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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  $\alpha$  and  $\beta$  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  $\alpha$ (aquomet)- $\beta$ (penta-coordinated Fe<sup>3+</sup>) 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 substrate 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<sub>2</sub> reversibly via the Fe<sup>2+</sup> 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)<sub>2</sub>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<sup>2+</sup> 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<sup>2+</sup>, Mn<sup>2+</sup>, Mg<sup>2+</sup> or Co<sup>2+</sup>, while the tight binding site (site 2) can be occupied by Zn<sup>2+</sup> or Co<sup>2+</sup>. We recently reported that introduction of Mn<sup>2+</sup> 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 sulphhydryl moiety was found to be