the synthesis, structures, binding and functional properties of these compounds to their target enzymes.
O035
Redox properties of the Fe-hydrogenase homologue Nar1 from Saccharomyces cerevisiae
Peter L. Hagedoorn1, G. P. H. van Heusden2, H. Y. Steensma3, W. R. Hagen1, 1Department of Biotechnology, Delft University of Technology, Delft, The Netherlands; 2Institute of Biology Leiden, Leiden University, Leiden, The Netherlands.
Contact e-mail: p.l.hagedoorn@tudelft.nl

Fe-hydrogenases are hydrogen producing and consuming enzymes that occur in bacteria and anaerobic unicellular eukaryotes. The catalytic site is a unique iron-sulfur cluster with organometallic CO and CN ligands that has been named the H-cluster [1]. Genomic information from higher eukaryotes revealed a family of proteins (Narf-family) that share significant homology with the Fe-hydrogenases. All the amino acids that are involved in the coordination of the H-cluster are conserved in the Narf-family. Recently convincing evidence has emerged of involvement of the S.cerevisiae Narf-family member, Nar1, in cytosolic iron-sulfur cluster biosynthesis [2]. In order to study the iron-sulfur clusters of Nar1 we have cloned and over-expressed the protein in S.cerevisiae and E.coli. EPR monitored redox titrations were performed to characterize the iron-sulfur cluster(s) and additional paramagnetic centers in the protein. Evidence of substoichiometric [3Fe-4S] cluster and stoichiometric mononuclear Fe and [4Fe-4S] cluster was found (Fig. 1). Furthermore evidence for oxidative Fe-cluster loss and subsequent protein aggregation was found.

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O036
Pre steady state and steady state kinetic studies into the reaction cycle of the W/Fe/S enzyme formaldehyde oxidoreductase from Pyrococcus furiosus.
Emile Bol, Nicolette Broers, Wilfred R. Hagen, Delft University of Technology, Delft, The Netherlands.
Contact e-mail: w.r.hagen@tudelft.nl

The hyperthermophilic archaean Pyrococcus furiosus has a highly specific ABC transporter for the internalization of tungstase [1], which it uses to make five tungstoenzymes with a wide range of aldehyde oxidizing activities [2]. One of the enzymes, formaldehyde : ferredoxin oxidoreductase (FOR) activates free (i.e. non-hydrated) formaldehyde [3]. In addition to a tungsto-bispterin cofactor these enzymes contain an electron transferring iron-sulfur cubane that heavily dominates the optical spectra making mechanistic studies through optical monitoring difficult. EPR monitoring of paramagnetic W(V) intermediates is not a viable alternative since substrate oxidation is essentially an electron pair reaction coupled to the one-step reduction of W(VI) to W(IV) [3]. However, molybdopterin and tungstopterin cofactors do exhibit relatively weak optical absorptions, typically $e_{\text{mole}} = 2 \text{ cm}^{-1} \text{ mM}^{-1}$, and this property has been used previously in a pre steady state kinetics study on DMSO reductase, an enzyme that has a single molybdopterin cofactor only. We have now identified substrate-induced weak optical changes of the tungstopterin in P. furiosus FOR, and we have used these for monitoring pre-steady state kinetics employing stopped-flow absorption spectrometry. The results, combined with tryptophan stopped-flow fluorescence and with data from steady-state kinetics and from EPR titrations, form the basis for a proposal for the catalytic cycle of this W/Fe/S enzyme.

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O037
UV resonance Raman investigation of the functional mechanism of Ec DOS protein: Utilization of the heme 6-propionate hydrogen bond network
Teizo Kitagawa1, Samir F. El-Mashtoly1, Hiroto Takahashi2, Toru Shimizu3, 1Okazaki Institute for Integrative Bioscience, Okazaki, Japan; 2Institute of Multidisciplinary Advanced Material, Tohoku University, Sendai, Japan.
Contact e-mail: teizo@iims.ac.jp

The direct oxygen sensor protein from Escherichia coli (Ec DOS) is a heme-based signal transducer protein responsible for phosphodiesterase (PDE) activity. Binding of O2 or CO to a heme enhances the PDE activity towards c-di-GMP. We report ultraviolet resonance Raman (UVRR) spectroscopic investigations of the reduced, O2- and CO-bound forms of its heme-bound PAS domain (Ec DOSH). The UVRR results show that heme discriminates different ligands, resulting in altered conformations in the protein moiety. Specifically, the environment around Trp53 that contacts heme 2-vinyl group, shifts towards more hydrophobic upon O2 binding, while towards more hydrophilic upon the CO-binding. The PDE activity of the O2- and CO-bound forms for W53F mutant is significantly decreased compared with that of WT, demonstrating the importance of Trp53 in its function. Binding of O2 or CO to the heme produces drastic changes in the Tyr126 at the surface of the sensor domain. Furthermore, we found that Asn84 forms a hydrogen bond with Tyr126 either in the O2- or CO-bound forms but not in the reduced form. The PDE activities of the ligand bound forms for Asn84Val and Tyr126Phe mutants are significantly reduced compared with that of WT, suggesting the importance of the hydrogen-bonding network from heme 6-propionate to Tyr126 through Asn84 in signal transmission.

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Figure 1. EPR spectra of S.cerevisiae Nar1.

![EPR spectra](image-url)
O038
The S = 3/2 [4Fe-4S]+ cluster of the nitrate reductase A (NarGHI) from Helicobacter pylori: Characterisation and implication during the maturation pathway of the complex
Pascal Lanciano1, Stéphane Grimaldi1, Alexandre Vergnes2, Adrien Sauvaget2, Axel Magalon2, Patrick Bertero1, Bruno Giugliarelli1, *Laboratoire de Bioénergétique et Ingénierie des Protéines, Marseille, France; 2Laboratoire de Chimie Bactérienne, Marseille, France.
Contact e-mail: lanciano@ibsm.cnrs-mrs.fr

Nitrate reductase A from Escherichia coli is a membrane-bound molybdoenzyme which catalyzes the reduction of nitrate to nitrite. This respiratory complex is composed of a catalytic subunit (NarG) containing a Mo-bisMDG cofactor (Moco), an electron transfer subunit (NarH) carrying four FeS clusters, and a membrane subunit (NarI) with two b-type hemes (1). The resolution of the 3D structure of NarGHI has revealed the existence of a fifth FeS cluster (FeS0) with a His-(Cys)3 coordination located close to the Moco (2). In the first EPR studies this cluster had escaped detection but recently, low intensity EPR lines around g = 5 and typical of a S = 3/2 species were proposed to arise from it (3). A detailed analysis of the EPR signal of this S = 3/2 species enabled us to quantify accurately its stoichiometry. Additionally, by mutating its amino acid ligands we assigned definitively the S = 3/2 signal to the FeS0 center. Moreover we have taken advantage of its peculiar characteristics to show that the FeS0 cluster and the Moco are strongly related during the metal insertion processes occurring in the biogenesis pathway of the enzyme (4). In particular, we demonstrate that the sequential insertion of these two metal centers is orchestrated by a single accessory protein.

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O039
DNA-binding properties of Helicobacter pylori NikR
Yanjie Li, Deborah B. Zamble, University of Toronto, Toronto, ON, Canada.
Contact e-mail: yli@chem.utoronto.ca

Helicobacter pylori (H. pylori) is a Gram-negative gastric pathogen that is responsible for the majority of peptic ulcer diseases and significantly increases the risk of gastric cancers in humans (1). H. pylori can survive severe acidic shock in the stomach. Nickel, as a major regulator of the protective response against acid (2), is responsible for the majority of peptic ulcer diseases and can survive severe acidic shock in the stomach. Nickel, as a nickel-responsive transcription factor that controls many important cellular processes such as urease expression and nickel uptake either directly or indirectly and was also proposed to be a nickel-binding protein (3). The investigation of the DNA-binding affinity of HpNikR on various promoters under different conditions is important for understanding the mechanism by which HpNikR regulates H. pylori physiological functions. Our previous work has shown that HpNikR nickel-dependently binds to different promoters (4).

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O040
Structures of intermediates in the oxygen activation mechanism of extradiol dioxygenases
Elena G. Kovaleva, John D. Lipscomb, University of Minnesota, Minneapolis, MN, USA.
Contact e-mail: lipsc001@umn.edu

The homotetrameric enzyme homoprotocatechuate 2,3-dioxygenase (HPCD) catalyzes proximal extradiol ring-cleavage of catecholic substrates. Substrate binding to the active site Fe2+ promotes binding and activation of O2 as well as the subsequent insertion of both oxygen atoms to yield the ring-opened product. It is shown here that the intermediates in the reaction mechanism can be structurally characterized by conducting the reaction in a crystal of HPCD. When the crystal is soaked in the slow substrate 4-nitrocatechol (4NC) in a low O2 atmosphere, 3 of the 4 nominally identical subunits in the asymmetric unit contain different intermediates, none of which have been previously characterized [1]. The high-resolution crystal structure shows that these are: (I) a side-on bound Fe2+-superoxo-4NC-semiquinone species, (II) an Fe2+-alkylperoxo species, and (III) the product complex. Species I shows that electron density is transferred from the aromatic substrate to oxygen via the iron, thereby activating both substrates. This first step in O2 activation may apply broadly in the 2-His-1-Carboxylate facial triad non-heme iron enzyme family. Intermediate II, shows that oxygen attack occurs before O-O bond cleavage and reveals the position of attack as 4NC-C2. This is the key intermediate in the extradiol dioxygenase mechanism. Similar alkylperoxo species have been proposed for many other oxygenases, but never observed. Supported by NIHGM24689. [1] Kovaleva, E. G., and Lipscomb, J. D. (2007) Science, in press.

O041
Catalase-peroxidase mutant KatG[Ser315Thr] from M. tuberculosis exhibits unique heme iron structure and catalytic function despite the remote locus of residue 315.
Richard S. Magliozzo1,2, Xiangbo Zhao2, Johannes Schelvis3, Shengwei Yu1, Kalina Ranguelova1, 1Brooklyn College, Brooklyn, NY, USA; 2The Graduate Center, New York, NY, USA; 3New York University, New York, NY, USA.
Contact e-mail: rmagl02@brooklyn.cuny.edu

Catalase-peroxidases (KatG) are Class I heme enzymes that exhibit dual activities. In M. tuberculosis, the organism causing TB, the peroxidase activity of KatG is responsible for activation of the old antibiotic isoniazid (INH), leading to formation of an acyl-NAD (INH-nicotinamide adenine dinucleotide) adduct regarded as the bactericidal molecule. The INH-NAD molecule inhibits M. tuberculosis InhA (enoyl-acyl carrier protein-reductase) and thereby, cell wall (mycolic acid) biosynthesis. The KatG mutant in which serine 315 is replaced by threonine is responsible for the majority of the world’s INH-resistant TB infections. According to optical, resonance Raman and EPR spectroscopy, KatG[Ser315Thr]...
exhibits stable 5-coordinate heme iron in HS and the unusual QS spin states, while wild-type KatG, after similar purification and brief storage, accumulates 6-c forms. These differences result from a structural change at the heme periphery nearby residue 315. A biomimetic approach was used to characterize the catalytic competence of KatG and KatG[S315T] in the activation of INH under physiologically relevant conditions, where a slow flux of H2O2 (from glucose/glucose oxidase) initiates peroxidase function. The mutant enzyme only produced a good yield of INH-NAD in the presence of high concentrations of INH, relative to those used exclusively by wild-type KatG, under conditions where the peroxidase intermediates should be produced equally. Isothermal titration calorimetry demonstrated low affinity of the mutant enzyme for INH. The 3-dimensional crystal structures revealed a narrowed substrate access channel in KatG[S315T] and the origin of antibiotic resistance due to the steric effects of the methyl group of threonine.

**O042**

**NO-Synthases as a direct source of oxidative and nitrative stress.**

Amandine Maréchal, Tony A. Mattioli, Dennis J. Stuehr, Jérôme Santolini, CEA IBi Tec-S, Gif-sur-Yvette, France; Cleveland Clinic Foundation, Cleveland, OH, USA.

Contact e-mail: amandine.marechal@cea.fr

Nitric oxide (NO) is a major biological mediator generated in mammals exclusively by proteins from the NO-Synthase family (NOS). To explain their implication in both processes of signalling and cytotoxicity, it has been proposed that after NO biosynthesis, NOSs can partition in two distinct catalytic cycles (signalling) i) Fe III NO complex, whose dissociation produces NO, and (cytotoxicity) ii) Fe IIINO complex, whose oxidation produces other reactive nitrogen species (toxicity) with transient formation of peroxynitrite (PN). To investigate NOSs' ability to generate oxidative stress, we studied the model reaction [Fe III + PN]. We showed by rapid kinetic measurements that the inducible isoform (iNOS) was activating PN, enhancing its nitrative potency, with potential damage for biomolecules. This PN activation led to NOS self-inhibition which could serve as a physiological feedback regulation.

Lately we investigated the activation of PN by other NOS isoforms, involved in different physiological pathways. Each isoform displays a distinct PN activation mechanism ranging from toxicity enhancement to detoxification.

Finally, we have been investigating the structure-reactivity relationship of NOS Fe III NO complex by combining spectroelectrochemistry, pulsed and classic EPR, and resonance Raman spectroscopy. We focus on parameters such as the heme structure and environment. Correlated with the kinetic analysis of Fe III NO oxidation, these results will lead us to suggest parameters that could promote in situ PN production.

This work open new perspectives for the study and the understanding of NOSs functioning and biological role.

Footnotes

1 Maréchal A., Mattioli T.A., Stuehr D.J., Santolini J., J Biol Chem. 2007, in press.

**O043**

**Mechanistic studies of the ethylene-forming enzyme ACC oxidase.**

Liviu M. Mirica, Judith P. Klinman, University of California, Berkeley, CA, USA.

Contact e-mail: liviu@berkeley.edu

Our studies have focused on 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase, an ascorbate-dependent non-heme iron enzyme that produces the plant hormone ethylene, important in many aspects of plant growth and development (i.e. germination, fruit ripening, and senescence). In order to study the mode of O2 activation by ACC oxidase, steady- and pre-steady-state kinetic analyses and 18O kinetic isotope effect measurements were employed to determine the order of substrate binding, the nature of the activated iron-oxygen species involved in substrate oxidation, and to characterize the intermediates that accumulate during catalytic turnover. Several ACC substrate analogues that can inhibit ethylene production have been kinetically investigated with the goal of designing a mechanism-based inhibitor. The use of radioactively labeled analogues has been employed to determine the fate of substrate breakdown products. Recent detection of radioactive labeling of specific residues in the active site and subsequent enzyme inactivation suggests an alternative mechanism for substrate oxidation in ACC oxidase.

**O046**

**Purification and structural analysis of Vanadium Nitrogenase activator in Azotobacter Vinelandii.**

Hiroshi Nakajima1, Takatani Nobuyuki, Iho Mitsuko, Shigetoshi Aono, Yoshihito Watanabe, Nagoya Univ., Nagoya, Japan; Institute of Molecular Science, Okazaki, Japan; Nagoya Univ. Research Center of Materials Science, Nagoya, Japan.

Contact e-mail: hnakajima@mbox.chem.nagoya-u.ac.jp

Azotobacter vinelandii harbors three genetically distinct nitrogenases whose expression are regulated by corresponding transcriptional activators. VnfA is the activator and known to bind an upstream sequence of vnf operons to express vanadium nitrogenase. Previous studies revealed several properties of VnfA based on in vivo activity assays, while investigation on the molecular structure including a sensing mechanism of an effector molecule was thwarted due to difficulty to purify VnfA. In the present study, we have achieved purification of wild type VnfA and preparation of the variants where Cys residues are replaced to alanine to inspect the role of Cys residues since Cys rich sequences in N-terminus of VnfA, Cys8-X-Cys10-XXXX-Cys15 has been suggested to bind any metals or metal clusters to form the sensory part. Spectroscopic analysis in vivo and in vitro found the presence of 3Fe-4S type clusters ([Fe3S4]2+) in transcriptionally active VnfA, and ICP-OES analysis indicated one [Fe3S4] in a VnfA monomer. Purified VnfA had a homo-tetrameric form regardless of the presence or absence of [Fe3S4], while VnfA bearing [Fe3S4] showed specific binding affinity to target DNA exclusively, suggesting that [Fe3S4] is responsible to activate VnfA. This is also evidenced by in vivo activity assays in which addition of metal chelator, α-phenanthroline to growth media of the assay strain decrease the transcriptional activity of VnfA by 80 %. Mutagenesis analysis supposes Cys10 and 15 serves as ligands of the cluster.
Superoxide reductase SOR is a novel class of non-heme iron proteins that catalyzes the one-electron reduction of $O_2$ to $H_2O_2$, providing an antioxidant defense in some bacteria. Its active site consists of an unusual non-heme $Fe^{3+}$ center in a [His 4 Cys1] square pyramidal pentacoordination, and was proposed to form $Fe^{3+}$-peroxo intermediate species during the catalytic cycle.

FTIR studies on the SOR from *Desulfoarculus bajersi* reveal that the protonated carboxylate group of Glu114 is in interaction with the cysteine ligand. Resonance Raman spectroscopy demonstrates that mutation of Glu114 into alanine results specifically in a weakening of the S-Fe bond and increases the extent of freeze-trapping of a $Fe^{3+}$-peroxo species after treatment with $H_2O_2$ by a specific strengthening of the Fe-O bond [1].

Crystals of the E114A mutant were reacted with $H_2O_2$ in order to trap $Fe^{3+}$-(hydro)peroxo species in the crystals. The structure of the $Fe^{3+}$-(hydro)peroxo species in the SOR active site was determined at 1.8 Å resolution. Three different conformations around the $Fe^{3+}$-(hydro)peroxo intermediates were characterized. The data highlighting the role of a key water molecule finely controlled by the enzyme dynamics and reveal the formation of transient hydrogen bond networks which assist the cleavage of the Fe-O bond in order to release the reaction product, hydrogen peroxide [2].

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O047

**Investigating the role of a highly conserved glutamate in reaction intermediate of superoxide reductase**

**Joao V. Rodrigues**, Lígia M. Saraiva, Bruno L. Victor, Harald Huber, Cândido M. Soares, Diane E. Cabelli, Miguel Teixeira, 1Instituto de Tecnologia Química e Biológica, Oeiras, Portugal; 2Lehrstuhl fuer Mikrobiologie der Universitaet Regensburg, Regensburg, Germany; 3Brookhaven National Laboratory, Upton, NY, USA.

Contact e-mail: joaovr@ibtb.unl.pt

Superoxide reductases (SORs) are antioxidant enzymes that eliminate the toxic superoxide anion solely by the reductive pathway in contrast with their counterparts, superoxide dismutases (SODs) that catalyze both reduction and oxidation of this radical. In this reaction SOR utilize cellular reducants to convert superoxide into hydrogen peroxide without forming molecular oxygen. These proteins are associated with anaerobic lifestyle since they are found only in anaerobic or microaerophilic prokaryotes, and in many cases where the canonical SODs are absent, they constitute the only known protecting system against superoxide.

The reaction mechanism of SOR from the hyperthermophilic archaean *Archaeoglobus fulgidus* was previously studied by pulse radiolysis, allowing the observation of a putative ferric-hydroperoxo intermediate, which decayed upon protonation to produce hydrogen peroxide. We also demonstrated the role of glutamate (E112) in the protonation of the $Fe^{3+}$-OOH intermediate; this residue also functions as the sixth ligand to the oxidized iron center. The glutamate ligand is highly conserved among known SORs with only a few exceptions. Here we study by pulse radiolysis the reduction of superoxide by a natural glutamate-lacking “mutant”, and show that this enzyme is fully functional. This suggests that the enzyme has evolved divergently to be able to efficiently protonate the intermediate in the absence of a glutamate.

O048

**Spectroscopic properties and reactivity of a nitric oxide synthase-like protein from Bacillus subtilis**

**Isabelle Salard-Arnaud**, Jean-Luc L. Boucher, Daniel Mansuy, Dennis J. Stuehr, Université R. Descartes Paris 5, UMR8601 CNRS, Paris, France; 2Lerner Research Institute, Cleveland, OH, USA.

Contact e-mail: isabelle.salard@wanadoo.fr

Mammalian nitric oxide synthases (NOS) are heme-thiolate proteins that catalyse the oxidation of L-arginine to citrulline and nitric oxide with N$^\text{•-}$hydroxy-L-arginine (NOHA) as an enzyme-bound intermediate. Recent genomes sequencing revealed that genes related to the oxygenase domain of mammalian NOSs exist in some gram-positive prokaryotes. Some of these proteins have been sequenced, cloned and purified. Recombinant bacterial NOS have been shown to produce nitrite and citrulline from NOHA and hydrogen peroxide [1]. In association with a mammalian reductase domain, NOS from *Bacillus subtilis* is able to form nitric oxide in single turnover experiments. Recently, a NOS-like protein from *Streptomyces turricacaides* has been implicated in the nitration of a dipeptide phytotoxin required for plant pathogenicity [2,3]. However, despite many studies, the role(s) and biological function(s) of these prokaryotic NOSs remain unclear.

We have performed a spectroscopic study of these new heme-proteins using UV-visible and EPR spectroscopy, and investigated their in vitro reactivity. We have demonstrated that a recombinant NOS from *Bacillus subtilis* catalyses the transformation of several hydroxyguanidines to the corresponding ureas and cyanamides and the nitration of phenol in a peroxodase catalytic pathway.

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**O050**

The 3-His ligated nonheme Fe(II)-centre in Diketone Dioxygenase Dkek1: probing the metallocentre's intrinsic reactivity and the role of the metal ion's second coordination sphere in catalysis.

Grit D. Straganz, Sigrid Egger, Bernd Nidetzky, Graz University of Technology, Graz, Austria.
Contact e-mail: grit.straganz@tugraz.at

Diketone cleaving dioxygenase Dkek1, which has been isolated from Acinetobacter johnsonii, shows unique catalytic properties, oxygenatively cleaving the C=C bond of enolizable diaxetones to the corresponding acid and aldehyde. Its active site shows an apical nonheme Fe(II) centre, which is not ligated via the 'classical' 2-His-1-carboxylate motif but via a facial triad of three histidines (1). The substrate's electronic properties govern the rate of dioxygen reduction and results from quantitative structure-activity relationship analysis lead to the proposal of a chemical mechanism of diketone cleavage in Dkek1 (2).

But what role does the protein environment play in catalysis? Investigating the impact of the metal's second coordination sphere on particular steps of catalysis by mutational and kinetic analysis, we identified a gate constituted of three amino acid residues that promotes diketone binding and oxidation. A basal reactivity of the metallocentre towards 'alien' (non-beta-dicarbonyl) substrates, on the other hand, is apparently insensitive towards alterations in the other metal's second sphere, and demonstrates the intrinsic reactivity of the 3-His metallocentre towards 'typical' dioxygenase substrates. Taken together, our findings give interesting insights into the interplay of metallocentre, protein environment and substrate structure in Dkek1.

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**O051**

Monitoring the redox state of the laccase type-1 and Cu3 copper centres by fluorescence

Armand W. J. W. Tepper1, Florian G. de Brouwer1, Erik Vlijgenboom1, Thijs J. Aartsma2, Gerard W. Canters1, 1Leiden Institute of Chemistry, Leiden, The Netherlands; 2Leiden Institute of Physics, Leiden, The Netherlands.
Contact e-mail: w.tepper@chem.leidenuniv.nl

Many redox active cofactors change color upon changing oxidation state or the binding of a ligand. For this reason, UV-Vis spectroscopy is a principal technique in studying the structure and function of redox proteins. Its downsides are the low sensitivity and selectivity. To circumvent these problems, we have used fluorescent labeling to translate these absorption changes into a fluorescence signal with the benefits of enhanced selectivity combined with single-molecule sensitivity1. Here we report on an extension of this methodology to study redox processes in SLAC, a multicopper laccase from *S. coelicolor*. It couples the four electron reduction of dioxygen with the oxidation of small organic substrates. Laccases contain two chromophores: a blue type-1 copper site, which accepts electrons from reducing substrate, as well as a green trinuclear Cu3 copper cluster, which is the site of O2 reduction (see figure). The optical absorption of these centers depends on their oxidation state. We show that the redox-state of the type-1 site and the Cu3 cluster can be simultaneously and independently monitored by following the emission of two different fluorescent reporters. Several examples illustrating the technique as well as potential applications will be discussed.

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The Role of SlyD in Nickel Incorporation in *Escherichia coli* [NiFe] Hydrogenase Biosynthesis Pathway

Jie Wei Zhang, Deborah Zamble, University of Toronto, Toronto, ON, Canada.

Contact e-mail: k.zhang@chem.utoronto.ca

E. coli expresses at least three [NiFe] hydrogenase enzymes, which catalyze the production or consumption of hydrogen gas and occupy a central place in the energy metabolism of anaerobic bacteria. The assembly of the hydrogenase metalloenzymes requires SlyD and the proteins encoded by the hyp genes (hyp:ABCDEF) [1, 2]. SlyD is a member of the FK-506 binding protein family of Peptidyl-prolyl Isomerase (PPIase) [3]. In addition to its PPIase domain, SlyD is composed of a molecular chaperone domain and a C-terminal tail rich in potential metal binding residues. SlyD directly interacts with HypB and the disruption of the SlyD-HypB complex results in deficient hydrogenase production in vivo [2, 4].

To examine the role of SlyD in hydrogenase biosynthesis, mutations that disrupt the PPIase activity were prepared. SlyD(D42S, F132Y) and SlyD(D42S), which displayed 1% and 40% of wild-type levels of PPIase activity respectively, could restore 61% ±10 and 41% ±9 of total hydrogenase activity in vivo. Both mutants interacted with HypB in vitro and accumulated the same level of cellular nickel under anaerobic growth condition. These results suggest that the PPIase activity of SlyD does not contribute to cellular nickel accumulation or hydrogenase activity. Additional experiments suggest that the SlyD-HypB complex may play a role in nickel storage in E. coli.

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POSTERS

**P099**

**Spectrophotometric determination of ascorbic acid with laccase oxidizing ABTS**

**Myriam Abad**, Virginia Tomas, Ramiro Martinez-Gutierrez, Francisco Garcia-Canovas, Jose Tudela, GENZ-Grupo de investigación Enzimología, Universidad de Murcia, Spain; Dept. Quimica Analitica, Universidad de Murcia, Spain; NOVOZYMES Spain S.A., Madrid, Spain; GENZ (http://www.um.es/genz), Universidad de Murcia, Spain.

Contact e-mail: myabad@um.es

Laccase (EC 1.10.3.1) is a four copper oxidase that catalyzes the oxidation by molecular oxygen of phenols up to phenoxyl radicals, which can evolve up to quinones. These are reduced by ascorbic acid (vitamin C) which oxidizes to dehydroascorbic acid. The spectrophotometric assays of laccase oxidizing ABTS in presence of ascorbic acid, they show a lag period proportional to the concentration of this vitamin. The assay conditions, reaction medium, concentration of enzyme and of chromogenic substrate have been optimized. A broad linear range with submicromolar limits of detection and of quantitation of ascorbic acid has been reached. The sensitivity of the method was higher for *Trametes versicolor* than for *Gliomaras bisporeus* laccase. The test is sensitive, precise and quick and requires low quantities of sample. The method has been applied to assays of quality control in drugs that contain vitamin C as active ingredient. Furthermore, the method is useful to evaluate the antioxidant activity of ascorbic acid. The assay does not require electrochemical neither spectrofluorometric instruments. The method is applicable on spectrophotometers (1mL cuvette) and on multwell microplate readers, which favours its mechanization for high throughput screen assays.

The authors are grateful to NOVOZYMES SPAIN S.A. by free samples of laccase. This work has been partially supported by grants from several Spanish organizations. (J.T.) Projects 00672/PI/04 Fundacion Seneca and BioCARM BIO-BMC 06/01/0004, CARM, Murcia; as well as Project BIO2006-15363, MEC, Madrid. (R.M.G.) NOVOZYMES SPAIN S.A. (M.A.) Doctorate fellowship BES-2004-3829, FPI-MEC, Madrid.

**P100**

**Rubredoxin as a paramagnetic shift-inducing probe**

Rui M. Almeida, Sofia R. Paulista, Isabel Moura, Jose J. G. Moura, REQUIMTE/CQFB, Caparica, Portugal.

Contact e-mail: rui.almeida@dq.fct.unl.pt

Rubredoxin (Rd) is a small protein (~5 kDa), containing a simple redox centre (one iron atom coordinated to four cysteinyl residues), present in all species of the *Desulfovibrio* genus. Rd has been assigned to play an important role in the oxygen detoxification pathway, donating electrons to enzymes such as superoxide reductase and rubredoxin:oxygen oxido-reductase. In the oxidized form, the inherent high-spin of the Fe(III) ion can be used to probe the interaction site of other metalloproteins, in particular using NMR methods, due to the induced pseudocontact shift and linewidth broadening. In this study, we used *Desulfovibrio gigas* Rd (both Fe and Zn-substituted forms) and cytochrome c5, a tetraheme containing protein, isolated from the same organism, as a model of a transient electron transfer complex. In order to determine these effects we have used 1H NMR titrations. We have observed that increasing amounts of rubredoxin induce selective heme methyl shifts and linewidth broadenings (only with the Fe form), and an apparent Kd of ~10 μM was determined. These results are in agreement with the higher ranked complexes obtained using a soft-docking algorithm program, BiGGER1, that predicted a cluster of solutions with Rd binding near the heme IV of cytochrome c5.

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**P101**

**Biochemical and catalytic characterization of Desulfoviridin from *Desulfovibrio desulfuricans* ATCC 27774 - towards the development of a sulfite biosensor**

Alexandra S. Serra, Isabel Moura, José J. G. Moura, Maria G. Almeida, REQUIMTE/CQFB, Dept. Quimica, Faculdade de Ciências e Tecnologia, Monte Caparica, Portugal; Escola Superior de Saúde Egas Moniz, Monte de Caparica, Portugal.

Contact e-mail: mga@dq.fct.unl.pt

Thanks to their antimicrobial and antioxidant activities, sulfur dioxide and sulfites have been used as food additives for many years. However, sulfites have been occasionally implicated as asthma triggers [1]. As a consequence, the EC Directive 95/2/EC includes sulfites in the list of preservatives and antioxidants endorsed in specified products with a Maximum Permitted Level [2]. The classical methods for sulfite determination in food products are time consuming and have a low sensitivity. Therefore, it is very important to develop a new analytical tool capable of easily...
quantifying sulfites in complex samples. With the aim of constructing an electrochemical sulfite biosensor based on a selective enzyme, we have been studying and characterizing the respiratory sulfite reductase from *Desulfovibrio desulfuricans* ATCC 27774 - Desulfoviridin (Dsv). The enzyme is composed by three subunits of ca. 50, 40 and 11 kDa, all of them showing a strong homology to Dsv from *Desulfovibrio vulgaris* at the N-terminal. The iron content, as determined by ICP-AES was slightly lower than expected (17±4). From the biosensor point-of-view, the low specific activity of Dsv (mmol·min⁻¹ range) and the instability of sulfites in solution constitute important drawbacks that should be overcome. In this regard, we also examined by cyclic voltammetry the ability of a set of electronic mediators alternative to methyl viologen (phenoxyaniline, meldonia blue/NADH, neutral red, benzyl viologen and bromophenol blue) to efficiently shuttle electrons to Dsv.

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**P102**

Novel non-heme iron lipoxygenases from cyanobacterium *Nostoc punctiforme*

**Alexandra Androu**, Imke Lang, Ivo Feussner, Dept. of Plant Biochemistry, Georg-August-University of Göttingen, Albrecht-von-Haller-Institute for Plant Sciences, Göttingen, Germany.  
Contact e-mail: androu@gvg.d.de

Lipoxygenases (LOXs) are a family of structurally related non-heme iron containing dioxygenases. They catalyse the insertion of molecular oxygen into polyunsaturated fatty acids that contain one or more 1,4-pentadiene moieties to give the corresponding hydroperoxides. LOX products can be further metabolized to yield signaling molecules such as aldehydes and jasmonate. The insertion of oxygen exhibits high regio- and stereo-specificity. This specificity is dependent on the primary sequence of the enzyme, which is predicted to determine the orientation and depth of substrate penetration into the active site.

Although there is an abundance of information on plant and animal LOXs, the number of prokaryotic LOXs that have been described is limited. Prokaryotic and eukaryotic algae are known as sources of biologically active compounds among which are alcohols, alkanes, aldehydes and branched hydrocarbons, which may derive from biologically active compounds among which are alcohols, alkanes, aldehydes and branched hydrocarbons, which may derive from

**P103**

Octaheme tetrahionate reductase (OTR) is involved in the reactions of the nitrogen cycle

**Sally J. Atkinson**, Vivek A. Jassal, Emma L. Rothery, Chris G. Mowat, Caroline S. Miles, Graeme A. Reid, Stephen K. Chapman, University of Edinburgh, Edinburgh, United Kingdom, 2Institute of Cell and Molecular Biology, Edinburgh, United Kingdom.  
Contact e-mail: S.J.Atkinson@sms.ed.ac.uk

*Shewanella oneidensis* MR-1 is a gram-negative *γ*-proteobacterium isolated from the freshwater Lake Oneda, USA. Genomic analysis of this metal-reducing bacterium has identified 39 genes encoding c-type cytochromes[1] (determined using the c-type heme attachment motif, CXXCH), a large proportion of which contain multiple heme groups. This profusion of cytochromes confers upon the organism a wide respiratory flexibility and the ability to utilise a diverse range of electron acceptors, including Fe(III) and Mn(IV), nitrite, nitrate, thiosulfate, sulfate and fumarate.

A putative octaheme cytochrome, containing eight CXXCH motifs, with no homologs of known function has been identified and overexpressed. This protein has been purified and identified as a tetrahionate reductase, and the structure has been solved to 2.2 Å[2],[3] Despite the presence of eight conventional heme-attachment motifs, the protein structure revealed the presence of seven normally ligated c-type heme groups and one unusual lysine-ligated CXXCK heme at the active site. This unconventional lysine heme ligation has been previously observed only in the pentaheme nitrite reductases, however in these cases lysine ligation was predicted by a CXXCK motif.

Assays to determine potential substrates for this enzyme have shown activity with a range of nitrogen-containing compounds, including nitrite, nitric oxide, nitrous oxide and hydroxylamine. Additionally, a mutant of the ligating lysine has been prepared and work is currently underway to determine its structure and kinetics.

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**P104**

Investigation of the catalytic cycle of laccase immobilized on gold electrodes

**Véronique Balland**, Christelle Hurcau, Erin Wallace, Thierry Tron, Benoit Limoges, Laboratoire d’Electrochimie Moléculaire, CNRS UMR 7591, Université Paris Diderot, Paris, France; Laboratoire BioSciences, FREG05, Université Paul Cézanne, Marseille, France.  
Contact e-mail: veronique.balland@univ-paris-diderot.fr

Laccases are four-copper containing enzymes exhibiting dual reactivity. Their ability to oxidize a large panel of substrates including recalcitrant environmental pollutants and to reduce molecular dioxygen into water makes them promising enzymes for the development of biosensors and biofuel cells, respectively. However, these applications require the challenging preparation of stable enzymes-coated electrodes with well-defined catalytic properties toward both substrates and dioxygen.

We report the immobilization of a recombinant *Trametes C30 LAC3* using the nitrol triacetic acid (NTA)-histidine-tag technology that requires mild protein modification. Analysis of the his-tagged LAC3 in solution indicates that addition of the polyhistidine moiety does not strongly affect its catalytic activity. Moreover, it allows formation of a stable enzyme monolayer on gold electrodes. The catalytic cycle of immobilized LAC3 was investigated by cyclic voltammetry and analysed using available theory for ping-pong mechanisms and immobilized enzymes.[2] To achieve a complete analysis of LAC3, the kinetic characteristics of the immobilized enzyme were compared with their homogeneous counterparts. Our results indicate that high surface coverage of fully functional LAC3 can be obtained by using this immobilization procedure.
Biochemical Characterization of Human COX11

Lucia Banci, Ivano Bertini, Adele Fantoni, Sayaka Inagaki, Magnetic Resonance Center CERM, Florence, Italy.
Contact e-mail: banci@cern.unifi.it

COX11 is a copper-binding protein, which plays a crucial role in the incorporation of copper into the CuB site of cytochrome c oxidase. In this study, we carried out the expression and characterization of human COX11 (hCOX11). The gene of hCOX11 codes 276 amino acids, parts of which are predicted to be present as trans-membrane helices. We cloned C-terminal soluble domain, Cys121-Asn276, in pPICZ B vector. The expression plasmid was used to transform into the methylotrophic yeast P. pastoris X-33. The hCOX11 was expressed as a soluble form in P. pastoris under control of the inducible alcohol oxidase 1 promoter. The purified hCOX11 was observed at ~ 18 kDa on SDS-PAGE. This result is consistent with the molecular weight calculated from the translated cDNA sequence. The far-UV circular dichroism spectrum of hCOX11 showed characteristic features of a folded protein with beta-strands and alpha-helical structures. Analytical gel filtration showed that hCOX11 was eluted as dimer with no effect induced by the reducing agent DTT. This behavior suggests that the dimerization would not involve formation of disulfide bonds. The mechanisms responsible for the dimerization and the interaction with copper are under study. The results will be present and discussed.

Thermal and conformational stability of lactoperoxidase and myeloperoxidase

Srijib Banerjee, Paul Georg Furtmüller, Christian Obinger, Department of Chemistry, Division of Biochemistry, BOKU University of Natural Resources and Applied Life Sciences, Vienna, Austria.
Contact e-mail: srijib.banerjee@boku.ac.at

Lactoperoxidase (LPO) and myeloperoxidase (MPO) belong to the peroxidase-cyclooxygenase superfamily. Both enzymes are critical elements of the innate, i.e. nonantigenic immune system. Lactoperoxidase is a monomeric protein consisting of a single polypeptide that binds a modified heme via two ester linkages. By contrast, MPO is a dimeric protein with a single disulfide bridge between symmetry-related halves, each of which contains two polypeptides. In MPO the heme is also covalently attached to the protein via two ester bonds, but it is the only heme protein with an additional sulfonium ion linkage. The mode of heme binding strongly affects the biophysical and biochemical properties of these metalloproteins. In order to elucidate the effect of heme to protein linkages on the thermal and conformational stability of LPO and MPO in their ferric forms, we have performed comparative electronic absorbance, electronic circular dichroism and intrinsic steady-state fluorescence studies on protein unfolding by urea and guanidine hydrochloride as well as with increasing temperature both in the absence and presence of dithiothreitol. Determination of thermodynamic (free enthalpies, enthalpies and entropies) as well as kinetic parameters of denaturation revealed differences between LPO and MPO, which will be discussed with respect to the known structures of human MPO and bovine LPO.
P108
Subunits Identification of the Complex I from
Rhodothermus marinus
Ana P. Batista, Andrea S. Fernandes, Ana Coelho, Manuela P. Pereira, Miguel Teixeira, Instituto de Tecnologia Química e Biomolecular, Oeiras, Portugal.
Contact e-mail: abatista@itqb.unl.pt

Complex I, NADH:quinone oxidoreductase, is the first and the biggest complex of all that constitute respiratory chain. This enzyme couples electron transfer from NADH to quinone to the proton translocation across the membrane, which contributes to the maintenance of electrochemical potential that is the driving force for ATP synthesis. Complex I is composed of multiple polypeptide subunits and contains several prosthetic groups involved in its activity. The fact that many mitochondrial diseases are related to complex I defects shows the need to know more about the structure and function of this large respiratory chain complex. Rhodothermus marinus complex I, our model system, is a NADH:menaquinone oxidoreductase and has been extensively characterized [1,2].

The genes coding for R. marinus Nqo subunits are clustering in two operons, nqoA-7 to nqoA-15 and two independent genes, nqo8 and nqo9.[3] Our recent results concerning the identification of the subunits are reported. By mass spectrometry, of the complex in solution, was possible identified all the subunits of the complex. Using this technique, with digest SDB-PAGE bands, were identified just the subunits that constitute the peripheral part of the complex. Nqo1-5 and Nqo9. The subunits Nqo1, Nqo3 and Nqo9 were also identified by immunoblotting with antibodies against subunits of Neurospora crassa Complex I[1].

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P109
Cyanobacterial terminal heme-copper oxides and their electron donors
Margit Bernroither, Doris Gussenbauer, Günter A. Peshchuk, Christian Obinger, BOKU - University of Natural Resources and Applied Life Sciences, Vienna, Austria; 2University of Vienna, Vienna, Austria.
Contact e-mail: margit.bernroither@boku.ac.at

Photosynthetic (PET) and respiratory electron transport (RET) are physically separated in eukaryotic cells but share common components (e.g. plastoquinone, cytochrome b6f, cytochrome c6, plastocyanin etc.) in cyanobacteria. As in most other phototrophic prokaryotes, the intracytoplasmic membrane contains a dual-function PET-RET system, whereas the cytoplasmic membrane contains only RET without photosynthetic reaction centres. In RET terminal respiratory oxidases (TROs) function as the final electron acceptor (“electron sink”) reducing O2 to water in a concerted four-electron transfer reaction. Genome and expression analysis suggest that cyanobacteria can contain one or more representatives of TROs from both the heme-copper oxidase superfamily and the non-copper or bd-type oxidase superfamily. A detailed genomic (29 strains, 19 completely sequenced genomes), sequence and structural (subunits I & II) analysis of cyanobacterial heme-copper oxides and their putative electron donor proteins (cytochrome c6, plastocyanin and cytochrome cM) revealed that heterocyst-forming nitrogen-fixing cyanobacteria usually have more than one gene for obligate cytochrome c oxidase (aa1-type) and cytochrome c6. Many cyanobacterial strains lack genes encoding heme copper quinol oxidases. All cyanobacteria have a single-copy gene for cytochrome cM and plastocyanin (exceptions Gloeobacter violaceus & Thermosynechococcus elongatus). These data are discussed with respect to known in vitro interactions between these metalloproteins and to physiological relevance.

P110
Conformational control of S100A2 by metal ions
Hugo M. Botelho, Michael Koch, Günter Fritz, Cláudio M. Gomes, 1 Instituto de Tecnologia Química e Biomolecular, Oeiras, Portugal; 2Department of Biology, University of Konstanz, Konstanz, Germany.
Contact e-mail: hbotelho@itqb.unl.pt

The S100 proteins are small, dimeric Ca2+-binding proteins involved in cell cycle regulation and cell growth, differentiation and mobility in vertebrates. Several studies indicate expression misregulation of these proteins in a number of cancers. The Ca2+ binding sites are EF-hands. As in other EF-hand proteins, Ca2+ binding to S100A2 elicits the displacement of an helix exposing a hydrophobic patch which is the docking site for proteins involved in downstream signalling. The most studied one is transcription factor p53.

Besides binding Ca2+, S100A2 also binds Zn2+ with high affinity. Each S100A2 monomer binds two Zn2+ ions in sites different from the Ca2+ ones. The physiological role of Zn2+ seems to be the modulation of the Ca2+ affinity, as Zn2+ binding to one of the sites decreases the binding affinity of the Ca2+; a unique mechanism in the S100 family so far.

Our current research goal is to characterize the conformational and stability properties of S100A2 discriminating the effect of the binding of each metal to its cognate site. To accomplish this, engineered forms of S100A2 with mutated Zn2+ cysteine ligands were used. Biophysical and biochemical techniques such as circular dichroism and fluorescence spectroscopies have been employed in assessing conformational changes dependent of temperature, chemical denaturant concentration and metal loading status. Here we report the effects of Zn2+ binding to the high affinity site and discuss its repercussions in the physiology and conformational tuning of S100A2.

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P111
Synthesis and evaluation of new amino-acids as arginase inhibitors
Jean-Luc Boucher1, David Christianson2, Sylvie Djole1, Yves Frapart1, Stephanie Pethé1, Daniel Mansuy1, CNRS-UMR R601, Paris, France; 2Roy and Diana Vagelos Laboratories, University of Pennsylvania, Philadelphia, PA, USA.
Contact e-mail: jean-luc.boucher@univ-paris5.fr

In mammalian cells, L-arginine is metabolized by two major pathways: arginases catalyze its hydrolysis to L-ornithine and nitric oxide. NO is an important biological molecule involved in vasodilation, neurotransmission and immune responses. Recent studies support the hypothesis that arginases may be essential in the regulation of NOS activities by modulating local L-arginine concentration and it is observed that inhibition of arginases led to an enhancement of NO production. The search for potent inhibitors of arginases is thus the focus of active studies. L-Arginine hydrolysis by arginases is achieved by a metal-activated hydroxide ion at the guanidinium carbon of L-arginine. Using X-ray crystallographic data and a model proposed to explain the inhibitory effects of L-arginine analogues, we have synthesized and tested new amino-acids as putative inhibitors of rat liver arginase. EPR spectroscopy has been used to study their positionings at the (MnII)2 active site. The present poster will resume results obtained in our search for arginase inhibitors.
P112  Biochemical and spectroscopic analysis of a bacteriophytochrome from *Pseudomonas aeruginosa*

Latifa Bouzhir, Marten H. Vos, Ursula Liebl, INSERM U696-CNRS UMR7645, Ecole Polytechnique, Palaiseau, France.

Contact e-mail: latifa.bouzhir@polytechnique.edu

Bacteriophytochromes (BphPs) are bacterial photoreceptors that sense red/far-red light and bind a biliverdin chromophore, an immediate breakdown product of heme (1). Bacteriophytochromes are known to regulate the biosynthesis of photosynthetic and nonphotosynthetic pigments and motility, but have a wider array of functions, only partially known. Here we describe the biochemical and spectroscopic analysis of a novel bacteriophytochrome, PA5442, from the opportunistic pathogen *Pseudomonas aeruginosa*.

The protein is composed of a PAS domain linked to a GGDEF and EAL output module that is involved respectively in synthesis (diguanylate cyclase) and degradation (phosphodiesterase) of the bacterial second messenger c-di-GMP. GGDEF-EAL-containing proteins studied to date displayed either diguanylate cyclase or phosphodiesterase activity, but not both. To get insight into the function of PA5442, the protein was expressed heterologously in *E. coli* and purified in the dark using affinity chromatography. Recombinant PA5442 has an apparent molecular mass of 106 kDa and shows the characteristic red/far-red-light-absorption peaks at 480 and 338 nm (2). The ORP was heterologously expressed in *E. coli* as an apo-protein. In this work we report the reconstitution of the Mo-Cu cluster using [MoS₄]²⁻ and CuCl₂ in the over-expressed apo-protein.

Biochemical characterization of the reconstituted protein was performed including UV-visible spectra, metal content and EPR spectroscopy and the results were compared with the native protein. We are also presenting a preliminary biochemical characterization of an orange protein isolated from *Desulfovibrio alaskensis* PA5442 and essential for its role in vivo.

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P113  CO-inhibition of NiFe hydrogenases monitored by EPR spectroscopy

Bénédicte Burlat, Fanny Leroux, Patrick Bertrand, Sébastien Dementin, Marc Rousset, Christophe Léger, Bruno Guigliarelli, Laboratoire de Bioénergétique et Ingénierie des Protéines, Marseille, France.

Contact e-mail: burlat@ibsm.cnrs-mrs.fr

Hydrogenases are enzymes which catalyze the reversible oxidation of dihydrogen into protons and electrons. *D. fructosovorans* NiFe hydrogenase consists of two subunits and contains a nickel-iron active site and three iron-sulfur clusters involved in electron transferring. Hydrophobic channels (1) connect the NiFe active site (deeply buried in the protein) to the surface of the protein and enables diffusion of substrate H₂ and small inhibitors (O₂, CO) towards the active site.

In this study, we focused on inhibition by CO of the NiFe active site by using EPR spectroscopy. From the so-called paramagnetic Ni-C species corresponding to an intermediate state on the catalytic mechanism, a paramagnetic CO-inhibited species can be trapped by incubation of hydrogenase under a controlled CO atmosphere. The resulting Ni-CO species displays light-sensitivity properties, as observed for the Ni-C species (2). Upon irradiation monitored by EPR, we carefully explored the photosensitivity of the Ni-CO species and subsequent thermal recombination processes. This relates to the photodissociation of the Ni-bound CO and the concomitant proton transfer between terminal cytochrome ligands of the Ni ion.

This strategy has been used with hydrogenase mutants for which intramolecular gas diffusion was hampered by mutating residues in the channel close to the active site.

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P114  Orange proteins from Desulfovibrio gigas and *Desulfovibrio alaskensis*

Marta S. Carepo¹, Americo Duarte², Sofia R. Pauleta¹, Anthony G. Wode², Alice S. Pereira¹, Jose J. G. Moura¹, Isabel Moura¹, Requinte J., Departamento de Química, QFIB, Faculdade de Ciências e Tecnologia – UNL, Monte da Caparica, Portugal; ²School of Chemistry, University of Melbourne, ParkVill, Australia.

Contact e-mail: martha.carepo@dq.fc.ul.pt

The orange protein (ORP) isolated from *Desulfovibrio gigas* has a mixed-metal sulphide cluster of the type [S₂Mo₆S₄Cu₂S₂Mo₄S₄]³⁺

(1) The protein is a monomer of 11.8 kDa and the cofactor contributes to a characteristic UV-visible spectra with maximum absorption peaks at 480 and 338 nm (2). The ORP was heterologously expressed in *E. coli* as an apo-protein. In this work we report the reconstitution of the Mo-Cu cluster using [MoS₄]²⁻ and CuCl₂ in the over-expressed apo-protein.

Biochemical characterization of the reconstituted protein was performed including UV-visible spectra, metal content and EPR spectroscopy and the results were compared with the native protein. We are also presenting a preliminary biochemical characterization of an orange protein isolated from *Desulfovibrio alaskensis* PA5442 and essential for its role in vivo.

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P115  Naphthalene dioxygenase-catalyzed cis-dihydroxylation of substituted 2-pyridones and 2-quinolones

Claude Chopard, Robert Azerad, UMR 8601 CNRS Univ. René Descartes, Paris, France.

Contact e-mail: claude.chopard@univ-paris5.fr

The bacterial metabolism of aromatic compounds often proceeds via an initial dioxygenase-catalyzed dihydroxylation to yield cis-dihydrodiol derivatives. A large range of mono- and polycyclic substrates have been reported to produce the corresponding enantiopure cis-dihydrodiol metabolites. However, to date, only a few data have been reported about the cis-dihydroxylation of compounds derived from heterocyclic amines. In the recent years, direct evidence for the cis-dihydroxylation of *N*-methyl-2-pyridone by NDOs has been reported in our laboratory (1), a major dihydroxylated metabolite, *N*-methyl-cis-5,6-dihydro-5,6-dihydroxy-2-pyridone was obtained from this substrate.

The study presented here was initiated to extend this observation by investigating the effect of new substituents on 2-pyridone and 2-quinolone rings in the NDO-catalyzed reaction. The potential asymmetric dihydroxylated derivatives may be used in the further synthetic elaboration (2) of a number of biologically important compounds, such as unnatural amino acids, azasugars, alkaloids, etc.

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Marta S. Carepo is supported by Grant SFRH/BPD/24150/2005-POCTI

The orange protein (ORP) isolated from *Desulfovibrio gigas* has a mixed-metal sulphide cluster of the type [S₂Mo₆S₄Cu₂S₂Mo₄S₄]³⁺
The major products obtained were fully characterized and revealed that for several substrates NDOS have catalyzed also extra-cyclic hydroxylations and N-dealkylation.

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P116
Magnetic circular dichroism of dicobalt model complexes of metallohydrolases
Seung-An Chyun, James A. Larrabee, Middlebury College, Middlebury, VT, USA.
Contact e-mail: schyun@middlebury.edu

The μ-hydroxy-μ-carboxylato-dimetal core is a common structural feature in metallohydrolase active sites such as aminopeptidase and glycerophosphodiesterase. An interesting feature of the Co(II)-substituted enzymes is the extent of magnetic exchange coupling between two high-spin Co(II) ions. Magnetic circular dichroism (MCD) can be used to measure the magnitude of exchange coupling between Co(II) ions. To demonstrate the principle, a study of two isostructural dicobalt complexes, \([\text{Co}^{II}(\mu-\text{O}_2\text{CCH}_3)\mu-\text{OH})_L\text{PF}_3\) (1) and \([\text{Co}^{III}(\mu-\text{O}_2\text{CCH}_3)\mu-\text{OH})_L\text{PF}_3\) (2) (\(L = \text{N}_2\text{N}_2^*\text{triazenylcyclopropane}\)), was conducted. In 1 both Co(II) ions are high-spin while in 2 the Co(III) is diamagnetic; there is no possibility of coupling in 2. The low temperature MCD spectra of 1 and 2 are nearly identical having peaks at 483, 506 and 535 nm which arise from the \(^3\text{T}_{1g} \rightarrow ^3\text{T}_{2g}(\text{P})\) d-d transition in Co(II). The MCD spectrum of 1 is twice as intense as that of 2 because diamagnetic Co(III) is MCD silent. The magnetization plots for 1 and 2 are very different, indicating that the ground states are different. Spin-orbit coupling and low symmetry combine to create a pseudo-Kramer’s doublet ground state in single-ion 6-coordinate Co(II), and the magnetization data from 2 fit this model very well with \(g \approx 4.8\). In coupled binuclear Co(II), spin-orbit coupling and low symmetry lead to a ground state manifold of two singlets and a doublet, separated by energies, \(E_1\) and \(E_2\). The doublet is lower in energy for ferromagnetic coupling. The magnetization data for 1 fit well to the ferromagnetically coupled model with a \(g = 9.6\), \(E_1 = 4.2\) cm\(^{-1}\) and \(E_2 = 8.5\) cm\(^{-1}\).

P117
Redox chemistry of low-pH forms of tetrahemic cytochrome c3
Margarida M. Correia dos Santos1, Maria Santos1, Maria de Lurdes Simões Gonçalves1, Cristina Costa2, José J. G. Moura2, Jorge C. Romão3, 1Centro de Química Estrutural, IST, Lisboa, Portugal; 2REQUIMTE-CQFB, FCT-UNL, Caparica, Portugal; 3Centro de Física Teórica de Partículas, IST, Lisboa, Portugal. Contact e-mail: mcsantos@ist.utl.pt

Desulfovibrio vulgaris Hildenborough cytochrome c3 contains four hemes in a low-spin state with bis-histidinyl coordination. High-spin forms of cytochrome c3 can be generated by protonation of the axial ligands [1,2]. In this work, high-spin forms of cytochrome c3 were generated by protonation of the axial histidine ligands in order to probe spin equilibrium (high/low), and to generate putative reactive species towards external ligands. The spin alterations occurring at extreme pH, the associated changes in redox potentials, as well as the reactivity towards external ligands (NO) were complementary studied by the conjunction of UV-visible, CD, NMR and EPR spectroscopies and electrochemistry. Since the square wave voltamgrams obtained correspond to four successive one-electron processes, the experimental data were fitted to the theoretical predictions for four similar but non-equivalent redox sites.

The binding of NO to the high-spin forms of D.vulgaris Hildenborough cytochrome c3 indicates that low-spin/high-spin equilibrium may enhance the reactivity of the hemes towards the binding and eventually catalysis of small molecules [3].

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P118
Radiolytic cryoreduction and cryooxidation of ferrous hemoproteins: EPR and ENDOR evidence for conformational substates of pentacoordinate ferrous hemoproteins
Roman Davydov, Brian M. Hoffman, Northwestern University, Evanston, IL, USA.
Contact e-mail: r-davydov@northwestern.edu

\(\gamma\)-Irradiation of frozen solutions of ferrous hemoproteins at 77K generates EPR active singly-reduced low-valent hemes and oxidized iron(III) heme centers, each trapped in nonequilibrium conformation of its ferrous precursor. The one-electron reduction products of pentacoordinate (S=2) ferrous globins, peroxidases, and cytochrome P450cam show EPR and ENDOR spectra characteristic of (3d\(^2\)) Fe(I) species. Cryogenerated low-valent deoxyhemoglobin and deoxymyoglobin also exhibit an S=3/2 spin state comprised of a porphyrin \(\pi\)-anion radical. The EPR spectra of cryoreduced deoxyhemoglobin and deoxymyoglobin reveal three spectroscopically distinct low-valent species, which indicates that the deoxy precursors adopt different conformational substates. Reduction products of hexacoordinate ferrous cytochrome c and b\(_3\) as well as those of Fe(II)-CO globins are Fe(II) porphyrin \(\pi\)-anion radical species. The data thus show that the presence of the 6-th axial ligand in the low-valent intermediates favors localization of an unpaired electron in the porphyrin \(\pi\)–orbitals, consistent with previous results for low-valent iron porphyrin models. The cryogenerated low-valent hemoprotein intermediates relax to the ferrous states at T > 200K. The spectroscopic properties of cryogenerated ferrous cytochrome C and b\(_3\) are similar to these for the resting ferric states. The products of cryooxidation of pentacoordinate ferrous globins and peroxidases as well as of the low-spin complexes of the ferrous globins with CO show EPR spectra with \(g_{\perp} \approx 6\) characteristic of the quantum-mechanically admixed S=3/2, 5/2 ground states. The trapped species relax to the equilibrium ferric states upon annealing at T > 190K.

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The enzyme $\text{N}_2\text{O}$ reductase ($\text{N}_2\text{OR}$) catalyses the final step of denitrification, the two electron reduction of $\text{N}_2\text{O}$ to N$_2$. The recently solved structures of $\text{N}_2\text{OR}$ from *Pseudomonas nautica* (Pn) and *Paracoccus denitrificans* revealed the presence of a functional homodimer containing two different multicopper sites: CuA and CuZ. CuA is a binuclear copper site that transfers electrons to the catalytic CuZ site, a novel mixed valence compound with a sulfide ion bridging a distorted tetrahedron of copper atoms. In order to clarify the complex mechanism of activation and catalysis of this enzyme we studied the interaction with the probable physiological electron donor: cytochrome $c_{552}$ from *Pn*. Activity tests show that the reduced cyt $c_{552}$ is able to transfer electrons to CuA promoting the catalysis of $\text{N}_2\text{O}$. The nature of the short-lived ET complex is investigated using docking analysis performed with BigGER. This study shows a strong affinity of this cytochrome to a hydrophobic patch located near CuA centre and putative complexes involving a small group of key residues are proposed. The recognition of the two partners is driven by electrostatic interaction, while hydrophobic contacts produce the fine orientation at short distances. In comparison, horse heart cytochrome $c$ isn’t able to transfer electrons to $\text{N}_2\text{OR}$ from Pn and the docking analysis of this non-physiological electrostatic ET complex doesn’t point to a specific interaction. Kinetic studies and protein-protein $^1$H-NMR titrations are in progress to completely characterize this ET complex.

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**Study on the structure-property-reactivity-function relationship of human neuronal growth inhibitory factor and its interaction with amyloid beta peptide**

Zhi-Chun Ding, Zhong-Xian Huang, Chemistry Department, Shanghai, China.

Contact e-mail: 051022004@fudan.edu.cn

Human metallothionein-3 (hMT3), also named human neuronal growth inhibitory factor (hGIF), can inhibit the outgrowth of neuronal cells in the presence of brain extracts, a property not shared by MT-1/-2. In order to study the structure-property-reactivity-function relationship, including the roles played by the particular residues and by particular structural elements and to illustrate the possible molecular mechanism of the bioactivity of hGIF a systematic site-directed mutagenesis on hGIF has been done. The structure of its $\beta$-domain of hGIF, which was mainly responsible for the bioactivity of the entire protein, was predicted by the molecular dynamics simulation for the first time. The properties of hGIF and its mutants were characterized by a series of spectroscopy, the reactions of hGIF and its variants with DTNB, EDTA and SNOC were examined, and finally the bioactivity of hGIF variants were assayed by neuron culture experiments.

From these studies we conclude that the bioactivity of hGIF is regulated by various factors, including the TCPCCP motif, $\beta$-domain interactions, the structure and stability of the metal-thiolate cluster and other unknown factors.

The interaction between hGIF and amyloid beta peptide (A$\beta$) is another part of our research. It was reported that the level of hGIF mRNA was down-regulated in AD brains and hGIF could inhibit the aggregation of A$\beta$ in vitro. However, no detailed mechanism analysis has been carried out till now. The aim of our research is to explore the mechanism how hGIF inhibits the aggregation of A$\beta$ and provide information for potential drugs design for AD therapy.

**Copper resistance proteins PcoA and PcoC from Escherichia coli: expression, characterisation and interaction study**

Karrera Y. Djoko, Zhiguang Xiao, Anthony G. Wedd, School of Chemistry and Bio21 Institute, University of Melbourne, Parkville, VIC, Australia.

Contact e-mail: kydjoko@unimelb.edu.au

Copper is a micronutrient required for correct cellular functions but is toxic in excess. However, a strain of *E. coli* isolated from the faeces of copper-fed pigs can survive in copper-rich environments (up to 10 mM CuSO$_4$). This resistance is attributed to a plasmid-borne pco operon. High copper levels induce expression of the operon proteins PcoABCDERS that interact in the periplasm to confer copper resistance. Both PcoA and PcoC are soluble proteins. We have demonstrated recently that PcoC (10.5 kDa) features two separate binding sites ($K_d \sim 10^{-13}$) specific for Cu(I) and Cu(II) and effects intermolecular transfers of bound Cu(I) and Cu(II) with or without a change in oxidation state. A$\gamma$O- and Cu($\gamma$O)+Cu(II) forms are intermediates in the two-step redox transfer processes.

We have also expressed and isolated PcoC (63.7 Da). Preliminary data suggest that it is a laccase-type multicopper oxidase that binds multiple copper ions, including a T1 blue copper and a trinuclear copper cluster at the active site. It catalyses the oxidation of common laccase substrates, including *para*-phenylenediamine and *para*-methoxyphenol, and requires excess copper for activity. Interactions between PcoA and PcoC under various conditions provide insight into how these two members of the pco operon proteins function cooperatively in the periplasm and contribute to this copper resistance in *E. coli*. Details of the expression, isolation and interaction study will be provided and discussed.

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assistance with mass spectrometry and Charlotte Lehmann for technical assistance with cell culturing and experiments.

P123
Spectroscopic and Mutagenesis studies on the Iron Sulfur Centers from the Fuscoredoxin of Desulfovibrio desulfuricans
Rui O. Duarte, Ana R. Ramos, Sara Costa, Alice S. Pereira, Pedro Tavares, Isabel Moura, Requimite/CQFB/FCT, Almada, Portugal. 1Estesi/LIP, Lisboa, Portugal. Contact e-mail: rod@dq.fct.unl.pt

Fuscoredoxin is an unusual Fe-S protein from D. desulfuricans ATCC 27774. This protein contains two types of Fe-S clusters: a [4Fe-4S] cubane cluster and a novel one, [4Fe-2S-20], which can attain four redox states. EPR and Mössbauer data reported some of the magnetic and redox properties, but they are still far from being understood. We cloned the gene in an expression vector (pGEX-6P-1) and performed site-directed mutagenesis aiming to correlate the biochemical properties of the aminoacid involved in the metal center coordination with the spectroscopic data obtained for the protein, in particular the data on the iron related the atypical center. We also have made a mutant consisting in the deletion of the first 25 amino acids residues (from the N-terminal) to result in the specific loss of the [4Fe-4S], simplifying the data analysis. EPR and Mössbauer studies are in progress to clarify the redox properties of this mutant. These studies will also be an important tool for revealing the biological function of fuscoredoxin.

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P124
Conception of myeloperoxidase inhibitors based on flufenamic acid structure
Pierre Van Antwerpen, Martine Prevost, François M. U. Dufrasne, Patrick Moreau, Karim Zouaoui Boujdjelta, Thierry Franche, Stephan Kohnen, Sajida Babar, Nicole Moguilevsky, Michel Vanhaeverbeek, Jean Ducobu, Didier Serteyn, Jean Nève, Laboratory of Organic Pharmaceutical Chemistry, Brussels, Belgium. Genomic and Structural Bioinformatic, Faculty of Applied Sciences, Université Libre de Bruxelles, Brussels, Belgium. Laboratory of Experimental Medicine, CHU-Vesale, Montigny-le-Tilleul, Belgium. Department of Clinical Sciences, Equipe Clinic, Université de Liège, Liège, Belgium. Center for Oxygen, Research and Development, Université de Liège, Liège, Belgium. Laboratory of Applied Genetics, IBMM, Université Libre de Bruxelles, Gosselies, Belgium. Technology Transfer Office, FUNDP, Namur, Belgium. Department of Internal Medicine, CHU-Tivoli, La Louvière, Belgium. Contact e-mail: dufrasne@ulb.ac.be

Myeloperoxidase (EC 1.11.1.7, MPO) is involved in several pathologies, especially in atherosclerosis through its binding to LDL and lipoproteins oxidation. Attention has been focused on the development of MPO inhibitors with no success so far. Flufenamic acid (1) has been reported as an in vitro MPO inhibitor. As a substrate of MPO compound 1, it prevents the formation of HOCl from the MPO/H2O2/Cl- system. It is metabolized into 5-chloro- and 5-carboxylic acids (2 and 3). Molecular docking predicts that (1) interacts with the enzyme through aromatic stacking with the heme and electrostatic bonds with binding site residues. A series of inhibitor candidates were synthesized, based on the structure of (1) with these expected characteristics: - no or little HOCl scavenging, - enhanced interaction with the heme and/or binding site residues, - penetration into MPO when enzyme is bound to LDL.

These compounds have been designed following a molecular docking analysis and included diacids (4), 4-substituted- and 5-substituted derivatives (5 and 6). Evaluation of these molecules shows that MPO inhibition is not improved. Compounds 2 and 6 (X = Br) have a slightly higher inhibitory effect. However, all the new synthesized compounds and, particularly those with an additional acidic group, are weaker inhibitors of LDL oxidation. This could originate from electrostatic repulsion between the acid groups of the ligands and negatively charged LDLs or from steric hindrance.

P125
Structural, biochemical and functional characterizations of PerR
Abdelnasser El Ghazouani, IRTSV/ LCBM, Grenoble, France. Contact e-mail: aelghazouani@cea.fr

Oxidative stress is generated by exposure to elevated levels of Reactive Oxygen Species (ROS), such as hydrogen peroxide. To avoid or counter the harmful effects of ROS, cells constitutively express proteins to protect themselves and repair the damages. These proteins are under negative control of specific regulators which are the central pieces of the inducible response to oxidative stress present in cells. In Bacillus subtilis the zinc protein PerR, a peroxide sensor that binds DNA in the presence of a regulatory metal Mn2+ or Fe2+, mediates the adaptive response to H2O2. It has been shown that Fe2+ or Mn2+ is essential for DNA binding and that inactivation of PerR-Zn-Fe in the presence of H2O2 proceeds via the metal catalyzed oxidation of at least 2 histidine residues that are supposed to coordinate the Fe2+.

The present study reports the crystal structure of the PerR-Zn protein without its regulatory metal. Molecular modelling of the PerR-Zn structure led us to propose a potential binding site for the regulatory metal. The structural model for the active PerR protein suggests a conformational change of the protein upon metallation. Taking advantage of the PerR-Zn structure, several mutations were carried out in order to introduce tryptophan residues as fluorescent probes to monitor the dynamics of the protein. The results obtained with the PerR-177W mutant are described. Recent work on the PerR-DNA interaction, in order to identify the components of the highly specific recognition is also presented in light of protein-DNA cross-linking experiments and site-directed mutagenesis studies.

P126
Zinc horse heart cytochrome c unfolding monitored by fluorescence resonance energy transfer
Amy A. Ensign, Iris Jo, Todd Krauss, Kara L. Bren, University of Rochester, Rochester, NY, USA. Contact e-mail: aensign@mail.rochester.edu

The physical basis for protein folding is one of the greatest unknowns in science.1 Fluorescence resonance energy transfer (FRET), when used as a ‘spectroscopic ruler’, is a method that gives information about intermolecular distances and is an attractive method for the study of protein folding. However, FRET generally requires the specific attachment of at least two dyes (a donor and an acceptor) to the protein; in practice, producing pure samples with two dyes attached to specific sites has proved to be challenging.2
In order to eliminate the need to attach two dyes to perform FRET experiments, we have chosen to study protein folding using horse cyt c in which the heme iron is replaced with Zn2+ (Zn-cyt c). Significantly, zinc porphyrin is an intrinsic fluorophore which can act as an acceptor of energy from a dye attached to the protein surface in FRET studies, eliminating the need to doubly label the protein and minimizing perturbation to the system. The present study examines folding probed by dyes attached to six different sites on Zn-cyt c. This study confirms that the folding of horse cyt c deviates from a two state folding model while making use of a novel fluorophore pair.

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P127

Kinetic studies of metal incorporation in Desulfovibrio gigas rubredoxin
Filipe Folgosa, Joana A. Santos, Alice S. Pereira, Isabel Moura, José J. G. Moura, Pedro Tavares, REQMTÉ, Faculdade de Ciências e Tecnologia, Caparica, Portugal.
Contact e-mail: filipe.folgosa@dq.fct.unl.pt

Rubredoxin (Rd) is a small iron containing protein (~5kDa), present in several organisms [1]. This protein is often linked to electron transfer in biologic systems, working as an electron donor to other proteins in many different pathways, like cellular detoxification for example [1,2]. Electron transfer studies between rubredoxin and superoxide reductases (SOR) were recently published [3]. To be able to accomplish these functions the presence of the iron atom is fundamental. However, it is possible to reconstitute Rd with different metals [4].

Our present work is focused in the mechanism of metal incorporation. Several metals, like cobalt, zinc and iron, were used in the reconstitution of Desulfovibrio gigas Rd. All the studies were preformed with overexpressed and purified protein.

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Chlorite as oxidant and reductant of heme peroxidases: a mechanistic study
Paul G. Furtmüller1, Christa Jakopitsch1, Holger Spaltelhofer2, Jürgen Arnhold2, Christian Obinger1, 2Department of Chemistry, Division of Biochemistry, BOKU – University of Natural Resources and Applied Life Sciences, Vienna, Austria; 2Institute of Medical Physics and Biophysics, School of Medicine, University of Leipzig, Leipzig, Germany.
Contact e-mail: paul.furtmueller@boku.ac.at

Heme peroxidases can use chlorite, ClO2−, in the absence of hydrogen peroxide to catalyze chlorination reactions. * We show that chlorite acts both as oxidant (mediating the oxidation of ferric enzymes to compound I) as well as one electron reductant (mediating the transformation compound I -> compound II -> ferric peroxidase) and that all rates of these reactions increase with decreasing pH. The corresponding reaction products, hypochlorite (ClO2−) and chlorine dioxide (ClO2), mediate the chlorination of monochlorodimedone, with ClO2 being much more effective than hypochlorite. Enzymes of both peroxidase superfamilies (myeloperoxidase, horseradish peroxidase and catalase-peroxidase) have been investigated in their reaction with chlorite by using the sequential-mixing stopped-flow technique. Bimolecular rate constants at various pH values and a general reaction scheme are presented and discussed with respect to known reduction potentials of enzyme intermediates (compound I/ferric peroxidase, compound II/ferric peroxidase) and of the couples ClO2−/ClO− and ClO−/Cl2−. H2O. In addition, chlorine dioxide is shown to mediate the oxidation of ferric peroxidase to compound I as well as inactivation of heme peroxidases.

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P129

Redox states of Ferredoxin II from Desulfovibrio gigas
Esther E. Grazina, Alice S. Pereira, Isabel Moura, José J. G. Moura, REQUIMTE, CQFB, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Caparica, Portugal.
Contact e-mail: raquel.grazina@dq.fct.unl.pt

Ferredoxins are simple iron-sulphur proteins that contain prosthetic groups composed of iron and sulphur atoms and they play a fundamental role in electron transfer processes relevant for several biologic systems. Four distinct types of ferredoxins are found in SRB, containing [3Fe-4S], [4Fe-4S], [3Fe-4S] plus [4Fe-4S] and 2×[4Fe-4S] clusters. Desulfovibrio gigas ferredoxin II (DgFdII) is a small tetrameric protein of 58 amino acids, which contains a single [3Fe-4S] cluster and a redox active internal disulphide bridge. DgFdII gene was cloned using the polymerase chain reaction (PCR), inserted in the expression vector pET-21c and over expressed in Escherichia coli (E.coli). Electrochemical tools can provide important information for the understanding of the redox and mechanistic/structural role of Fe-S clusters and to the interconversion process occurring between 3 Fe and 4 Fe clusters, as well as to the effect of the addition of an extra metal to form heterometal clusters of the type [M3Fe-4S]. Characterization of the recombinant protein will be presented. Direct electrochemistry (cyclic voltammetry and differential pulse voltammetry) will be used in order to detect all the redox transitions attainable by the core and to probe cluster interconversion. EPR will be used in complementary way.

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P130

Functional studies on a bacterioferritin from the anaerobe Desulfovibrio vulgaris
Marcia Guilherme, Cristina G. Timoteo, Pedro Tavares, Alice S. Pereira, REQUIMTE, CQFB, Dep. de Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Caparica, Portugal.
Contact e-mail: marcia.guilherme@dq.fct.unl.pt

Iron is an important nutrient, required in almost every aspects of cellular function. However, at physiological pH and under oxidizing conditions, the predominant form is Fe3+, which is highly insoluble. In the presence of O2, free Fe3+ ions are extremely toxic. Ferritins are a broad superfamily of iron storage proteins present in all type of organisms. Ferritin (Ft) and bacterioferritin (Bfr) are unique in the sense that they perform dual functions of iron detoxification, by oxidizing the Fe3+ ions in solution and iron sequestration by storing the oxidized Fe3+ ions in its inner protein cavity in the form of
ferricyanide mineral (1,2). They are large spherical proteins composed of 24 subunits with a nanocavity of about 80 Å. Bfrs are heme containing proteins with a b-type heme per dimer. A Bfr encoding gene was isolated from the Delftia acidovorans genome and overexpressed in Escherichia coli. Biochemical, kinetic and spectroscopic studies on the recombinant D. acidovorans Bfr and a variant (E95A) were presented.

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P131
Magnetic circular dichroism study of a cobalt derivative of Enterobacter aerogenes GpdQ
Kieran S. Hadler¹, Mark J. Riley¹, Lawrence R. Gahan¹, Gerhard Schenk¹, James A. Larrabee², The University of Queensland, St Lucia, Australia; ²Middlebury College, Middlebury, VT, USA. Contact e-mail: k.hadler@uq.edu.au

The binuclear metalloenzyme glyceraldehyde-3-phosphate dehydrogenase (GpdQ) is the only enzyme known that is capable of catalysing the hydrolysis of phosphoester bonds in mono-, di- and triesters. The protein binds one metal tightly and one loosely in the active site and thus activity is dependent on the concentration and identity of metals in the local environment. Magnetic circular dichroism (MCD) was used to probe the binding and coordination of cobalt(II) in the active site. The spectra reveal that addition of two equivalents of cobalt to the apo-enzyme gives rise to only one protein MCD transition corresponding to a six-coordinate site. Addition of a further 48 equivalents leads to a second transition, corresponding to a partially occupied 5-coordinate site. The observed transitions and coordination number were verified by angular overlap model (AOM) calculations. MCD of the phosphate inhibited species allows visualisation of the transition state involved in catalysis. Analysis of these spectra indicates that the coordination of each metal is conserved, supporting the proposal that the phosphate displaces the bridging hydroxide during the phosphorolysis. In addition, intensification of the 5-coordinate transition suggests that the phosphate aids in assembly of the catalytic centre by anchoring the loosely bound metal in the second site. Magnetisation behaviour of the cobalt ions were studied and indicates an uncoupled system of the cobalt ions were studied and indicates an uncoupled system.

P133
Delay time-resolved X-ray crystallographic study of Fe-type nitrile hydratase
Koichii Hashimoto, Masafumi Yohda, Masafumi Odaka, Faculty of Technology, Tokyo University of Agriculture and Technology, Tokyo, Japan. Contact e-mail: kounda@bel.bio.tuat.ac.jp

Nitrile hydratase (NHase) from Rhodococcus sp. N771 is a non-heme iron enzyme having post-translationally modified cysteine ligands, cCys112-3SO₂⁻ and cCys114-3SO⁻. NHase is widely used for industrial production of acrylamide, but its catalytic mechanism is very limited. tert-Butylisocyanide (t-BuNC) is a strong competitive inhibitor with a Ki of 5 uM. Recently, we found that NHase catalyzes the conversion of t-BuNC to tert-butyamine. Since the turnover rate for t-BuNC is much lower than that for nitriles, t-BuNC is likely to be a good substrate for studying the reaction mechanism of NHase. Here, we performed the time-resolved crystal structure analysis of NHase using t-BuNC as a substrate. t-BuNC was soaked to crystals of the nitrosylated inactive NHase. The catalytic reaction was started by photo-induced denitrosylation. At 0 min, a t-BuNC molecule was located in the reaction cavity but its -NC group was not reacted with NHase. At 70 and 120 mins, the carbon atom of the NC group in t-BuNC was coordinated to the iron at a distance of 2.07 Å. Interestingly, two electron densities maps were appeared at a distance of 1.26 Å from the carbon atom of the -NC group in the 440 min complex. The structure is likely to represent the enzyme-reaction intermediate complex. Based on the results obtained, the catalytic mechanism of NHase will be discussed.

P134
Peroxidase activity of hemoprotein reconstituted with iron porphycene
Takashi Hayashi, Takashi Matsuo, Department of Applied Chemistry, Osaka University, Suita, Japan. Contact e-mail: thayashi@chem.eng.osaka-u.ac.jp

Iron porphycene (FePc), a structural isomer of iron porphyrin, is an attractive prosthetic group for hemoproteins. In our previous study, we have reported that myoglobin reconstituted with FePc had...
extremely high O₂ affinity, whereas unfavorable autooxidation was remarkably slow due to the small rate constant of O₂ dissociation. The interesting behavior may be due to the decrease of the macrocyclic ligand framework. Recently, we also focused on the peroxidase and peroxidase activity of the hemoproteins reconstituted with FePc. The H₂O₂-dependent oxidations of guaiacol, thiocyanate and styrene catalyzed by myoglobin reconstituted with FePc gave the corresponding oxidation and/or oxygenation products with ca. 10-fold acceleration compared to the native protein. In addition, the large amounts of H₂O₂ led to the conversion of compound II-like species of FePc into the compound III-like species, oxygenated FePc, in the myoglobin heme pocket. Furthermore, horseradish peroxidase (HRP) reconstituted with FePc was also prepared by the conventional method. The peroxidase activity of the reconstituted protein toward thiocyanate oxidation was larger than that of native HRP by 10-fold. The highly oxidative intermediates, compound I and II of FePc were successively detected by stopped-flow technique. In this presentation, we will discuss the reactivity of FePc in the hemoprotein pocket.

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P135
Identification of a heme axial ligand of heme-regulated
eukaryotic initiation factor 2 alpha kinase (HRI): Role of the
Cys-Pro motif in heme regulation
Jotaro Igarashi, Aya Iizuka, Motohiko Murase, Marketa
Martinkova, Toru Shimizu, Institute of Multidisciplinary Research
for Advanced Materials, Tohoku University, Sendai, Japan.
Contact e-mail: jotaro@tagen.tohoku.ac.jp

Heme-regulated eIF2α kinase (heme-regulated inhibitor [HRI]) plays a critical role in the regulation of protein synthesis by heme iron. HRI senses and mediates responses to changes in the heme concentration. However, little is known regarding how heme binds to the catalytic domain and regulates catalysis, the stoichiometry of heme binding, and which residues are axial ligands for the heme iron. From our spectroscopic studies of an N-terminal truncated mutant lacking the first 145 amino acids, it was suggested that one of the heme axial ligands should be a Cys residue located in the C-terminal catalytic domain. To determine which Cys residue is responsible for the heme binding, we generated site-directed mutants at six Cys residues. Heme binding abilities of the Cys mutants were clearly different from that of the wild-type protein. The role of the Cys-Pro motif in the heme binding will be dissected. Relationships between the heme binding and catalytic regulation will also be discussed.

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P136
The crystal structure of the all cysteinyl coordinated
D14C mutant of [4Fe-4S] Pyrococcus furiosus ferredoxin
Monika N. Johannessen, Michael S. Nielsen, Pernille Harris, Børre L. Ooi, Hans E. M. Christensen, Department of Chemistry, Technical University of Denmark, Kgs. Lyngby, Denmark.
Contact e-mail: s021830@student.dtu.dk

The structure of the all cysteinyl coordinated D14C mutant of [4Fe-4S] ferredoxin (Fd) from the hyperthermophilic archaeon Pyrococcus furiosus has been determined to 1.7 Å resolution from a crystal belonging to space group C222, with two molecules in the asymmetric unit. The structure has been solved with molecular replacement using the [3Fe-4S] ferredoxin from P. furiosus [1] as a search model. The two molecules in the asymmetric unit are different. The A molecule contains a double conformation of the disulfide bond which exists in a 60 % occupancy of the left-handed and a 40 % occupancy of the right-handed spiral conformation, while molecule B has a disulfide bond only in the right-handed spiral conformation. Furthermore, the A molecules create dimers through a β-sheet interaction, while the B molecules are packed as homomers in a less rigid position.

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P137
Alterations in the thermostability of apo and zinc-bound
wild type and ALS mutant CuZnSOD
Matthew H. S. Clement, Edith B. Gralla, Lindsay E. Kane, Kevin W. Sea, Joan Silverstone Valentine, University of California, Los Angeles, Los Angeles, CA, USA.
Contact e-mail: lkane@chem.ucla.edu

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease that results in death within two to five years. The homodimeric antioxidant enzyme Cu-Zn superoxide dismutase (SOD1) has been implicated in two percent of ALS cases. Over 100 mutations leading to ALS have been documented in SOD1. Our laboratory has characterized biophysically a number of these mutant proteins and found that they fall into two general classes, pseudo-wild type or metal-binding region mutants. We have found that many of the pseudo-wild type ALS mutations destabilize the apo-protein. Previous studies have indicated that major structural rearrangements occur upon the binding of one zinc to the wild type apo protein dimer. Here we extend our studies of the ALS mutant proteins to include the thermal stability of the zinc bound forms using differential scanning calorimetry. Surprisingly, the mutant proteins with one zinc bound have similar stability to wild type, despite their differing (lower) stability in the apo form. With two zinc ions bound, the ALS mutant proteins once again exhibit a broader range of thermal stability. Identifying causes of the differing thermal stabilities of the apo and metallated forms of the proteins will help us better understand the nature of the toxicity of the mutant proteins.

P138
Direct electrochemistry of amicyanin derived from
Paracoccus denitrificans immobilized on a electrode via a
phenylglyoxal derivative
Ayako Kaneko, Nobuhumi Nakamura, Hiroyuki Ohno, Tokyo University of Agriculture & Technology, Koganei, Tokyo, Japan.
Contact e-mail: 50006641204@st.tuat.ac.jp

The oriented immobilization of redox proteins on an electrode is expected to control a electron transfer reaction between the protein and the electrode. There are methods for the oriented immobilization using specific amino acids within protein. A keto aldehyde group of phenylglyoxal selectively reacts with a guanidino group of an arginine residue. In this study, amicyanin (Ami) from Paracoccus denitrificans have been immobilized on the electrode via Arg by using p-carboxyethylphenylglyoxal (CEPG). Ami is a blue copper protein which mediates electron transfer between methylamine dehydrogenase and cytochrome c₅₅₃. Furthermore, to
investigate the effect of the orientation of Ami toward an electrode on its electrochemical properties, we constructed three Ami mutants (R48A, R99A, R48A/R99A). The gold electrode was immersed into 2-mercaptoethanol (ME), and then, immersed in CEPG solution including NHS and EDC. The modified electrodes were soaked in the protein solution. The redox responses using ME-Ami and ME/CEPG-Ami electrodes were observed by cyclic voltammetry. This result suggests that Ami adsorbed nonspecifically on the ME-modified electrode. Therefore, to remove the nonspecifically absorbed Ami, Ami-immobilized electrodes were immersed in phosphate buffer containing 1 M NaCl. As a result, the redox response using the ME-Ami electrode was diminished. When the ME/CEPG-Ami electrode was used, the redox response was still observed. The results suggest that CEPG is a useful compound to immobilize protein via Arg on the electrode surface. We will also report the electrochemistry using the Ami mutants immobilized on the electrode.

**P139**

Environment of "proximal" heme pocket in myoglobin multiple mutants as revealed by resonance Raman spectroscopy

Keihei Kiyota, Yoshikazu Tomisugi, Tadayuki Uno, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Japan.

Contact e-mail: k-kiyota@phs.osaka-u.ac.jp

Porcine myoglobin (Mb) mutants having a ligand binding pocket in the heme proximal side was investigated by absorption and resonance Raman spectroscopic methods. Mb is an oxygen storage protein which has been the subject of intense research over the last few decades. The heme iron in wild-type (WT) Mb is coordinated to the protein through the proximal His93, and exogenous small ligands including oxygen molecule binds to the distal iron coordination site. We focused on a quadruple mutant, H64V/V68H/H93A/H97F (VHAA), which uniquely has an endogenous distal His68 ligand and a binding pocket for an exogenous ligand in the iron proximal side1). In order to make the proximal pocket for exogenous ligands to access easily, we further constructed a quadruple mutant, H64V/V68H/H93A/H97F (VHAA), in which the proximal Phc97 in VHAA is replaced by less bulky Ala. Stability of the bound heme, pH dependence, and ligand (CN-, CO) affinity to VHAA were studied using UV-vis absorption spectroscopy. The VHAA mutant was revealed to be a six-coordinate high-spin species in the ferric state and five-coordinate high-spin species in the ferrous state. The Fe-CO stretching frequency of VHAA was higher than that of VHAF, suggesting more polar proximal environment similar to that in WT. Mb. The Fe-CO stretching frequency of VHAA, however, differed substantially from that of WT. Thus, VHAA was revealed to have a unique binding pocket for exogenous ligands.

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**P140**

Metal-chelated forms of adenylyl kinase

Anna V. Kladova, Sergey A. Bursakov, Olga Yu. Gavel, Isabel Moura, Jose J. G. Moura, Universidade Nova de Lisboa, Caparica, Portugal.

Contact e-mail: anna-kladova@yandex.ru

Adenylyl kinases (AK) catalyze the reversible transfer of the terminal phosphate group from MgATP to AMP and contribute to the maintenance of constant level of cellular adenine nucleotides. The adk gene from D. gigas was cloned and the protein overproduced in E. coli BL21(DE3) strain using M63B1 minimal medium. The method for metal containing recombiant AK production was developed for in vitro conditions, for the purpose to understand the role of metals for activity and stability of AK. The optimal conditions (concentrations of metals and inducer IPTG, time and temperature of incubation) were established. Homogenous AKs with the ratio metal/protein equal 1/1 (where metals were Co2+, Zn2+, Fe3+) were obtained. Apo-AK was obtained in vivo conditions in inclusion bodies by following refolding in presence of detergent NDSB256 as well as in vitro conditions using phenylmercuricdioxide as substituted agent and DTT and TCEP as reducing agents. Metal chelated and apo-AK forms were spectroscopically (UV/Visible, EPR) and kinetically characterized and compared. For metal containing forms Vmax for ADP and MgADP can be represented in following order Zn-AK > Co-AK = Fe-AK. In the same time Km values for MgADP for all metal chelated forms are the same but Kd values for ADP are vary (Co-AK > Zn-AK > Fe-AK). The kinetic properties of apo-AK are different with comparison to metal chelated forms. Thus specific activity of it is only 20% from metal chelated forms.

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**P142**

A ferredoxin apo-state with molten globule characteristics: a role in iron-sulfur center assembly?

Sónia S. Leal, Cláudio M. Gomes, Instituto de Tecnologia Química e Biológica, Oeiras, Portugal.

Contact e-mail: leal@itqb.unl.pt

The biological insertion of Fe-S involves the interaction of (metallo) chaperons with a partly folded target polypeptide. In this respect, the study of non-native protein conformations in iron-sulfur proteins is relevant for the understanding of the folding process and cofactor assembly [1-6]. We have investigated the formation of a molten globule state in a prokaryotic [3Fe4S]4+Fe4S4 ferredoxin which also contains a structural Zn2+ site [6]. Biophysical studies have allowed to dissect the sequence of thermal unfolding events: Fe-S disassembly, loss of tertiary contacts and dissociation of the Zn2+ site which is simultaneous to alterations on the secondary structure. Upon cooling, an apo-ferredoxin state is obtained, with characteristics of a molten globule: compactness identical to the native form; similar secondary structure evidenced by far-UV CD; no near-UV CD detected tertiary contacts; and an exposure of the hydrophobic surface [6].
The possible physiological relevance of molten globule states in Fe-S proteins and the hypothesis that their structural flexibility may be important to the understanding of metal centre insertion are discussed.

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P143
Impact of the first coordination sphere of Fe2+ in Dke1 on cofactor affinity
Stefan Leitgeb, Grit Straganz, Bernd Nidetzky, Institute of Biotechnology and Biochemical Engineering, Graz University of Technology, Graz, Austria.
Contact e-mail: s.leitgeb@tugraz.at

Diketone cleaving enzyme (Dke1) from Acinetobacter johnsonii is a non-heme iron dependent dioxygenase that degrades its natural substrate acetylacetone by oxidative C-C bond cleavage. The metal binding site of this enzyme is similar to other dioxygenases that show a cupin fold and consists of three histidine-residues. Since the majority of analogous non-heme metal-dependent dioxygenases possess a 2-His-1-carboxylate facial triad we performed mutagenesis experiments to replace the first-coordination sphere histidine residues by carboxylate and carboxylamine residues to investigate the effects on activity and metal binding. Microdialysis equilibrium experiments combined with fluorescence titrations showed the removal of a high affinity iron binding site in Dke1, consistent with the loss of diketone-cleaving activity. A second, low-affinity, iron binding site was identified that is not relevant for catalysis. In contrast, the other divalent ions that were tested in terms of binding - Cu2+, Ni2+, Zn2+, Mn2+ - only showed minor effects upon exchange of the first coordination sphere residues. We therefore found that by disrupting the 3-histidine coordination motif in Dke1 we could selectively lower the enzymes affinity for Fe2+.

P144
Novel cluster structure and histidine coordination in a plant metallothionein from wheat embryos
Oksana I. Leszczyszyn, Claudia A. Blindauer, University of Warwick, Coventry, United Kingdom.
Contact e-mail: o.leszczyszyn@warwick.ac.uk

Gene mining and expression studies have shown that plant metallothionein-like proteins display significant variation in sequence and biological function. However, information about their biochemical properties, metal-binding interactions and solution structure is considerably underrepresented in metallothionein research. We present a comprehensive characterisation of one plant MT exclusively expressed in the embryos of pre-germinating wheat seeds (E C). Unlike other metallothioneins, the CXC motifs of E C MT exclusively expressed in the embryos of pre-germinating wheat seeds (E C) are arranged into three regions along the primary sequence, and as such the E C sequence cannot be aligned with those of mammalian origin. In addition, E C has two histidine residues, which are not present in mammalian metallothioneins, but have been shown to coordinate metal ions in bacterial metallothioneins. It is therefore not surprising that the solution properties and structure differ from previously characterised metallothioneins.

Using various multinuclear and multidimensional NMR experiments, we demonstrate the involvement of histidine residues in zinc ion coordination and their likely modes of binding, together with analysis of backbone dynamics and support for a novel cluster composition. 111Cd NMR and [1H,1H] TOCSY spectra reveal interesting exchange behaviour in one of the clusters and also offer structural insights.

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P145
Novel Nitric Oxide Dioxygenase, SHP (Sphaeroides Heme Protein), and New detoxification mechanism
Bor-Ran Li1, J. L. Ross Anderson1, Caroline S. Miles2, Christopher G. Mowat3, Graeme A. Reid2, Stephen K. Chapman1, School of Chemistry, University of Edinburgh, United Kingdom; 2School of Biology, University of Edinburgh, United Kingdom.
Contact e-mail: Li.Borran@ed.ac.uk

SHP (Sphaeroides Heme Protein) is an oxygen-binding cytochrome, typically found in photosynthetic and metal-reducing bacteria. Like other oxygen-binding cytochromes, it not only binds oxygen but also nitric oxide, with similar “on-rates”. SHP is found in 16 different species of bacteria and, exceptionally, is the only known e-type cytochrome to have asparagine as a heme ligand. The 3-dimensional structure of SHP was solved in 2000 [1]. The redox partner for SHP is thought to be another cytochrome known as DHC (DiHeme Cytochrome c) [2]. In previous studies, we have shown that DHC transfers electrons efficiently to SHP. DHC also binds tightly to SHP under low ionic strength conditions. However, the biological function of SHP is not clear. An investigation of the Integrated Microbial Genomes data base revealed that the genes for SHP/DHC/Cyt b are side by side in a gene neighbourhood, and their mRNAs are up-regulated when nitrite is the electron acceptor. Using biochemical methods, we have demonstrated that the oxyferrous form of SHP can react rapidly with nitric oxide to form nitrate. Therefore, we propose that one function for SHP is as a nitric oxide dioxygenase.

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P146
Metal binding properties of Heat-shock protein A (HspA)
Hongyan Li, Shujian Cun, Wei Xia, Ruiguang Ge, Marie C. M. Lin, Hongzhe Sun, Department of Chemistry, The University of Hong Kong, Hong Kong, China.
Contact e-mail: hylichem@hkuce.hku.hk

The Heat-Shock Protein A (HspA) is a member of GroES chaperonin family, and it consists of 118 amino acids with a unique histidine/cystein-rich C-terminus, which is completely absent from most other GroES members. When being introduced into Escherichia coli with urease gene cluster together, HspA has been observed to be able to increase urease activity greatly [1], indicating that the protein may play an important role for the process of nickel transportation. Histidine-rich proteins are known for their abilities to bind to nickel [2-11] although their biological functions are not well understood.
The complete coding sequence of hspA was cloned from the genomic DNA of _Helicobacter pylori_ (strain 26695) and the protein was expressed in _E. coli_ and purified by nickel-affinity column and was verified by mass spectrometry. The aggregation behavior was studied via gel filtration chromatography. The metal binding properties of both HspA and its C-termini histidine/cystein rich peptide were examined using UV-vis spectroscopy and the results showed that HspA binds to nickel and bismuth with a stoichiometry of 2:1, similar to that of nickel binding to the peptide. Binding of both Ni^{2+} and Bi^{3+} to the protein is reversible and can be released either by chelate ligands such as EDTA or by lowing pH.

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P147

Directed evolution of tyrosinase for improved activity towards chlorinated phenols

Timothy E. Machonkin1,2, Min-Sun Park3, Brian O’Mara3, Taylor L. Weiss4, 1Whitman College, Walla Walla, WA, USA; 2University of Rochester, Rochester, NY, USA. Contact e-mail: machonte@whitman.edu

Tyrosinase holds great potential for the bioremediation of phenols in wastewater, since it reacts with such compound to form minimally soluble pigments that can be removed readily. It is unique in being capable of catalyzing the hydroxylation and subsequent oxidation of phenols using only O_{2} and substrate without additional reducing equivalents or co-substrates.

We have developed an expression system for _Streptomyces glaucescens_ tyrosinase that produces enzyme that is active _in vivo_ in high yield, and have designed a strategy for the directed evolution of tyrosinase mutants with improved activities towards non-native phenol substrates. This approach yielded a series of mutants of tyrosinase with improved activity towards chlorinated phenols, specifically 4-chlorophenol. The first round of directed evolution yielded 25 mutants that were 50-150% more active towards 4-chlorophenol. A second round of directed evolution yielded two double-mutants with an approximately 5-fold increase in _kcat_ towards 4-chlorophenol. Detailed steady-state kinetic studies also showed modest improvements in _kcat_ towards other chlorophenols, and decreased activity towards methylphenols. The mutations identified are mostly in the second-coordination sphere, and provide an interesting basis for future studies of structure-function correlations in this enzyme.

P148

Elucidating the functional properties of metalloenzymes: design and characterization of DF3 an artificial di-iron protein

Ornella Maglio1, Rafael Torres Martin de Rosales2, Flavia Nastri1, Marina Faella1, Vincenzo Pavone1, William F. DeGrado3, Angela Lombardi1, 1University of Naples “Federico II”, Naples, Italy; 2Imperial College London, London, United Kingdom; 3University of Pennsylvania, Philadelphia, PA, USA. Contact e-mail: ornella.maglio@unina.it

A major objective in protein science is the design of novel enzymes with properties tailored to specific applications. This goal is becoming increasingly possible as the growing number of protein sequences and known three-dimensional structures are providing essential information, which contribute to a deeper understanding of the relationship between protein sequence, structure, and function. In this perspective, metalloprotein design provides a powerful approach, contributing to a more comprehensive understanding of the way metalloproteins function in biology, with the ultimate goal of developing novel biocatalysts and sensing devices.

We approached the challenge of reproducing metalloprotein activities through the _de novo_ protein-design. We centered our attention on iron-containing proteins, and we developed the DF (Due Ferri) family of artificial proteins, as models of di-iron proteins.

A first subset of the DF family, DF1, consisted of two non-covalently associated helix-loop-helix motifs, which bound the di-metal cofactor near the center of the structure. DF1 analogues showed limited solubility in aqueous solution, probably due to an inter-helical turn with strained conformation, and an active site relatively inaccessible. To enhance the properties of this subset, we refined the structure by designing a new symmetric helix-loop-helix dimer. DF3 contains changes into the sequence to improve metal ion access, and a new inter-helical loop.

It shows improved solubility and active site accessibility, while retaining the unique native-like structure.

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P149

Role of the Threonine 101 residue on the stability of the heme active site of cytochrome P450cam: Multi-wavelength Circular Dichroism Studies

Soumen K. Manna, Shyamalava Mazumdar, Tata Institute of Fundamental Research, Mumbai, India. Contact e-mail: soumen@tifr.res.in

The role of the Thr101 residue in the conformational property and the stability of the active site of cytochrome P450cam were studied using site-directed mutagenesis and multi-wavelength circular dichroism (CD) techniques. The T101V mutation did not change the substrate binding affinity of the enzyme but it was associated with subtle changes in the conformational anisotropy around the heme active site. Thermolability of the mutant and the wild type enzyme have been studied by temperature dependence of the far-UV CD as well as that of the visible CD bands that arise from the heme moiety along with differential scanning calorimetry. The analysis of the thermal unfolding data showed that the thermal stability of the secondary structure of the enzyme was unaffected by the mutation. But the mutation was found to decrease the thermostability of the active site in presence of the substrate. On the other hand, this mutation had no apparent effect on the thermostability of the substrate-free enzyme. These results suggested that the threonine residue stabilizes the heme centre in case of the substrate-bound species, possibly by hydrogen bonding with one of the heme-propionate side chains. Such hydrogen bonding of the heme-propionate with threonine is absent in the substrate-free enzyme.

\[ \text{Ornella Maglio} \]

\[ \text{Rafael Torres Martin de Rosales} \]

\[ \text{Flavia Nastri} \]

\[ \text{Marina Faella} \]

\[ \text{Vincenzo Pavone} \]

\[ \text{William F. DeGrado} \]

\[ \text{Angela Lombardi} \]

\[ \text{University of Naples “Federico II”, Naples, Italy} \]

\[ \text{Imperial College London, London, United Kingdom} \]

\[ \text{University of Pennsylvania, Philadelphia, PA, USA} \]

\[ \text{Contact e-mail: ornella.maglio@unina.it} \]

A major objective in protein science is the design of novel enzymes with properties tailored to specific applications. This goal is becoming increasingly possible as the growing number of protein sequences and known three-dimensional structures are providing essential information, which contribute to a deeper understanding of the relationship between protein sequence, structure, and function. In this perspective, metalloprotein design provides a powerful approach, contributing to a more comprehensive understanding of the way metalloproteins function in biology, with the ultimate goal of developing novel biocatalysts and sensing devices.

We approached the challenge of reproducing metalloprotein activities through the _de novo_ protein-design. We centered our attention on iron-containing proteins, and we developed the DF (Due Ferri) family of artificial proteins, as models of di-iron proteins.

A first subset of the DF family, DF1, consisted of two non-covalently associated helix-loop-helix motifs, which bound the di-metal cofactor near the center of the structure. DF1 analogues showed limited solubility in aqueous solution, probably due to an inter-helical turn with strained conformation, and an active site relatively inaccessible. To enhance the properties of this subset, we refined the structure by designing a new symmetric helix-loop-helix dimer. DF3 contains changes into the sequence to improve metal ion access, and a new inter-helical loop.

It shows improved solubility and active site accessibility, while retaining the unique native-like structure.

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This study also indicated that the multi-wavelength CD studies can help to identify the weak interactions in the active site of metalloenzymes.

**P150**

*Redox states of Nitric Oxide Reductase from Pseudomonas nautica: Kinetic and Spectroscopic characterization*

Carlos E. Martins¹, Alice S. Pereira², Pedro Tavares¹, Cristina M. Cordas¹, Filipe Folgosa¹, Cristina G. Tamoteo¹, Sunil Naik³, Boi Hanh Huynh², José J.G. Moura¹, Isabel Moura¹

¹REQUIMTE/CQFB, Dept Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Caparica, Portugal; ²Department of Physics, Emory University, Atlanta, GA, USA.

Contact e-mail: carlos.martins@dq.fct.unl.pt

Nitric Oxide Reductase (NOR) from *Pseudomonas (Ps.) nautica* 617 is a membrane bound enzyme which belongs to the cNOR class. The as-isolated form is a heterodimer with a ~17 kDa (NORC) and a ~54 kDa (NORB) subunits. While NORC is a c-type monohemic subunit, NORB contains two b-type hemes and a non-heme iron atom.

The protein can be stabilized in three different redox forms: the as-isolated, ascorbate reduced and dithionite reduced, that exhibit different spectroscopic and kinetic behavior. The application of an array of spectroscopic (UV/Visible, EPR and Mössbauer spectroscopies) in conjunction with visible stopped-flow and electrochemistry to the study of the different redox forms, allowed the identification of the active form. Cytochrome c552 from the same organism was, also, identified as the putative physiologic electron donor in the nitric oxide reduction reaction catalyzed by NOR.

Data will be shown on the bifunctionality of Ps. nautica NOR, capable of NO reduction, as well as O₂ reduction.

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**P151**

*Ring opening mechanism of verdoheme catalyzed by heme oxygenase*

Toshihisa Matsui, Hiromichi Jin, Masao Ikeda-Saito, Tohoku University, Sendai, Japan.

Contact e-mail: matsui@tagen.tohoku.ac.jp

Heme oxygenase (HO) catalyzes catabolism of heme to biliverdin, CO and a free iron through three successive oxygenation steps. The third oxygenation, ring opening of verdoheme to yield biliverdin, has been the least understood step in spite of its importance in regulating the HO activity. In this study, we have performed reactions of a verdoheme-HO complex with alkyl hydroperoxides to assess three possible pathways previously proposed for the verdoheme opening (Fig.). The reaction with ethyl hydroperoxide, but not t-butyl and cumyl hydroperoxides, under reducing conditions afforded two linear tetrapyrroles without forming significant amount of biliverdin. The new products could also be prepared by chemical opening of verdoheme with sodium ethoxide, and were identified by HPLC and ESI-MS analysis as 1-ethoxy- and 19-ethoxy-deoxybiliverdins. The formation of the ethoxy-biliverdins unambiguously identifies the Fe-OOR species as a key intermediate in the EtOOH reaction, and strongly suggests the corresponding Fe-OOH mechanism for the physiologically relevant O₂ and H₂O₂-dependent biliverdin production. This is similar to the well-established mechanism of the first oxygenation, meso-hydroxylation of heme. HO appears to be evolved to control the reactivity of the Fe-OOH complexes to allow addition of their terminal oxygen moieties to the porphyrin macrocycle.

Cytochrome P450s are heme monooxygenases involved in a variety of oxidative metabolic reactions, which constitute a large heme-thiolate protein family. The enzymes mainly catalyze the monooxygenation of a variety of hydrophobic substrates. We reported overexpression and purification of cytochrome P450 from a thermocacidophilic crenarchaeon, *Sulfolobus tokodaii* strain 7 (P450st), previously.¹ It has been reported that the electronic environment around the heme group moiety and proximal heme ligand, Cys, affects the redox potential of cytochrome P450s.² In this study, we have constructed the P450st mutants substituted Phc310 to Ala and Ala320 to Glu, respectively. The double-point mutant changed at both F310A and A320Q has also been constructed. Phc310 and Ala320 in P450st reside closely at the conserved cysteine ligand, Cys317 in P450st, which coordinates to heme iron (Fig. 1). We have been investigated the effects of the substituents on the electrochemical properties and thermostability. The reduction potential and the catalytic activity with shunt pathway of P450st and mutants have also been investigated.

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and the results compared to our findings for TauD.

Hydroxylases, taurine/2-ketoglutarate (2KG) dioxygenase (TauD) and the results of parallel studies of TauD variants, may provide important information on how active site protein residues work in concert with the Fe(II) center to catalyze the specific hydroxylation of taurine. Parallel studies on XanA will be presented.

The HYSCORE cross-peaks from this 1H hyperfine spectrum showed a hyperfine tensor of axial symmetry characterized by a dipole-dipole distance of 3.2 Å and an isotropic contribution of 1.1 ppm. These facts indicate that the DET reaction occurs at the carbon black modified electrode and that the type 1 Cu site is a primary electron acceptor from the electrode. The dependences of the electrocatalytic currents on the solution pH, for values ranging from pH 4.0 to 8.0, have been investigated. The difference in the pH-optima for ASOM and ZAO was observed. ASOM and ZAO showed the pH-optima about 4.5 and 6.0, respectively. These pH-optima are good agreement with previously reported own pH-optima in the reaction with ascorbate. In addition, the catalytic current obtained from ASOM modified electrode was higher than that from ZAO at each pH. This could reflect the stability of the enzymes on the electrode surface.

However, further studies are required to define the precise causes of the difference in their activity.

One- and two-dimensional Electron Spin Echo Envelope Modulation (ESEEM) experiments have been used to study the coordination chemistry at the Fe(II) site of two non-heme Fe(II)/2-KG dioxygenases, taurine-2-ketoglutarate (2KG) dioxygenase (TauD) and xanthine hydroxylase (XanA). For both systems, Fe(II)-NO derivatives of the enzymes were studied. The 4-pulse HYSCORE method was used to resolve contributions from bound histidine nitrogens, coordinated H2O, and ambient H2O. For TauD, HYSCORE spectra collected for samples in aqueous buffer and 60% D2O-buffer showed changes in H2O and histidyl coordination upon substrate addition. HYSCORE spectra collected for samples in aqueous buffer and 60% D2O-buffer showed substantial distribution of exchangeable, 1H hyperfine couplings. When 2KG is a substrate addition, HYSCORE spectra show a substantial distribution of exchangeable, 1H hyperfine couplings. When 2KG is added, the 1H HYSCORE is considerably altered with the dominant hyperfine coupling arising from an exchangeable, strong-coupled proton of rhombic symmetry. Subsequent addition of substrate taurine to yield the ternary, Fe(II)-NO/2KG/taurine, complex at the active site showed a new, 1H hyperfine interaction that was not exchangeable in D2O. The HYSCORE cross-peaks from this 1H show a hyperfine tensor of axial symmetry characterized by a dipole-dipole distance of 3.2 Å and an isotropic contribution of 1.1 MHz. Comparison of these data with the X-ray crystal structure of TauD and the results of parallel studies of TauD variants, may provide important information on how active site protein residues work in concert with the Fe(II) center to catalyze the specific hydroxylation of taurine. Parallel studies on XanA will be presented and the results compared to our findings for TauD.

Direct electrochemistry of ascorbate oxidases on carbon black modified electrodes has been investigated with cyclic voltammetry. The catalytic current was observed at the potential which was close to the formal potential of a type 1 Cu site at pH 7.0. On the other hand, in the absence of enzymes no catalytic current was observed. These facts indicate that the DET reaction occurs at the carbon black modified electrode and that the type 1 Cu site is a primary electron acceptor from the electrode.

The dependences of the electrocatalytic currents on the solution pH, for values ranging from pH 4.0 to 8.0, have been investigated. The difference in the pH-optima for ASOM and ZAO was observed. ASOM and ZAO showed the pH-optima about 4.5 and 6.0, respectively. These pH-optima are good agreement with previously reported pH-optima in the reaction with ascorbate. In addition, the catalytic current obtained from ASOM modified electrode was higher than that from ZAO at each pH. This could reflect the stability of the enzymes on the electrode surface. However, further studies are required to define the precise causes of the difference in their activity.

Human porphobilinogen synthase (PBGS) [EC.4.2.1.24] catalyzes the condensation of two molecules of 5-aminolevulinic acid to produce porphobilinogen. The oligomeric state of PBGS is homoocotamer, which consists of microheterogenous subunits in the tertiary structure under air-saturated conditions. Two subunits form a minimal structural unit; in one subunit, Cys^{322}, Cys^{324}, and Cys^{312} nearly a catalytic site coordinates a proximal zinc ion, which is catalytically essential, and in the other subunit, Cys^{317} at the orifice of the active center coordinates a reservoir distal zinc ion. We elucidated that, in enzymatic activation by 2-mercaptoethanol, the distal zinc ion was transferred, and bound to the proximal binding-site (1). In this study, analytical ultracentrifugation, negative staining electron microscopy, and native PAGE were performed to determine the oligomeric state of PBGS under physiologic conditions. Furthermore enzyme activity staining and enzyme kinetic study were also carried out to investigate the structural change in enzymatic activation. It is concluded that PBGS exists in octamer under physiological conditions, and that the metamorphosis of the four sets of dimer units, resulting from the decrease in the Stokes radius of the oligomer, occurs in enzymatic activation. Metal- and redox-regulated metamorphism of the oligomer is an essential process for PBGS activation.

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Iron-sulfur proteins are found in virtually all life forms including archaebacteria, eubacteria, plants, and animals, and play various important physiological roles in electron transport, enzyme reactions, gene regulation, and DNA/RNA metabolisms. While a number of factors involved in iron-sulfur cluster biosynthesis in bacteria and in yeast have been identified and characterized extensively, molecular mechanism of iron-sulfur cluster biosynthesis and delivery in the plant cell still remains poorly
understood. In Arabidopsis thaliana, iron-sulfur cluster biosynthetic machineries have shown to be present both in the mitochondrion and in the chloroplast. In the chloroplast, two essential factors have already been identified as important participants, namely CnfU and Hcf101. Previously we identified and characterized the chloroplast-localized iron-sulfur cluster biosynthetic scaffold named CnfU whose function is essential for biogenesis of ferredoxin and photosystem I in Arabidopsis. We have now determined the crystal structure of CnfU and also gained the precise Fe-S cluster structure assembled on this scaffold protein in order to elucidate detailed molecular mechanisms of Fe-S cluster assembly and delivery. Moreover, we have characterized several additional Arabidopsis proteins which are likely involved in iron-sulfur cluster biosynthesis in the chloroplast or outside the chloroplast, the cytosol. We are now studying their detailed in vivo functions by analyzing Arabidopsis T-DNA insertion mutants and by using yeast complementation assay. Biochemical characterization of respective recombinant proteins purified from E.coli cells is also underway.

P156
Expression and characterization of iron containing alcohol dehydrogenase from hyperthermophilic archaeon, Pyrococcus horikoshii OT3
Yumi Kariya, Hirotoshi Matsumura, Nobuhumi Nakamura, Masafumi Yohda, Hiyoriuki Ohno, Department of Biotechnology and Life Science, Tokyo University of Agriculture and Technology, Koganei, Tokyo, Japan.
Contact e-mail: nobu1@cc.tuat.ac.jp

Alcohol dehydrogenases (ADHs; EC 1.1.1.1) belong to the oxidoreductase family, which catalyze the interconversion of alcohols with the corresponding aldehyde or ketone, using the nicotinamide cofactors, NAD(H) or NADP(H). The NAD- or NADP-dependent ADH can be divided into three groups according to their structures: Group I is a zinc-dependent, long-chain ADH family. Group II is a zinc-independent short-chain ADH. Group III is an iron-activated ADHs. The iron-activated ADHs have been little studied due to their structural instability. In this study, we have constructed the overexpression system of iron containing ADH derived from hyperthermophilic archaea, Pyrococcus horikoshii OT3 (PhADH) with Escherichia coli. The catalytic activity and thermostability of PhADH have been investigated. The PhADH gene was amplified by PCR using P. horikoshii OT3 genomic DNA. The gene subcloned into the pET23d was transformed into E. coli BL21 (DE3). Cells were cultured at 37 °C in LB medium containing ampicillin and FeSO₄. After thermostability of PhADH have been investigated. OT3 (PhADH) with derived from hyperthermophilic archaeon, constructed the overexpression system of iron containing ADH little studied due to their structural instability. In this study, we have focused on the axial coordination behavior in NiSOD, so three types of Ni(II) complexes with N2S2 square-planar coordinations (Figure) as active site models of NiSOD were synthesized. Addition of imidazoles to the acetone solution of Ni(II) complexes, 1 and 2, showed absorption spectral changes due to their axial coordination, but, in the case of Ni(II) complex 3, no spectral changes were observed under the same condition. EPR studies of an oxidized Ni (III) complex of 3 revealed that an imidazole molecule bound to the axial position of the Ni(III) center, judging from the appearance of hyperfine-splitting of its axially coordinating nitrogen atom in the parallel region. Based on these spectroscopic analysis data of these model systems, we will discuss about relationships between the axial coordination behaviors and electrochemical properties of the nickel center of NiSOD.

P157
Spectroscopic and electrochemical properties of Ni(II) complexes with axial coordination as an active site model of NiSOD.
Daisuke Nakane, Tatsuya Fuji, Yasuhiro Funahashi, Tomohiro Ozawa, Hideki Masuda, Graduate school of materials and engineering, Nagoya, Japan.
Contact e-mail: funanitech@yzhio.co.jp

Ni-containing superoxide dismutase (NiSOD) is an enzyme catalyzing disproportionation of superoxide anion through the cycles of Ni(II) and Ni(III) states. The active site of NiSOD contains the nickel ion with the equatorial plane of monoamine-monoamide-dithiolate type coordination, and such a planar N2S2 coordination geometry is necessary for the most suitable redox reaction of superoxide-dismutation. The crystal structural analysis of NiSOD has showed that the central nickel ion is changed from the square-planar Ni(II) to square pyramidal Ni(III) by coordination of axial imidazole of His1. In this study, we focused on the axial coordination behavior in NiSOD, so three types of Ni(II) complexes with N2S2 square-planar coordinations (Figure) as active site models of NiSOD were synthesized. Addition of imidazoles to the acetone solution of Ni(II) complexes, 1 and 2, showed absorption spectral changes due to their axial coordination, but, in the case of Ni(II) complex 3, no spectral changes were observed under the same condition. EPR studies of an oxidized Ni (III) complex of 3 revealed that an imidazole molecule bound to the axial position of the Ni(III) center, judging from the appearance of hyperfine-splitting of its axially coordinating nitrogen atom in the parallel region. Based on these spectroscopic analysis data of these model systems, we will discuss about relationships between the axial coordination behaviors and electrochemical properties of the nickel center of NiSOD.

P158
The mechanism of arsenic binding to one and two-domain metallothioneins
Thanh T. Ngú, Moira K. Rushton, Martin J. Stillman, The University of Western Ontario, London, ON, Canada.
Contact e-mail: tmsgu@uwo.ca

The number of reported cases of chronic arsenic poisoning is on the rise throughout the world, making the study of the long-term effects of As exposure critical. As³⁺ binds to biological thiols, including mammalian metallothionein (MT), which is an ubiquitous sulfur-rich metalloprotein that coordinates a wide range of metals. The two-domain mammalian protein binds divalent metals (M) into two metal-thiolate clusters with stoichiometries of M₂S₉ (β) and M₂S₁₀ (α). We report that As³⁺ binds with stoichiometries of As₂S₈ (β) and As₂S₁₀ (α) to the recombinant human metallothionein (rhMT) isoform 1α protein. Further, we report the complete kinetic analysis of the saturation reactions of the separate 1α and 1β domains of rhMT with As³⁺. Speciation in the metalation reactions was determined using time- and temperature-resolved electrospray ionization mass spectrometry (ESI-MS). The binding reaction of As³⁺ to the α and β MT domains is shown to be noncooperative and involves three sequential, bimolecular metalation steps. The time-dependent As-metalation to the two-domain 16-cysteine seaweed Fucus vesiculosus metallothionein is also described. The analyses allow for the first time, the complete simulation of the experimental data for the step-wise metalation reaction of MT showing the relative concentrations of the metal-free, apo-MT and each of the As-MT intermediate species as a function of time and temperature.
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P159
Arylhaloallylamine Complexes of Copper-Containing Amine Oxidases
Yen H. Nguyen1, David B. Langley1, Ping Ying1, David M. Dooley1, Hans C. Freeman1, Ian A. McDonald2, J. M. Grass3, 1University of Sydney, Sydney, Australia; 2Pharmacia Ltd, Frenchs Forest, Australia; 3Montana State University, Bozeman, MT, USA.
Contact e-mail: ynguyen@usyd.edu.au

Semicarbazide-sensitive amine oxidase (SSAO, AOC-3) is a member of the family of copper-containing amine oxidases and encompasses a variety of roles that include detoxification in mammals, wound healing in plants, and provision of essential nutrients in bacteria. The human SSAO, also called vascular adhesion protein-1 (VAP-1), is of particular interest, owing to its adhesion and enzymatic properties that have been proven to be implicated in inflammation-related diseases, such as rheumatoid arthritis, multiple sclerosis, Crohn’s disease, diabetes, and atherosclerosis.

We are undertaking structural studies of various arylhaloallylamine derivatives with *Arthrobacter globiformis* amine oxidase (AGAO), which possesses structural and enzymatic similarities to VAP-1. These inhibitors have been previously reported to be mechanism-based inhibitors of monoamine oxidase (MAO) and SSAO. These compounds consist of a primary amine moiety which poses as the substrate and allows the inhibitors to form a Schiff-base intermediate with the topaquinone cofactor (TPQ, a modified tyrosine residue) during the catalytic cycle. This hypothesis is supported by preliminary data indicating the inhibitor-TPQ adduct is trapped in the active site.

P160
Oxygen sensing mechanism of signal transducer protein HemAT
Muneto Nishimura1, 2, Hideaki Yoshimura1, 2, Kazumiichi Ozawa1, Shiro Yohsioka1, Minoru Kubo2, Teizo Kitagawa2, Shigetoshi Aono3, 1The Graduate University for Advanced Studies, Okazaki, Japan; 2Okazaki Institute for Integrative Bioscience, Okazaki, Japan.
Contact e-mail: muneto@ims.ac.jp

HemAT is a heme-containing signal transducer protein that functions as an O2 sensor in aerotaxis control system in some bacteria and archaea. HemAT should discriminate its physiological effector O2 from other gas molecules, for which the specific interaction between the heme-bound ligands and the surrounding amino acid residues play an important role. Resonance Raman spectroscopy and mutagenesis studies reveal that HemAT from *Bacillus subtilis* (HemAT-Bs) has three conformers in O2-bound form with different hydrogen bonding pattern on the O2, where Thr95 is involved in the hydrogen bonding interaction.

Recently, it is reported that HemAT homologues exist in some bacteria. In some HemAT homologues, Thr residue is not conserved at the corresponding position to Thr95 in HemAT-Bs in the distal heme pocket. This suggests that the hydrogen bonding interaction between Thr residue and the heme bound O2 is not a mandatory requirement for the selective O2 sensing mechanism. Essential factors for O2 sensing by HemAT would be clarified by comparing the detail properties including the hydrogen bonding pattern on the heme-bound ligand among HemAT homologues.

In this study, we characterized the hydrogen bonding pattern on the heme-bound O2 in HemAT from *Halobacterium salinarum* (HemAT-Hs) and *Rhodospirillum rubrum* (HemAT-Rs) by using resonance Raman spectroscopy. O2-bound HemAT-Hs and HemAT-Rs have three conformers but the hydrogen-bonding pattern in the distal heme pocket is different from HemAT-Bs. Based on these results, we will discuss the essential factors for the selective O2 sensing by HemAT.

P161
Spectroscopic and Electrochemical Studies of Pseudoazurin Met16X Variants
Yuji Obara1, Doreen E. Brown2, David M. Dooley2, Takamitsu Kohzuma3, 1Ibaraki Univ., Ibaraki, Japan; 2Montana State Univ., Bozeman, MT, USA.
Contact e-mail: 06nd603s@hcs.ibaraki.ac.jp

Noncovalent weak interactions play important roles in many varieties of biological systems. Very recently, we reported the spectroscopic and electrochemical studies of M16F, M16Y, and M16W mutants of blue copper protein, pseudoazurin (PAz) to investigate the effects of the n-n interaction on the active site structure [1]. The Met16 substituted mutants of PAz, in which several alkyl group has been introduced in the vicinity of the His81 imidazole ligand, have been constructed and characterized in order to elucidate the effects of the indirect weak interaction on the structure and function of the active site. Electronic absorption spectra of M16L, M16V, M16A, M16L mutants and WT showed the absorbance ratio, A ~460/A ~600 were 0.70, 0.61, 0.60, 0.43, and 0.46 respectively. The ratio values except M16L variant are significantly higher than that of WT PAz. The higher value suggests the increasing of rhomic structure population.

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P162
Rate limiting event for Paracoccus pantotrophus CCP catalysis as revealed by direct electrochemistry
Patricia M. Paes de Sousa1, Sofia R. Pauleta1, David Rodrigues2, M. Lurdes Simões Gonçalves, 1, 2Graham W. Pettigrew, 1, Isabel Moura1, 2Margarida M. Correia dos Santos2, José J. G. Moura1, 1REQUIMTE-CQFB, FCT-UNL, Monte da Caparica, Portugal; 2CQE-IST, Lisboa, Portugal; 2Royal (Dick) School of Veterinary Studies, University of Edinburgh, Edinburgh, United Kingdom.
Contact e-mail: patricia.sousa@dq.fct.unl.pt

Reactive oxygen species like hydrogen peroxide are toxic compounds leading to cell damage or death. Peroxidases reduce peroxide to H2O using a variety of oxidisable substrates, small mono haem c type cytochromes and/or copper proteins, like pseudoazurin [1,2].

As to the catalytic mechanism, with the exception of *Nitrosomonas europaea*, all bacterial cytochrome c peroxidases (CCP) are isolated in a resting state. The high potential haem needs to be reduced by one electron to allow the conversion from resting to active protein, triggering the loss of one of the coordinants from the low potential haem allowing the hydrogen peroxide to bind.

We report on the direct electrochemistry of *Paracoccus pantotrophus* CCP in non turnover and catalytic conditions at a pyrolytic graphite membrane electrode. The results show that the enzymatic activity is mediated and limited by the structural and spin state changes, following the electron transferring haem reduction. The estimated turnover number is in clear agreement with the rate constant determined for the rate limiting step.

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P163
Microwave-Assisted Solid-Phase Synthesis, Cellular Uptake Studies, and Cytotoxicity of Cymantrene-Peptide Bioconjugates
Harmel Willfried P. Peindy Ndongo, Ulrich Schatzschneider, Lehrstuhl für Anorganische Chemie I – Bioanorganische Chemie, Ruhr-Universität Bochum, Universitätsstr., Bochum, Germany. Contact e-mail: Harmel.PeindyNdongo@rub.de

Incorporation of metallocenes into biological molecules has attracted the attention of several researchers. Thus, steroids, penicillins, antibiotics, and saccharides have been functionalized with metallocenes. The rationale to prepare such bioconjugates lies in the increased lipophilicity brought by the metallocene structure and the use of the covalently bound metal-label for sensing applications. Despite many advances in peptide chemistry and the synthesis of peptide-metallocene bioconjugates, cymantrene derivatives have received relatively little attention so far. In this work, the neuropeptide pseudoneurotensin H-Lys-Lys-Pro-Tyr-Ile-Leu-0H, a 99mTc-labelled analogue of which shows enhanced tumor uptake, was chosen to be part of metal-peptide bioconjugates. We report on the microwave-assisted solid phase conjugation between cymantrene carboxylic acid and the biologically active neuropeptide part (Scheme). Microwave irradiation was applied at different stages of the synthesis, including amide bond formation, removal of Fmoc protective groups, and acidolytic release of the metal-bioconjugate from the resin. The use of microwave irradiation in all steps of the solid-phase peptide synthesis and for coupling of cymantrene allows to significantly reduce reaction times while leading to high purity of the crude product. The resulting metal-peptide bioconjugate (1) was purified by reverse-phase HPLC and characterized with ESI mass spectrometry. Additionally, the biological activity of this new cymantrene-peptide will be reported. This work was supported by the Deutsche Forschungsgemeinschaft (DFG) within FOR 630 (“Biological Function of Organometallic Compounds”) through grant SCHA 962-3.

P164
Wheat Ec-1 MT: A detailed view and a comparison with other plant metallothioneins
Estevao Peroza, Eva Freisinger, Institute of inorganic chemistry, Zurich, Switzerland. Contact e-mail: peroza@aci.unizh.ch

Metallothioneins (MTs) are small proteins found in a wide range of living organisms with a characteristic high cysteine content and affinity for d^{10} metals ions. There is evidence supporting their participation in the homeostasis of essential metal ions, e.g. Zn^{II} and Cu^{II}, sequestration of toxic elements (Cd^{II} or Hg^{II}) and in the redox conditions with each other. The early cysteine-labelled protein (Ec-1) from wheat is a member of the plant MT pec sub-family and was the first MT identified in living organisms with a characteristic high cysteine content and affinity for d^{10} metals ions. There is evidence supporting their participation in the homeostasis of essential metal ions, e.g. Zn^{II} and Cu^{II}, sequestration of toxic elements (Cd^{II} or Hg^{II}) and in the redox conditions with each other. As another of our current interests lies in the redox state controlled mobilization of metal ions in plant MTs, we will further compare Zn^{II}-release rates from Ec-1 and other plant MT sub-types under varying redox conditions with each other.

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P165
Does the deprotonation of CuB ligands play a role in the reaction mechanism of Cytochrome c oxidase?
M. Punnagai, G. Matthias Ullmann, University of Bayreuth, Bayreuth, Germany. Contact e-mail: Punnagai.Munusami@uni-bayreuth.de

Recent studies on the cytochrome c oxidase (CcO) CuB center suggested that the deprotonation of the CuB-bound imidazolate ring of histidine (His291 in mammalian CcO or His294 in Rhodobacter sphaeroides CcO) as a key element in the proton pumping mechanism. The central feature of the proposed mechanism is that pKa values of the imidazolate vary significantly depending on the redox state of the metals in the binuclear center. We report the pKa values of the His291, His290 and water molecules both in the oxidized and reduced state of CuB center, where the the pKa values of the His291 was considered to play a significant role in the reaction mechanism of cytochrome c oxidase. We use hybrid density functional theory in combination with continuum electrostatics to calculate the pKa values, successively with aqueous solution and with in the protein, of the CuB-bound imidazolates in oxidized and reduced states respectively. In the present study we considered all the possible protonation equilibrium reactions in the CuB center to understand the deprotonation reactions of His290, His291 and water. The pKa shifts are calculated in protein environment.

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P166
The complex III from Rhodobacter marinus
Manuela M. Pereira1, Patricia N. Refojo1, Gudmundur O. Hreggvidsson2, Sigridur Hjorleifsdottir2, Miguel Texeira3, 1Instituto de Tecnologia Quimica e Biologica, Oeiras, Portugal, 2Prokaria Ltd, Sudurgata, Iceland. Contact e-mail: refojo@itqb.unl.pt

Rhodobacter marinus is a thermohalophilic bacterium, whose respiratory chain has been extensively studied. The biochemical, spectroscopic and genetic search for a bc_{1} complex was always fruitless; however a functional equivalent complex was purified from the membranes and characterized. Now, with the sequencing of the R. marinus genome it was possible to assign the N-terminal sequences from several proteins of this complex to its coding genes. It was observed that the bc complex has the same genomic organisation of the so called MFC compounds, which have been proposed to be oxidoreductases participating in the respiratory and in the photosynthetic electron transfer chains. Furthermore it was observed the presence of this complex in several genomes in which the genes coding for the bc_{1} complex are absent and in which a quinol:cytochrome c oxidoredutase has to be present. R. marinus be complex is coded by a six gene cluster. Three of these genes
codify for peripheral proteins; two cytochromes c, a pantetheanolic and a monohaemoc, and a large protein containing iron sulphur centres. The other three genes code for transmembrane proteins: two are predicted to have ten transmembrane helices with putative quinone binding sites motifs and are homologous to membrane subunits present in several members of the DMSO reductase family; the third gene codifies a two transmembrane helices protein.

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P167 Identification and analysis of cadmium protein targets in Escherichia coli: a 2D electrophoresis approach maintaining protein-metal interactions performed in vivo
Stéphanie Renaud1, Nicolas Bremond1, Céline Henry2, Catherine Berthomieu1, LIPM, IBEB, CEA cadarache, Saint Paul Lez Durance, France; 2APSS, BIOBAC, INRA, Jouy en Josas, France. Contact e-mail: stepharenud@yahoo.fr

Despite numerous studies, the molecular mechanism of cadmium (Cd) toxicity in cells is not totally understood. Our aim is to detect protein targets of Cd at low Cd doses (10 μM) to unravel some aspects of cadmium toxicity. Although Cd-protein interactions have been evidenced in vitro, only few data are available concerning these interactions in vivo, at low Cd doses.

To identify Cd protein targets in vivo, cells were grown in the presence of radiolabeled 109Cd. Two successive native electrophoreses were performed to separate soluble proteins of the cell extract. This method preserves metal-protein interactions established in vivo. Autoradiography revealed fourteen soluble protein targets for cells cultured in the presence and absence of bound metal ions. The results are interpreted in light of the metal ion binding sites proposed in our model. We also consider the effects of increasing temperature and metal ion binding on the far-UV CD spectrum of Hx and find that Zn2+ and Cu2+ influence the tertiary structure of the protein the most by reducing the 2a by 4 and 5 °C, respectively. Because these metal ions also destabilize the structure of the Hx-heme complex, we studied their effect on the spectroscopic properties and CO recombination kinetics of the Hx-Fe(II)-heme complex. These results are consistent with binding of Cu2+ in the linker region between the two domains of the protein as recently proposed. Supported by a CHIR/CSB partnership grant and a Canada Research Chair (to A.G.M.).

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P168 Characterization of an iron(IV) intermediate from the Fe(II) and alpha-ketoglutarate-dependent halogenase CytC3
Pamela Rigg-Gelasco1, Danica P. Galonic2, Eric W. Barr3, Megan L. Matthews4, Gretchen M. Koch5, Joseph R. Yonce1, Joseph M. Bollinger3, Christopher T. Walsh5, Carsten Krogh1, College of Charleston, Charleston, SC, USA; 2Harvard Medical School, Cambridge, MA, USA; 3The Pennsylvania State University, University Park, PA, USA. Contact e-mail: rggelasco@cofc.edu

The enzyme CytC3 from Streptomyces halogenates the γ-methyl group of L-amino acid to produce α-chloro and α- dichloroaminobutyric acids, of which the latter shows antibacterial activity.1 To achieve halogenation of the unactivated methyl carbon, CytC3 and other non-heme iron halogenases utilize α- ketoglutarate in an O2 dependent reaction to generate a high-valent Fe(IV)-oxo species. The intermediate abstracts a hydrogen atom from the substrate, allowing the incorporation of a metal-bound halide into the substrate radical.2,3 We describe here the structural characterization of the CytC3 Fe(IV)-oxo intermediate that was generated in the presence of bromide rather than chloride using x-ray absorption and Mössbauer spectroscopies. Iron x-ray absorption spectroscopy demonstrates an Fe-Br interaction in the reactant CytC3:Fe(II) α-KG:Br:L-Ab-L-oxa-5-CytC2 complex of 2.5 Å. When this complex is reacted with oxygen for 2 s, a reaction intermediate is formed in high yield (80%). Mössbauer spectroscopy demonstrates that the novel species contains a high-spin Fe(IV) center. The enhanced preedge feature in the Fe-EXAFS absorption spectra and the short Fe-oxygen and Fe-Br interactions of 1.62 Å and 2.43 Å, respectively, support the formulation of this intermediate as a Br(Fe(IV))-oxo complex.
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P169 Metal ion binding and structural dynamics of human hemopexin
Federico I. Rosell, Marcia R. Mauk, A. Grant Mauk, Dept. of Biochemistry and Molecular Biology and Centre for Blood Research, University of British Columbia, Vancouver, BC, Canada. Contact e-mail: fir@interchange.ubc.ca

The heme-scavenging plasma protein hemopexin (Hx) binds a variety of divalent metal ions and may, therefore, participate in metal ion homeostasis in vivo. Recently, we used homology modeling to propose a structure for human Hx based on the structure reported for rabbit Hx, and we identified several putative metal ion binding sites. Here, we use thermally-induced near-UV difference spectroscopy to study human Hx lacking bound heme (the form normally in circulation) in the presence and absence of bound metal ions. The results are interpreted in light of the metal ion binding sites proposed in our model.2 We also consider the effects of increasing temperature and metal ion binding on the far-UV CD spectrum of Hx and find that Zn2+ and Cu2+ influence the tertiary structure of the protein the most by reducing the tan by 4 and 5 °C, respectively. Because these metal ions also destabilize the structure of the Hx-heme complex, we studied their effect on the spectroscopic properties and CO recombination kinetics of the Hx-Fe(II)-heme complex. These results are consistent with binding of Cu2+ in the linker region between the two domains of the protein as recently proposed. Supported by a CHIR/CSB partnership grant and a Canada Research Chair (to A.G.M.).

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2. Mauk, M. R.; Rosell, F. I.; Mauk, A. G. Nat. Prod. Rep. in press.
to examine the C\(\text{O}\) binding site on Cyt \text{c}. Based on the chemical shift changes and line broadening of the NMR signals, we successfully determined that the Cyt \text{c} binding site is located near heme vinyl group and showed the possibility that, in addition to electrostatic interactions, hydrophobic interactions are also involved in the specific binding with Cyt \text{c}. Upon oxidation of ferrous Cyt \text{c} to ferric Cyt \text{c}, the signal perturbation by binding Cyt \text{c} is reduced for some NMR signals from polar residues such as Glu4 and Lys8, suggesting that the electrostatic interactions with Cyt \text{c} would be weakened to facilitate the replacement of ferric Cyt \text{c} with ferrous Cyt \text{c}. Thus, our NMR analysis for the Cyt \text{c}-Cyt \text{c} complex clearly identified the Cyt \text{c} binding site on Cyt \text{c} and we can propose a mechanism for the reox-dependent affinity change of Cyt \text{c} to Cyt \text{c}, leading to the specific and effective electron transfer between Cyt \text{c} and Cyt \text{c}.

P171

Iminodiacetic Carboxylate/Hydroxamate-Sulfonamide Derivatives as Potential Dual Target Antitumor Drugs

Maria Amelia Santos, Centro de Química Estrutural, Lisboa, Portugal.

Contact e-mail: masantos@ist.utl.pt

Inhibitors of Zinc-containing enzymes, including Matrix Metalloproteinases (MMPs) and some tumor-associated Carbonic Anhydrases (CAs) isoenzymes are attractive therapeutic targets in the treatment of numerous diseases. Therefore their inhibition is supposed to slow down or prevent disease progression. The inhibition of each of these enzymes needs special structural requirements, not only in corresponding zinc-binding groups but also in the different scaffolds to assure strong interaction within the cofactor-binding region of the enzymes.

A series of new compounds, based on iminodiacetic (IDA) scaffolds with different sets of alkyaryl and sulfonylaryl groups as well as two types of zinc binding groups, namely hydroxamate and primary sulfonamide have been designed, developed and tested \textit{in vitro}, with the aim of improving potency and selectivity for the inhibition of some tumor-associated MMPs and some CAs isoenzymes. These non-peptidic compounds were easily prepared, mostly from IDA, and they were assayed as inhibitors of seven MMPs (MMP1, MMP2, MMP7-9, MMP14, and MMP16) and three CAs (hCA I, II and IX). Some of the compounds show excellent specificity for the CA IX as well as MMP2, MMP9 and MMP13 with \(K\text{d} \text{cat}\) values in nanomolar order.

P172

Histidine\(^{131}\) is essential for metal- and redox-regulated activation of human porphobilinogen synthase

Nori Sawada\(^1\), Noriyuki Nagahara\(^1\), Fumino Arisaka\(^2\), Yoko Endo\(^1\), Yoshiaki Nakajima\(^1\), Masayasu Minami\(^1\), Tomoyuki Kawada\(^1\), Yoshiaki Nakajima\(^3\), Masayasu Minami\(^1\), Tomoyuki Kawada\(^1\), Yoshiaki Nakajima\(^3\), Masayasu Minami\(^1\), Tomoyuki Kawada\(^1\), Yoshiaki Nakajima\(^3\), Masayasu Minami\(^1\), Tomoyuki Kawada\(^1\), Yoshiaki Nakajima\(^3\), Masayasu Minami\(^1\), Tomoyuki Kawada\(^1\).

\(^1\)Dept. of Environmental Medicine, Nippon Medical School, Tokyo, Japan; \(^2\)Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Yokohama, Japan; \(^3\)Clinical Research Center on Occupational Poisoning, Tokyo, Japan.

Contact e-mail: pnori@mms.ac.jp

Human porphobilinogen synthase (PBGS) \([\text{EC}.4.2.1.24]\) has two zinc binding sites; one site, referred to as a proximal zinc-binding site, consists of three cysteines near two catalytic sites. The other site, referred to as a distal zinc-binding site, consists of His\(^{131}\) and Cys\(^{232}\), which are located at the orifice of the catalytic cavity. To elucidate the role of His\(^{131}\) and Cys\(^{232}\) in the catalysis, electrostatic vectors for wild type PBGS, H131A and C223A were constructed. Each protein was then overexpressed in \textit{E. coli} and purified to homogeneity. Kinetic study, zinc ion analysis, native-PAGE and analytical ultracentrifugation were performed. The \(K\text{d}\) and \(K\text{d}/K\text{cat}\) values for C223A were similar to those of wild type PBGS. When His\(^{131}\) was substituted with Ala or Arg, the \(K\text{d}/K\text{cat}\) values were decreased to 1/10 or 1/17, respectively. The profiles of pH-dependency of \(K\text{d}\) showed that the optimum pH and \(pK\text{a}\) values were shifted toward alkaline pH. Zinc contents of wild type PBGS, H131A and C223A were 1, 1 and 0.5 Zn/subunit, respectively. The Zn concentration dependence of enzymatic activities revealed that Cys\(^{232}\) contributed to maintenance of the catalytic efficiency at low concentration of zinc ion in the reaction mixture. Native-PAGE showed that a structural change, which was required for enzymatic activation by 2-mercaptoethanol and zinc ions, was not observed in H131A. These findings suggest that His\(^{131}\) contributes to the structural change in enzymatic activation without coordination of zinc ions.

P173

The catalytic mechanism of purple acid phosphatases

Nata \(\text{a}\) Mitic\(^1\), Robynn S. Cox\(^2\), Maurizio Lanzmester\(^3\), Lawrence R. Gahan\(^1\), Ademir Neves\(^3\), Alvan C. Hengge\(^2\), Gerhard Schenk\(^1\), The University of Queensland, St Lucia, Australia; \(^2\)Utah State University, Logan, UT, USA; \(^3\)Universidade Federal de Santa Catarina, Florianópolis, Brazil.

Contact e-mail: schenk@uq.edu.au

Purple acid phosphatases (PAPs) belong to the family of binuclear metallohydrodrolases that catalyze the hydrolysis of numerous phosphoester substrates at acidic pH.\(^1\) PAPs from different sources (mammalian, plant, fungal) have been isolated and characterized, and generally have an Fe(III) and a M(II) ion in the active site (M = Fe, Mn, Zn). Despite low overall sequence homology between kingdoms structural conservation in their active sites is observed. The seven metal-coordinating residues are invariant. The precise details of the catalytic mechanism employed by PAPs remains subject to debate.\(^1\) Here, we investigate the mode of substrate binding in pig PAP (uteroferrin) using stopped-flow spectroscopy, and we demonstrate, for the first time, that PAPs are also effective diesterases. Specifically, interactions between the leaving group and residues in the second coordination sphere are essential for the rapid and efficient binding of the substrate to both metal ions in the active site. Furthermore, using a diester substrate we demonstrate that PAP sequentially hydrolyses both ester bonds without releasing the initially formed monoester. The most likely mechanism is initial attack by a terminal Fe(III)-bound hydroxide and then a metal-bridging hydroxide as nucleophile. A similar mechanism has also been proposed for the \([\text{L(OH}_2\text{Fe(III)}\text{O}])\) PAP mimic (where \(\text{L} = 2\text{-bis}[2\text{-pyridyl-methyl}]-\text{aminomethyl}]\-6\text{-[(2-pyridyl-benzyl)-(2-pyridyl-methyl)]-aminomethyl]-4-methylphenol). Mechanistic implications are discussed.

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P174
Isolation, characterization, and monoxygenase activity of the minimal functional unit of octopus hemocyanin
Chizu Shimokawa1, Kenji Suzuki1, Shigetoshi Aono2, Shinobu Itoh1, Department of Chemistry, Graduate School of Science, Osaka City University, Osaka, Japan; 1Okazaki Institute for Integrative Bioscience, National Institutes of Natural Sciences, Okazaki, Japan. Contact e-mail: chizu@sci.osaka-cu.ac.jp

Hemocyanin and tyrosinase are the members of type-3 copper proteins, which involve a common side-on peroxo species at their dinuclear copper reaction center (Chart 1). Despite having the same side-on peroxo dicopper(II) complex, these copper proteins exhibit different chemical reactivity toward external substrates. Namely, tyrosinase catalyzes the \( \alpha \)-hydroxylation of phenols to catechols (phenolase activity) as well as the oxidation of catechols to \( \alpha \)-quinones (catecholase activity). In contrast, hemocyanin has essentially no redox reactivity toward external substrates, but exhibits only reversible dioxygen binding ability. We have recently found that octopus hemocyanin exhibits efficient monoxygenase activity (phenolase activity) when it is treated with 8 M urea (J. Am. Chem. Soc. 2006, 128, 6788-6789). In order to get more detailed insight into the monoxygenase activity of octopus hemocyanin, we herein examined the reactivity of the minimal functional unit, containing a single dicopper active site, existed at the C-terminal domain of the large subunit (\(-350 \) kDa). The expected minimal functional unit with 47 kDa molecular weight was obtained by the treatment of native octopus hemocyanin with V8-protease and following column chromatographic treatments using Sephacryl S-100 HR. We have also tried to develop a gene expression system of the minimal functional unit of octopus hemocyanin to study the molecular mechanism of the monoxygenation activity.

Chart 1

P175
UV Resonance Raman Spectroscopic Studies of Cytochrome \( \text{c}^\prime \) from Alcaligenes xylosoxidans NC1MB 11015
Naoko Shiono, Institute of Applied Beam Science, Mito, Ibaraki, Japan. Contact e-mail: cherrypie381@yahoo.co.jp

Cytochromes \( \text{c}^\prime \) are a class of \( \varepsilon \)-type cytochrome, which consists of four \( \alpha \)-helix bundle structure. Cyt \( \text{c}^\prime \) has been considered to be a NO binding protein in the denitrifying systems. Cyt \( \text{c}^\prime \) showed a well defined quasi-reversible faradic response at a 6-mercaptopurin/Au electrode, and the redox potential of cyt \( \text{c}^\prime \) was evaluated to be 184 mV [1]. The pH and ionic strength dependability of the structure and properties of cyt \( \text{c}^\prime \) has been reported. UV resonance Raman (UVRR) spectroscopic measurements of cyt \( \text{c}^\prime \) (ex. at 244 nm) were performed to elucidate the pH and ionic strength effect on the structural transition of cyt \( \text{c}^\prime \). Most of prominent Raman bands are readily assigned based on the previous paper [2]. The Raman bands at 1621 (Y7a), 1208 (Y7a), and 1175 (Y9a) cm\(^{-1} \) are contributed from tyrosine residues, and the Raman bands at 1551(W3) and 1343/1358(W7) Fermi (doublet) cm\(^{-1} \) are contributed from tryptophan residues [3]. UVRR spectra of cyt \( \text{c}^\prime \) at different pH values indicated that the environment of tryptophan residues was affected by pH. The effect of ionic strength on the UVRR of cyt \( \text{c}^\prime \) will be reported and discussed.

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P176
Drastic alteration of the substrate specificity based on the substrate misrecognition of cytochrome P450\( \text{BS}^\alpha \)
Osami Shoji1, Takashi Fujishiro1, Takuya Hirose1, Hiroshi Nakajima1, Misa Kim2, Shingo Nagano2, Yoshitsugu Shiro2, Yoshihito Watanabe1, Graduate School of Science, Nagoya University, Nagoya, Japan; 1RIKEN SPring-8 Center, Harima Institute, Sayo, Japan. Contact e-mail: osami@mbox.chem.nagoya-u.ac.jp

Cytochrome P450\( \text{BS}^\alpha \) isolated from \( \text{Bacillus subtilis} \) utilizes hydrogen peroxide to catalyze exclusively the hydroxylation of long alkyl-chain fatty acid such as myristic acid. The X-ray crystal structure shows that the interaction of the terminal carboxyl group of the fatty acid with Arg\( ^{242} \) located nearby the heme is indispensable for the generation of the active species. Owing to this unique catalytic mechanism, P450\( \text{BS}^\alpha \) never oxidizes substrates except for the natural substrate. However, the substrate specificity of P450\( \text{BS}^\alpha \) was altered drastically by the misrecognition of the substrate induced by a decoy molecule having the structural similarity to that of the natural substrate such as heptanoic acid. In the presence of the decoy molecule, P450\( \text{BS}^\alpha \) started to catalyze oxidation reactions of non natural substrates including hydroxylation of ethylbenzene (Scheme 1). The X-ray crystal structure of P450\( \text{BS}^\alpha \) including heptanoic acid showed that heptanoic acid are located in the heme pocket with the interaction between its carboxyl group and Arg\( ^{242} \) similarly to the natural substrate and it keeps the substrate access channel open for the access of non-natural substrates.

Scheme 1. Schematic representation of our strategy for the oxidation of non-natural substrates by P450\( \text{BS}^\alpha \)

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P177
Comparative study of energy metabolism in Bilophila wadsworthia and an environmental sulfate-reducing bacterium
Sofia Silva, Filipa Valente, Inês Pereira, Instituto de Tecnologia Química e Biológica, Oeiras, Portugal. Contact e-mail: ssdalviva@itqb.unl.pt

Bilophila wadsworthia is a Gram-negative bacterium and a member of the human gut microbial ecosystem. This bacterium is isolated from a variety of pathogenic infections, being the third most common anaerobe present in patients with perforated and gangrenous appendicitis. Although related to Desulfovibrio organisms, which are sulfate reducers, it has a particular metabolism adapted to the human body since it uses taurine, an...
organsulfonate that is one of the major solutes in mammals. *Desulfovibrio vulgaris* Hildenborough is an environmental bacterium that is a model organism for sulfate reducers. Its genome, which was recently sequenced, codes for several hydrogenases and formate dehydrogenases, which are enzymes that play an important role in *Desulfovibrio* energy metabolism. Since *Bilophila wadsworthia* is a pathogen whose physiology is still poorly understood, we performed a comparative activity study of these enzymes in both organisms to achieve a better understanding of the energy metabolism of *B. wadsworthia*.

**P178**

EPR and ENDOR characterization of pMMO from *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporum* OB3b.

Stephen M. Smith1, Amanda S. Hakeman1, Joshua Telske1,2, Brian M. Hoffman1, Amy C. Rosenzweig1,1, Northwestern University, Evanston, IL, USA; 2Roosevelt University, Chicago, IL, USA. Contact e-mail: s-smith@northwestern.edu

Particulate methane monoxygenase (pMMO) from *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporum* OB3b is a copper-containing enzyme that converts methane to methanol. As-isolated pMMO from both species contains a mixture of Cu(I) and Cu(II). Crystallographic characterization of both pMMOs reveals the presence of several metal centers, but details of the coordination, including the possible presence of exogenous ligands, remain unclear. In addition, the active has not been identified. In this study, the copper centers in highly active crude pMMO membranes and purified pMMO samples from *M. capsulatus* (Bath) and *M. trichosporum* OB3b were investigated by electron paramagnetic resonance (EPR) and 1H, 15N, and 19F electron nuclear double resonance (ENDOR) spectroscopies. The EPR spectra show a typical type 2 Cu(I) signal for both pMMOs that accounts for 40–60% of the total copper. The ENDOR data indicate the presence of an exchangeable solvent ligand associated with the type 2 Cu (II) signal. Various substrates and products were incubated with pMMO from *M. capsulatus* (Bath) to probe the active site.

**P179**

Spectroscopic insight into the Fe(II) environment at the ferroxidase site in Ferritins

Jennifer K. Schwartz2, Xiaofeng S. Liu1,3, Elizabeth C. Theil1,3, Edward I. Solomon1,1 Stanford University, Stanford, CA, USA; 2CHORI (Children’s Hospital Oakland Research Institute), Oakland, CA, USA; 3University of California-Berkeley, Berkeley, CA, USA. Contact e-mail: edward.solomon@stanford.edu

Ferritin, an essential protein in humans, plants, bacteria, and other animals, is able to concentrate large amounts of iron as hydrated ferric oxides for iron-protein synthesis and minimize free radical consequences of mutations on the spectral features, redox thermodynamics of the ferric/ferrous couple and inter-conversion of redox intermediates of the halogenation cycle. Disruption of the Asp ester bond had no effect on the kinetics of binding of low spin ligands (cyanide and nitrite) as well as on the formation of compound I mediated by either hydrogen peroxide or hypochlorous acid. Additionally, its impact on the reduction of compound I by iodide and thiocyanate was negligible. By contrast, rates of compound I reduction by chloride and bromide were substantially decreased compared with the wild-type protein. Disruption of the Glu ester bond significantly increased heme flexibility, blue-shifted the UV-Vis spectrum decelerated both cyanide binding and compound I formation. The apparent bimolecular rate constants at

**P180**

Gas-sensing hemeproteins in *Mycobacterium tuberculosis*: a role for oxygen in pathogenesis

Eduardo H. S. Sousa, Jason R. Tuckerman, Gonzalez Gonzalez, Marie-Alda Gilles-Gonzalez, University of Texas Southwestern Medical Center, Dallas, TX, USA. Contact e-mail: eduardo.sousa@utsouthwestern.edu

During this past decade, heme-based sensor proteins have been discovered in all kingdoms of life at a very fast pace. These sensors usually bind O2, CO and NO while engaging in signal transduction with one of these gases. FixL- FixJ are responsible for sensing oxygen levels and promoting gene expression for bacterial adaptation. Similar behavior could be assumed for *Mycobacterium tuberculosis* (Mt). Mtb entrance into latency is thought to be linked to hypoxia in the granuloma, and recently, to NO. Genetic and biochemical experiments have supported that the main regulator of hypoxia-induced genes of Mtbb is the response regulator DevR. Two histidine kinases called DosT and DevS are responsible for DevR activation. In 2005, DevS was shown to contain heme, but there was no characterization or functional investigation of the holoheme protein. We have expressed and characterized full-length holo DosT and DevS proteins. DosT is also a heme containing protein. Both proteins present very similar electronic spectra but quite distinct heme properties. Kinetic experiments for binding of O2, CO and NO were carried out by flash-photolysis, along with Kd measurements. DosT and DevS autophosphorylation reactions were clearly inhibited by O2 while NO and CO did not alter the initial rates. Our results show DosT and DevS are oxygen sensors and explain why NO was mistakenly assumed as signal. We present our working model for this system.
pH 7.0 of compound I reduction by chloride, bromide, iodide and thiocyanate were 500-, 25-, 21- and 63-times decreased compared with recombinant wild-type MPO. The effects of exchange of Asp94 and Ghu242 on electron transfer reactions are discussed with respect to the known structures of MPO and the MPO-cyanide and MPO-halide complexes.

P182
Valence delocalization in the [2Fe-2S]⁺ centers of mutant forms of Aquifex aeolicus Ferredoxin 4
Sowmya Subramanian, Jacques Meyer, Michael K. Johnson, Takuya Kurahashi, Hiroshi Fujii.

Aquifex aeolicus Ferredoxin 4 (AaeFd4) is the best structurally characterized member of the class of the thioredoxin-like [2Fe-2S] Fds. High-resolution crystal structures are available for the wild-type (WT) protein as well as the C55S and C59S variants with Fe-S/Fe-O-Fe-Fe distances accurate to ±0.01 Å. [Unable to Display Character: &g;]506,1] (1). The reduced forms of the corresponding C56S and C60S variants of the closely related Clostridium pasteurianum [2Fe-2S] Fd are currently the only known samples of valence-delocalized [2Fe-2S]⁺ clusters (2). Such cluster fragments constitute a fundamental building block of all higher nuclearity Fe-S clusters and understanding the origin of valence-delocalization is central to the understanding of biophysical properties of Fe-S clusters in general. In this work, the spectroscopic properties of the [2Fe-2S]⁺⁻ center in WT and the C55S, C59S, C55S/R13E and C59S/R13E mutant forms of AaeFd4 have been studied by UV-Visible absorption, EPR, resonance Raman, and variable temperature MCD spectroscopies in an effort to understand the origin of valence delocalization. Valence delocalized species with ferromagnetically coupled S = 9/2 ground states were found to exist as minority species in frozen samples of each of the dithionite-reduced AaeFd4 mutants at pH 11. However, variable temperature UV-visible absorption and MCD studies at pH 11 indicate that the mutants exclusively contain valence-delocalized [2Fe-2S]⁺⁻ centers with S = 9/2 ground states at room temperature and only become valence-delocalized with antiferromagnetically coupled S = 1/2 ground states on freezing. The origin of valence-delocalization in [2Fe-2S]⁺⁻ centers will be discussed in light of these results.

P183
A Thermodynamic Analysis of Epoxidation versus Allylic C-H Hydroxylation on Cyclohexene Oxogenation by High-Valent Iron-Porphyrin Complexes Related to Cytochrome P450
Akihiro Takahashi, Takuya Kurahashi, Hiroshi Fujii.

It has been recognized that oxoiron(IV) porphyrin π-cation radical species (the so-called compound I) is involved as a reactive intermediate in catalytic cycles of cytochrome P450, peroxidase, and catalase. The compound I in Cytochrome P450 can catalyze alkane epoxidation and hydrocarbon hydroxylation. Interestingly, cyclohexene, which contains the C=C and allylic C-H functionalities in one molecular, is converted to cyclohexene oxide (epoxidation product) and cyclohexen-3-ol (hydroxylation product) by the compound I in cytochrome P450. It is reported that the chemoselectivity of epoxidation versus allylic hydroxylation of cyclohexene oxygenation are controlled by various factors such as reaction temperature, solvent, electronic nature and axial ligand of iron porphyrin. At present, however, it remain unclear how these factors control the chemoselectivity of cyclohexene oxygenation. In this study, we carried out that detailed kinetic analysis of C=C epoxidation and allylic C-H hydroxylation of cyclohexene. Herein, we reveal that entropy of activation, ΔS, is more important thermodynamic factor to control the chemoselectivity than activation energy, ΔEa. This result clearly explains drastic temperature effect on chemoselectivity. In additional, the present result suggests re-consideration of the discussion based on ΔEa calculated by the density functional theory (DFT).

P184
“Effector” roles of the electron donor protein in P450cam-catalyzed oxygenation reaction
Kenta Tanemura, Takeshi Uchida, Koichiro Ishimori.

P450cam is a heme-containing enzyme that catalyzes the hydroxylation of D-camphor by using dioxygen and two electrons. To accept electrons from the redox partner, Pdx, the specific structural changes by binding of Pdx are required in P450cam, indicating that the Pdxs have ‘effector’ functions for the catalytic cycle of P450cam. Our previous study suggested that the steric interaction mediated by the side chain of Leu-358 plays a key role in the propagation of the structural change from the Pdx binding site to the heme moiety, but details of this interaction are still unclear. In this study, we newly prepared a mutant, L358A, in which Ala is introduced into the position of 358 to perturb the static interaction with heme, and examined the heme environmental structure by using the resonance Raman spectroscopy. The Fe-C stretching (νFe-CO) and C-O stretching (νOCO) modes in carbonmonoxo L358A, which serve as the sensitive markers for the heme environment, were observed at 481 cm⁻¹ and 1940 cm⁻¹, respectively. In addition, the binding of Pdx to L358A upshifted νFe-CO by 2 cm⁻¹ and downshifted νOCO by 8 cm⁻¹ as found for wild type P450cam. Based on these results, we can conclude that the L358-mediated steric interaction would not be so crucial for the effector functions of Pdxs, which allows us to speculate that the Pdx binding induces the conformational change of the main chain rather than the side chain of Leu358 which enables P450cam to accept electrons for the catalytic reaction.

P185
Tryptophan 2,3-dioxygenase; structural and mechanistic studies
Sarah J. Thackray, Ross J. L. Anderson, Chris Mowat, Chiara Bruckmann, Stephen K. Chapman.

Tryptophan 2,3-dioxygenase (TDO) is a heme-containing enzyme from a family of enzymes including indoleamine 2,3-dioxygenase (IDO). TDO is a homotetrameric enzyme and has a high specificity for L-tryptophan (L-Trp) and related derivatives such as 6-F-Trp. TDO is monomeric, and shows activity toward a range of substrates, including L-Trp, D-tryptophan and tryptamine. TDO and IDO have been implicated in a number of physiological conditions, including...
A membrane bound desaturase from Arthrobacter thialana was cloned and overexpressed in E. coli BL21 (DE3) cells. Biochemical and spectroscopic studies will be presented which establish the presence of an iron centre in this enzyme.

**P187**
Ferritin-specific di-iron site ligands control rapid iron mineralization pathways

Takehiko Toshio1, Elizabeth C. Theil1 2, 1 Council on Bioiron at Children's Hosp. Oakland Res. Inst., Oakland, CA, USA; 2Dept. Nutri. Sci. & Toxicol., UC-Berkeley, Berkeley, CA, USA.
Contact e-mail: ttosia@chori.org

Ferritin initiates iron mineralization by catalytic coupling of ferrous iron and molecular oxygen at multiple, di-iron sites A and B containing some of ligands common to diiron oxygenases where iron is a cofactor, in contrast to ferritin where iron is a substrate. Site A, Glu, Glu-x-x-His is common to all the di-iron proteins (e.g. methane monoxygenase, ribonucleotide reductase, ferritin) but site B, Gln-x-x-Glu/His/Ala is ferritin-specific. Here we report the catalytic effects of engineering ferritin site B to mimic the oxygenase with the following changes: Site B: EED-Glu137Glu, site B-EQH-Asp140His, and site B-EEH-Gln137Glu/Asp140His. Substitution of His for Asp, whether in B site EQH or EEH mutant, inactivated catalysis with the initial rates of the ferrous oxidation decreased to 1.0-2.0% of WT ferritin. However, for B-site EED, with the conservative Gln137Glu substitution, catalysis was only slightly inhibited (73% of that of WT). In spite of the similar initial rates, the reaction pathway was altered by the Glu/Glu mutation in Site B-EED since the di-ferric peroxy intermediate was undetectable and, using entry into the slower phases of mineral formation as a reporter, dissociation of the diferric oxy (hydroxyo) product and mineral precursor appeared to be inhibited. These results indicate that ferritin-specific Site B iron ligands control the reaction pathway for rapid iron oxidation/coupling and release of diferric oxo/hydroxo mineral precursors in rapid iron mineralization of ferritin.

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**P188**
Heme Transfer Mechanism from Heme ChaperonE, CcmE, to Apocytochrome c

Takeshi Uchida1, Yuka Kondo1, Ayako Tamura1, Julie M. Stevens2, Stuart J. Ferguson2, Koichiro Ishimori1, 1Hokkaido University, Sapporo, Japan; 2Department of Biochemistry, University of Oxford, Oxford, United Kingdom.
Contact e-mail: uchida@sci.hokudai.ac.jp

c-type cytochromes are electron transfer proteins that are essential for the life of virtually all organisms. They characteristically carry covalently-bound heme via thioether bonds to two cysteines in the protein. In Gram-negative bacteria, biogenesis of c-type cytochrome is conducted by a multiprotein complex system known as a cytochrome c maturation (Ccm) system. This system consists of 8 gene products (ccmA-cmcf). CcmE, which binds heme and delivers it to apocytochrome c, is called as a heme chaperonE. In the previous study, covalent attachment of heme to cytochrome c was achieved in the presence of only CcmE, heme, and dithiothreitol. However, the precise mechanism remains to be clarified. In this study, we applied UV/vis absorption and resonance Raman spectroscopies to the reaction of CcmE with apocytochrome c to understand the mechanism of heme transfer and covalent bond formation.

After addition of apocytochrome c into CcmE in the presence of sodium dithionite and dithiothreitol, the Soret maximum shifted to 417 nm from 400 nm, and two new bands appeared at 527 and 533 nm. The positions of these bands were different from those of neither CcmE nor cytochrome c in the reduced form. Unfortunately, resonance Raman spectrum of the mixture of CcmE and apocytochrome c under the same condition was hindered by strong fluorescence probably caused by the dissociated heme. The results of the absorption spectral changes suggest that heme is not transferred from CcmE to apocytochrome c simultaneously, but an unspecified transient form is formed during the heme transfer.
P189
Crystal structures of the hydroperoxo-heme- and α-meso-hydroxyheme intermediates in heme oxygenase catalysis
Masaki Unno, Masao Ikeda-Saito, Tohoku University, Sendai, Japan.
Contact e-mail: unno19@tagen.tohoku.ac.jp

Heme oxygenase (HO) catalyzes the O₂-dependent conversion of heme to biliverdin, Fe, and CO through three successive oxygenation steps in which the heme participates both as a substrate and as a cofactor. The first oxygenation of heme occurs regiospecifically at the α-meso position to afford α-meso-hydroxyheme. A ferric hydroperoxo intermediate (Fe³⁺-O=O-H) has been identified as the meso-hydroxylating species, different from other heme enzymes which employ compound I as their reactive species. To gain further insight into the HO catalytic mechanism, we determined the crystal structures of the hydroperoxo-heme and the α-meso-hydroxyheme intermediates of HmuO, a bacterial HO, at 1.9 Å and 1.5 Å resolutions, respectively. The hydroperoxo intermediate was generated by X-ray-induced photoreduction of the oxy heme at cryogenic temperature. This intermediate was highly susceptible to the incident X-ray radiation, and only merging the independent diffraction data from 20 crystals attained successful structure determination. The Fe-O distance is slightly longer and Fe-O-O angle is smaller by 5° than those found in the oxy structure. The diffraction data set for the hydroxyheme intermediate was collected after brief annealing the crystal of the hydroperoxo intermediate near room temperature. The hydroxyheme shifts along the heme α-axis, and the distal helix moves in the opposite direction compared with the hydroperoxo-heme complex. The proton, required for activation of hydroperoxo, could be delivered to the proximal oxygen from nearby water through this rearrangement of the active site.

P190
Metal-free SOD1 forms amyloid-like oligomers: a possible general mechanism for familial ALS
Miguelina Vieru1, Lucia Bencző1, Ivano Bertini1, Stefania Girotto1, Manuele Martinelli1, Julian P. Whitelegge2, Armando Durazo2, Joan Sertverstone Valentine1, 1Magnetic Resonance Center, Florence, Italy; 2University of California, Los Angeles, CA, USA.
Contact e-mail: vielur@cern.unifi.it

Amyotrophic lateral sclerosis (ALS) is a progressive degenerative disease of motor neurons. The inherited form of the disease, familial ALS, represents almost 10% of the cases reported and there is strong evidence that these are due to mutations in the gene encoding copper–zinc superoxide dismutase (SOD1). Recent studies showed that SOD1 mutations, more than the loss of normal function, induce gain of a toxic function. The finding of proteinaceous cytoplasmic inclusions containing SOD1 in motor neurons of postmortem familial ALS patients, suggests that aggregation of mutant SOD1 produces a pathogenic species, and the insoluble aggregates may represent only the final form of a previous pathological state, which is eliminated by cells inducing its aggregation. We have characterized the WT SOD1 protein in the apo and metallated states with respect to its ability to form aggregates. Fluorescence studies using the Thioflavin T probe, coupled with gel chromatography, show that a soluble aggregate forms in vitro during incubation at 37 °C of an apo sample of SOD1 at physiological conditions (pH 7, 100 μM). We have further characterized this aggregate. This species might constitute the first seed for a subsequent insoluble aggregate formation. Further studies will focus on investigating the propensity of a selected set of pathogenic SOD1 mutants to form soluble aggregates.

P191
Looking for peroxidase substrates and binding site(s) in bifunctional catalase peroxidases
Jutta Vlasits, Marcel Zamocky, Christa Jakopitsch, Christian Obinger, University of Natural Resources and Applied Life Sciences, Wien, Austria.
Contact e-mail: jutta.vlasits@boku.ac.at

Catalase-peroxidases (KatGis) exhibit peroxidase and substantial catalase activities similar to monofunctional catalases. Crystal structures of four different prokaryotic KatGis reveal the presence of a peroxidase-conserved proximal and distal heme pocket together with features unique to KatG. Marked differences in the structural role of conserved amino acids and hydrogen-bond networks in KatG with respect to other plant-type heme peroxidases were found. Typically, the catalatic but not the peroxidatic activity was very sensitive to mutations that disrupted the KatG-typical extensive hydrogen-bonding network. By contrast, the endogenous peroxidase substrate(s) and binding site(s) in KatG are unknown. Typical for KatG is a unique distal Trp-Tyr-Met adduct with a joining arginine that can move between two conformations. The side-chain of this Arg is located on the surface of a cleft with a well-defined U-shaped region. Structural analysis and sequence alignment revealed the presence of several highly conserved residues in this region that could play a role in binding and oxidation of one-electron donors. Here, we present data on Synechocystis KatG and the effect of exchange of R509, W459, K435, N278 and E281 on the bifunctional activity and interconversion and spectral properties of redox intermediates.

P192
Magnetic circular dichroism of Aminopeptidase from Aeromonas proteolytica
Adam S. Volwiler, James A. Larrabee, Middlebury College, Middlebury, VT, USA.
Contact e-mail: avolwile@middlebury.edu

Aminopeptidase from Aeromonas proteolytica (AAP) serves as an example of a metallohydrolase in which the active site structure has a μ-hydroxo-μ-carboxylato-dimetal core. This core is a common structural motif in many metallohydrolases which are active in the dicothal(II) forms including methionine aminopeptidase (MetAP) and glycerophosphodiesterase (GpdQ). One interesting feature of the dicothal(II) active site is the extent of magnetic exchange coupling between the Co(II) ions. Magnetic circular dichroism (MCD) can be used to determine magnetic exchange coupling, and the AAP active site is particularly well suited for MCD study because three different metal-substituted forms, [CoZn(AAP)], [ZnCo(AAP)], and [CoCo(AAP)], can be prepared. Any magnetic exchange coupling between Co(II) ions is turned off in either the [CoZn(AAP)] or [ZnCo(AAP)] form. Furthermore the spectroscopic properties and electronic ground state of each Co(II) ion can separately be probed, and changes in the ground state properties in the [CoCo(AAP)] form can be attributed to exchange coupling. The study of AAP provides a basis for the more general understanding of all dinuclear metallohydrolases.
Flavo-Diiron Enzymes: Nitric Oxide and/or Dioxygen Reductases?
David A. Wampler, Angela-Nadia Albetel, Donald M. Kurtz, Jr., Department of Chemistry, University of Texas at San Antonio, San Antonio, TX, USA; Department of Chemistry, University of Georgia, Athens, GA, USA.
Contact e-mail: david.wampler@utsa.edu

A widespread group of bacterial and archaeal flavo-diiron enzymes (FprAs) contain an active site consisting of a flavin juxtaposed with a non-heme His, Glu, Asp-ligated diiron site. FprAs have no detectable amino acid sequence homology to any other class of non-heme iron proteins. Some FprAs clearly function as scavenging nitric oxide reductases (S-NORs) under anaerobic growth conditions. In vitro, however, some FprAs are reported to have dioxygen reductase (O2R) activity, either instead of or in addition to NOR activity. We are undertaking a systematic survey of these two activities, both in vivo and in vitro, of FprAs from various bacteria and archaea. Attempts to correlate the relative levels of these two activities with spectroscopic and structural differences among the FprAs will be described, as will progress in understanding the catalytic mechanisms.

Typical and untypical metal ion binding modes in Cicer arretinum metallothionein 2
Xiaoqiong Wan, Eva Freisinger, Institute of Inorganic Chemistry, Zürich, Switzerland.
Contact e-mail: xwan@aci.unizh.ch

Metallothioneins (MTs) are small cysteine-rich metal ion binding proteins found in a huge number of species. Nevertheless, only few attempts have been performed to study MTs in plants until now leaving the knowledge about their functions and the related mechanisms still mostly in the dark. C. arretinum (chickpea) MT2 shows the characteristic cysteine distribution pattern of the p2 subfamily of plant MTs [1] including the high affinity to d10 metal ions typical for all MTs. We will present the metal ion binding affinities of MT2 to essential or nonessential metal ions, e.g. ZnII, CdII, HgII, CuI and CoII, which were studied using a plethora of spectroscopic as well as (bio-) chemical methods. Specifically, changes in coordination geometry upon stepwise addition of the different metal ions will be shown. We were further able to identify an additional metal ion binding site with weaker affinity, which seems to be independent of the metal-thiolate clusters formed.

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Regulation of myoglobin functions by self-aggregates of an anionic porphyrin
Kenji Watanabe, Yoshiyuki Ishida, Koji Kano, Doshisha University, Kyotanabe, Kyoto, Japan.
Contact e-mail: efr1501@mail4.doshisha.ac.jp

In chemical biology, modification of protein functionality is one of the major subjects. Synthetic molecules that complex with a specific protein and alter a conformation of the protein have a potential to regulate the functions of the target protein. We studied complexation of self-aggregates of 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrin (TPPS) with metmyoglobin (metMb) leading to change in the secondary structure of metMb and increase in the binding affinity of metMb for the azide anion. TPPS is known to form extended J-aggregates in an aqueous acidic solution. However, under neutral conditions, TPPS exists as the monomer and/or the dimmer. In this study, we found that J-aggregates of TPPS can exist stably for several hours even in an aqueous neutral solution when the aggregates are bound to metMb (Figure 1). Circular dichroism study indicates that binding of the J-aggregates leads to a change in the secondary structure of metMb. The binding constants (K) for association of the azide anion to metMb in the absence and the presence of the J-aggregates as well as the monomer of TPPS were determined. The results revealed that the J-aggregates enhance the binding of the azide anion to metMb, while the TPPS monomer does not show significant effect (Figure 2).
Rhizobia have exploited the oxygen sensing two-component system, FixL/FixJ (histidine kinase/response regulator), to express the nitrogen fixation enzymes under anaerobic conditions. FixL, is a heme-containing histidine kinase, and is divided into sensor, dimerization, and catalytic domains. Under anaerobic conditions, FixL phosphorylates histidine residue by using ATP. The phosphoryl group is subsequently transferred to the conserved asparagin residue in FixJ. However, the kinase reaction is inhibited in high oxygen concentrations. Structural analyses of sensor domain of FixL revealed the intra-domain conformational change upon oxygen binding. However, it is still unknown how the signal is transferred to the downstream domains, because no full-length structure of FixL is available.

Then, we determined the crystal structure of the structural homolog of FixL/FixJ, ThkA/TrrA from hyperthermophile. The structures of sensor, dimerization, and catalytic domains and TrrA were determined at 1.6-1.9 Å resolution. The determined structures were fitted into the ThkA/TrrA complex obtained at 3.7 Å resolution. The determined structure revealed new insights as follows: (1) the inter-domain interaction between sensor and catalytic domain regulates the kinase activity, (2) the ATP binding site of catalytic domain changes the conformation after the kinase reaction, and (3) the inter-domain interaction between sensor and FixJ regulates the ligand affinity in the sensor domain.

Dynamic roles of specific heme-ligand-residue interactions in the oxygen sensor proteins Dos and FixL

Dynamic roles of specific heme-ligand-residue interactions in the oxygen sensor proteins Dos and FixL

Heme-ligand photodissociation can in principle be used to trigger ‘switching’ of the sensor and identify intermediates in the intra-protein signaling pathway. Ultrafast spectroscopic experiments indicate that unusual heme-ligand interactions are maintained after dissociation and that the heme pocket acts as an oxygen trap. With the aim of exploring the role of specific heme-ligand-residue interactions in functional motions, we have constructed mutants proteins (in particular with modified Met 95 in E. coli). Heme-ligand photodissociation can in principle be used to trigger ‘switching’ of the sensor and identify intermediates in the intra-protein signaling pathway. Ultrafast spectroscopic experiments indicate that unusual heme-ligand interactions are maintained after dissociation and that the heme pocket acts as an oxygen trap. With the aim of exploring the role of specific heme-ligand-residue interactions in functional motions, we have constructed mutants proteins (in particular with modified Met 95 in E. coli). Heme-ligand photodissociation can in principle be used to trigger ‘switching’ of the sensor and identify intermediates in the intra-protein signaling pathway. Ultrafast spectroscopic experiments indicate that unusual heme-ligand interactions are maintained after dissociation and that the heme pocket acts as an oxygen trap.
P199
Fungal catalase-peroxidases - a novel group of bifunctional oxidoreductases
Marcel Zamocky, Christa Jakopitsch, Jutta Vlasits, Christian Obinger. Division of Biochemistry, Department of Chemistry BOKU, Wien, Austria.
Contact e-mail: marcel.zamocky@boku.ac.at

Catalase-peroxidases (KatGs) are bifunctional heme b containing oxidoreductases exhibiting catalase (2 H2O2 → 2 H2O + O2) and peroxidase activity (H2O2 + 2 AH → 2 H2O + 2 •AH). All known catalase-peroxidases belong to class I of the superfamily of bacterial, fungal, and plant heme peroxidases. Four crystal structures of prokaryotic KatGs are known revealing several peculiar structural features. Sequence similarity searches and phylogenetic analysis of 134 eukaryotic genomes revealed the presence of 23 fungal and 3 protist KatG genes that form a novel and distinct group within the abundant catalase-peroxidase protein family. Mainly in the catalytic domain their sequence similarity with bacterial KatGs is rather high but multiple sequence alignments clearly suggest differences between prokaryotic and eukaryotic proteins.

In phytopathogenic fungi KatGs are probably involved in host-pathogen interactions. Thus, investigations of fungal KatGs have a great practical impact for developing tools that prevent plant affection by these pathogens. Here, we focus on the cloning and heterologous expression of catalase-peroxidases from the two phytopathogenic fungi Gibberella zeae and Magnaporthe grisea both having two katG paralogs. In the case of Gibberella zeae katG1 a rare fusion of katG gene with a region coding for a cytochrome P450-like domain was detected thus forming a putative unique trifunctional multidomain heme containing oxidoreductase.

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P200
Peroxidase affinity towards hydrogen peroxide: Determination from a conversion time method.
Rosa Zamora1, Virginia Tomas2, Ramiro Martinez-Gutierrez2, Francisco Garcia-Canovas1, Jose Tudela1. 1GENZ-Grupo de investigacion Enzimologia, Universidad de Murcia, Spain; 2Dept. Quimica Analitica, Universidad de Murcia, Spain; 3NOVOZYMES Spain S.A., Madrid, Spain; 4GENZ (http://www.um.es/genz), Universidad de Murcia, Spain.
Contact e-mail: rzamora@um.es

Peroxidase (POD, EC 1.11.1.7) is located in humans, animals, plants and microorganisms, and it is involved in a number of peroxidative processes with biotechnological interest. The enzyme catalyzes the oxidation by hydrogen peroxide of reductant substrates to radicals, which decompose by means of non-enzymatic reactions up to polymers. POD has great affinity towards hydrogen peroxide, thus it may be difficult to determine the value of its Michaelis constant using steady state rate assays. An alternative method consists of the use of progress curves of assays with hydrogen peroxide depletion.

The analysis of the data points of a progress curve needs its complex fitting by non-linear regression to an implicit equation, the integrated equation of Michaelis. A simpler method is based on the derivation of the analytical expressions of the conversion time. There have been obtained linear and hyperbolic useful expressions for the determination of Michaelis constant. Besides, there have been established equations that allow predicting the duration of the reactions of substrate depletion. The method has been applied successfully for the determination of the Michaelis constants of POD towards hydrogen peroxide and 4-tertbuthylcatechol as substrates.

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P201
Tryptophan-to-dye fluorescence energy transfer applied to oxygen sensing using type-3 copper proteins.
Gerhild Zauner1, Thijs J. Aartsma2, Gerard W. Canters1, Armand W. J. W. Tepper1, 1Leiden Institute of Chemistry, Leiden, The Netherlands; 2Leiden Institute of Biophysics, Leiden, The Netherlands.
Contact e-mail: g.zauner@chem.leidenuniv.nl

A fluorescence-based system to sense oxygen is described. The method exploits the sensitivity of the fluorescence of type-3 copper proteins towards oxygen by translating the near-UV emission of the protein to label fluorescence in the visible range through a FRET mechanism (Figure 1). The main protein in this study, a recombinant tyrosinase from the soil bacterium Streptomyces antibioticus, has been covalently labeled with a variety of fluorescent dye molecules with emission maxima spanning the visible wavelength range. The emission of the label varied considerably between O2-bound and O2-free protein with a contrast exceeding that of the Trp emission for some labels. Different constructs may be simultaneously observed using a single excitation wavelength. Next to oxygen sensing, the method may be applicable to any protein showing variations in tryptophan fluorescence, for example as a function of ligand binding or catalysis.

Figure 1. Structure of Streptomyces antibioticus tyrosinase (left), modelled after [PDB 1WX3] showing the type-3 Cu atoms, the coordinating His residues, the 12 Trp residues and Thr1, corresponding to the attachment point of the label. The principle of the FRET based O2 sensing is shown at the right.
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Overexpression and characterization of a histidine- and glutamine-rich protein, Hpn-like
Yibo Zeng, Dongmei Zhang, Hongzhe Sun, Department of Chemistry and Open Laboratory of Chemical Biology, The University of Hong Kong, Hong Kong, China.
Contact e-mail: h0492040@hkusua.hku.hk

Helicobacter pylori is a micro-aerophilic, Gram-negative, spiral-shaped organism. [1-2] Similar to Hpn, Hpn-like (Hpnl), a histidine- and glutamine-rich (25% and 40%) protein in H. pylori, may play a putative role in nickel detoxification and influence the active levels of Ni-activated urease [3-4]. We cloned gene from H. pylori 11637 strains, over-expressed in E. coli BL21(DE3), and purified the protein. Our UV-vis spectra reveals Hpnl binds 2 nickel ions per monomer at pH 7.5. This binding was reversible: metal could be released from Hpnl in the presence of the chelating ligand EDTA. Ni<sup>2+</sup> also induced conformation changes of the protein, with α-helix content decrease from 26% to 20% and β-sheet increase from 14% to 22%. Growth curves of E. coli both with and without hpnl gene implied a role for Hpnl to protect the cells from higher concentrations of outside Ni<sup>2+</sup>. Therefore Hpnl may serve multiple roles inside the bacterium: storage of Ni<sup>2+</sup> ions; donation Ni<sup>2+</sup> to metalloenzymes or other proteins; and detoxification via sequestration of excess Ni<sup>2+</sup>.

Keywords: Helicobacter pylori, Hpnl

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Molecular characterisation of metal transporters in the plant Arabidopsis Thaliana
Matthias Zimmermann, Zhiguang Xiao, Anthony Gordon Wedd, School of Chemistry and Bio 21 Institute, The University of Melbourne, Parkville, Australia.
Contact e-mail: m.zimmermann@pgrad.unimelb.edu.au

Fourteen metals are known to be essential for plants and animals. However, their concentrations in cells have to be strictly regulated at their optimum levels, since too much or too little is often lethal. The molecular details of these control mechanisms are poorly understood in most cases.

The simple plant Arabidopsis thaliana has eight P1B-type ATPase (HMA 1-8) responsible for uptake and elimination of various metals. Phylogenetic analysis shows that HMA 1-4 are likely to transport divalent cations and that HMA 5-8 are likely to transport monovalent cations. However, the molecular basis of such metal transporting specificity is not known.

The N-terminus of each HMA protein, HMAn comprises one or more metal binding domains that may contribute to the metal specificity. For example, a metal binding motif (CXXC; C, cysteine; X, another amino acid) typical for Cu(I) was present in HMA7n while a cysteine pair (CC) was found in both HMA2n and HMA4n and may promote Zn(II) binding. To understand the possible roles of the N-terminus in metal binding and selection, we have cloned, expressed and purified the N-terminal domains of the HMA4 and HMA7 with selected mutants. Metal binding affinity and specificity have been studied, together with structural aspects. The results will be presented and discussed.

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