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Cu,Zn superoxide dismutases (Cu,ZnSOD) are metalloenzymes that catalyze the dismutation of the superoxide anion into oxygen and hydrogen peroxide. These enzymes, for a long time considered peculiar of eukaryotic organisms have been found to be present also in bacteria. From an analysis of their protein sequences we can observe that, with few exceptions, the ligands of metal sites are conserved. Among the bacterial proteins the only one which does not conserve two of the residues able to bind copper is the protein from *Bacillus subtilis*.

The BsSOD protein may be thought as a step of the evolution line from a no-Cu,ZnSOD world to the fully active Cu,ZnSODs. With this in mind we have tried to reconstitute SOD's activity through an artificial evolution obtained by introducing the copper ligands with site-directed mutagenesis. We have cloned the wild type, the two mutants P104H and Y88H-P104H which reintroduce one or both of the copper binding histidines respectively, reestablishing in the first case the ability to bind copper and in the second case the standard copper site of Cu,ZnSOD. We report the structural and biochemical characterization of the three proteins showing the restoration in the double mutant of a partially active Cu,ZnSOD and the resulting mechanistic and physiological implications.

Keywords: bacillus subtilis SOD, CuZn SOD, SOD mutants

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Structural Characterization of the Oxidation Pathway of Antarctic Fish Hemoglobins

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Antarctic fish hemoglobins (AF-Hbs) exhibit a peculiar oxidation process. Our previous crystallographic and spectroscopic investigations have demonstrated that, upon oxidation, these proteins show a remarkable propensity to evolve toward the formation of low-spin hexa-coordinated species [1,2]. The crystal structures of the fully oxidized forms of AF-Hbs, isolated from *Trematomus newnesi* and *Trematomus bernacchii,* have also shown that α and β chains follow different oxidation pathways. Interestingly, the quaternary structures of these forms are intermediate between the physiological R and T hemoglobin states [1,2]. In order to obtain additional information on the structural features of the intermediate species along the oxidation pathway, we are currently characterizing AF-Hbs exposed to air for different time periods. Preliminary data reveal the presence of novel forms with unexpected structural properties. In particular, we detected (1) the presence of partially liganded forms with structures that are intermediate between the R and the T state, (2) the existence of hybrid α(aquomet)-β(penta-coordinated Fe^{3+}) forms, and (3) the occurrence of novel subunit-subunit interactions at the $\beta^1\beta^2$ interface.

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Keywords: hemoglobin, protein oxidation, protein cooperativity

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Crystal Structure of Human Indoleamine 2,3-dioxygenase

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Indoleamine 2,3-dioxygenase (IDO) catalyzes the cleavage of the pyrrole ring of indoleamines by the insertion of two oxygen atoms from molecular oxygen. This reaction is the first and the rate-limiting step in the kynurenine pathway, the major Trp catabolic pathway in mammals. IDO is a 45 kDa cytosolic protein containing heme as the prosthetic group that is essential for enzymatic activity. The crystallographic analysis of human IDO revealed that its polypeptide folds into two helical domains with unique folds. The heme is sandwiched between two domains. The heme iron is coordinated by His346 on the long helix in the proximal side of heme. A large pocket on the distal side of the heme is composed of hydrophobic residues, suggesting that the indole ring in the substarte are recognized only through hydrophobic interactions. It is unlikely that any amino acid group can interact with iron-bound oxygen. These findings suggest that the dioxygenase reaction would be triggered by subtracting the proton from the nitrogen atom in the 1-position of substrate indoleamine by the iron-bound oxygen.

Keywords: heme proteins, oxygenase, metalloenzymes

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High Resolution Structure of Cytoglobin Reveals the Extra Helix in N-terminus

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Cytoglobin (Cgb), a recently discovered member of vertebrate globin family, binds O_2 reversibly *via* the Fe^{2+} ion of a heme group. Sequence comparison shows that some key residues close to the active site related to ligand binding have been highly conserved among globin family. Cgb was found to be expressed in a broad range of mammalian tissues.

In the present study, we determined the structure of the ferric state of human Cgb in two different space groups at 2.4 Å and 1.68 Å resolution. The overall backbone structure of Cgb exhibits a traditional globin fold with an additional helix in the pre A-helix region and ordered loop structure in the C-terminal region. Cgb forms a homo dimer by the interaction between the E-helices and AB corners in these crystals. A similar dimeric arrangement is found in Lamprey Hemoglobin, whose ligand affinity is regulated by dimerization coupled with a movement of the distal residues. Therefore it might be possible that the structure on the dimerization interface of Cgb is affected by the ligand binding.

Keywords: heme, cytoglobin, myoglobin

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The Crystal Structure of the (Zn/Zn)bLAP/Zofenoprilat Complex

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Bovine leucine aminopeptidase (bLAP) is an exopeptidase that cleaves N-terminal hydrophobic residues from polypeptide substrates. It is a hexameric enzyme made up of six identical monomers. Each subunit contains two Zn^{2+} in the active site, which are fundamental for catalytic activity. They may be replaced by other divalent cations with different exchange kinetics. The readily exchangeable site (site 1) can be occupied by Zn^{2+}, Mn^{2+}, Mg^{2+} or Co^{2+}, while the tight binding site (site 2) can be occupied by Zn^{2+} or Co^{2+}. We recently reported that introduction of Mn^{2+} into site 1 generates a novel activity of bLAP toward Cys-Gly, which in contrast is not hydrolysed by the (Zn/Zn) enzyme. To clarify the influence of the metal present in site 1 on enzyme interaction with sulphur-containing derivatives, we have undertaken functional and structural studies on (Zn/Zn) and (Zn/Mn)bLAP forms. Here we report the kinetic analysis of various sulphur-containing derivatives with both enzyme forms and the crystal structure of (Zn/Zn)bLAP in complex with Zofenoprilat. This peptide-mimetic derivative containing a sulphydryl moiety was found to be

also a potent inhibitor of (Zn/Zn)bLAP. This combined approach provided insights on interaction of bLAP with sulphydryl-containing compounds, showing that metal exchange in site 1 modulates binding to these molecules that, depending on metal nature, may result as enzyme substrates or inhibitors.

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Keywords: biocrystallography of protein, proteins-inhibitor complexes, metalloenzymes

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The Crystal Structure of Human CA II Bound to a Strong Benzenesulfonamide Inhibitor

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Carbonic anhydrases (CAs) are ubiquitous metalloenzymes present in prokaryotes and eukaryotes, which catalyze the reversible hydration of CO_2. In previous studies we have investigated by means of X-ray crystallography the rational design of sulfonamide/sulfamate/sulfamide inhibitors of this enzyme, which is involved in a multitude of physiological and pathological processes [1]. At least fourteen different CA isozymes are presently known in humans, and many of them are targets for the design of inhibitors with potential use as antiglaucoma, anti-obesity, or anticancer drugs among others. A class of CA inhibitors which showed very promising applications are the thioureas obtained from isothiocyanato sulfonamides and amines, hydrazines or amino acids. Such compounds generally showed potent inhibitory activity against the human cytosolic isozyme CA II as well as the transmembrane, tumor-associated isozyme CA IX, being thus interesting candidates for developing antiglaucoma/antitumor therapies based on them.

Here we report the first X-ray crystal structure of a thioureido-benzensulfonamide derivative in complex with human CA II as well as its inhibitory properties against isozymes I, II and IX [2].

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Keywords: biocrystallography of protein, protein-inhibitor complexes, rational inhibitor design

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Role of the Non-protein Ligand at the Ni-Fe Active Site of [NiFe] Hydrogenase

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Hydrogenases catalyze oxidoreduction of molecular hydrogen and have a potential value for a use of dihydrogen as an energy source.

[NiFe] hydrogenase possesses two oxidized states, Ni-A (inactive) and Ni-B (active). The pure Ni-A state was successfully prepared from the solution of the as-purified enzyme (mixture of the Ni-A and Ni-B states), and the crystal structures of both the Ni-A and Ni-B states have been determined at ultra-high resolution. The shape and size of the electron densities show that Ni-B possesses a monatomic non-protein bridging ligand between the Ni and Fe atoms at the active site and the cysteine sulfur ligand (Cys546) was modified by unknown atomic species (X546). Whereas Ni-A has a diatomic ligand at the bridging site and two systein sulfur ligands (Cys546 and Cys84) were also modified by unknown species (X546 and X84). X546 of Ni-A was shifted towards the Ni atom about 1.0 Å compaired to that of Ni-B. Diatomic bridging ligand and X84 of Ni-A seem to block the pathway of dihydrogen.

The essential features of the enzyme structure at the resting state and the transition mechanism from Ni-B to Ni-A are proposed.

Keywords: [NiFe] hydrogenase, Ni-A and Ni-B, non-protein ligand

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Crystal Structure of Mouse Carnosinase CN2 at 1.8 Å Resolution

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L-Carnosine, β-alanyl L-histidine, is found as a bioactive dipeptide which affects autonomic neurotransmission and blood pressure through histamenergic nerves and is present in mammalian tissues including the central nervous system. In mammals, two types of carnosinases, CN1 and CN2, both of which catalyse the hydrolysis of L-carnosine, with different properties are known. The mouse carnosinase CN2 was found to be highly concentrated in the parafascicular nucleus of the thalamus and so on in the brain, which suggests carnosine is degraded by CN2 to supply the substrate of histamine-synthesizing enzyme, histidine decarboxylase. We started crystallographic study of CN2 from mice to understand its enzyme mechanisms on a structural basis.

The MAD data were collected on beamline BL6A of the Photon Factory using an ADSC Quantum 4D CCD detector. The protein phases were determined with the program Sharp and improved with the program dm using non-crystallographic symmetry. The peptide model was built with the program ARP/wARP. The structure is now being refined with the program Refmac5.

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Keywords: metalloproteinases, enzyme active site, protein structure determination

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Class III Superoxide Reductase from *Treponema pallidum*

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Superoxide reductase of *Treponema pallidum* (*Tp* SOR) is a metalloprotein responsible for the scavenging of superoxide radicals in the cell [1]. SORs can be divided into three classes according to amino acid sequence alignment: Members of class I have only the catalytic domain. Class II and III SORs present an additional N-terminal domain that, in the case of class II, has an additional non-heme iron center ($Fe(Cys)_4$) of the rubredoxin type [2]. The active site, common to all three classes, is an iron center, ($Fe(Cys)(His)_4$) that reacts with superoxide in the reduced state.

*Tp*SOR is the first member of class III to be structurally characterized. Blue crystals of the oxidized form diffracted beyond 1.55 Å. A highly redundant in-house data set allowed solving the structure and synchrotron data led to phase improvement.

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Keywords: superoxide, soft X-rays, iron

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Crystal Structures of Cytochrome c Peroxidases from *Ps. nautica* and *Ps. stutzeri*

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