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Crystal Structure and Stability of Red Alga *Porphyra yezoensis* Cytochrome c_6

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The *c*-type cytochromes (Cyts) are characterized by consensus Cys-X-X-Cys-His heme binding motif by which the heme is covalently bonded to the two Cys residues, and the axial His and Met ligands are generally coordinated to the heme iron as its fifth and sixth ligands, respectively. In addition, conformational stability of Cyt *c* is known to be extremely high through its strong heme C-protein contacts. However, role of heme axial ligands of *c*-type Cyts in the conformational stability still remains unknown. In this work, we investigated crystal structure and the effect of heme axial ligands in the conformational stability of Cyt c_6 from the red alga *Porphyra yezoensis*. The crystal structure was determined at 1.57Å resolution. X-ray diffraction data were collected at the BL44B2 station at SPring8, Japan. The overall structure of Cyt c_6 follows the topology of class I *c*-type Cyts in which the heme prosthetic group covalently binds to Cys14 and Cys17, and the heme iron has an octahedral coordination with His18 and Met58 as the fifth and sixth ligands, respectively. Moreover, we constructed M58C and M58H mutants of the Cyt c_6 in which sixth heme iron ligand (Met58) was replaced with Cys and His residues, respectively. The Gibbs free energy change for unfolding of the wild type, M58H and M58C were 2.43, 1.48 and 5.45 kcal/mol, respectively. These results indicate that the heme axial ligand is important key to determine the conformational stability in *c*-type Cyts.

Keywords: cytochromes, mutagenesis, structural stability

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Structure of the Intermediates in the Myoglobin-peroxide Reaction

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The biological conversions of O_2 and peroxides to water as well as certain incorporations of oxygen atoms into small organic molecules can be catalyzed by metal-ions in different clusters or cofactors. The catalytic cycles of these reactions pass through similar metal-based complexes. We have previously reported high resolution structures of the myoglobin compound II intermediate at pH 5.2 [1], and the state has been confirmed by microspectrophotometry in the pH range 5.2 to 8.7. These structures show a relatively long Fe···O distance of 1.9 Å compared to the 1.6 Å distance of the commonly proposed oxo-ferryl [Fe^{IV}=O] species. This long Fe···O bond is supported by the newly observed Raman Fe-O mode below 700 cm^{-1}. Quantum refinement best fit either a $Fe^{III}OH^-$ or a $Fe^{IV}OH^-$ state [2], while the Mössbauer spectroscopy indicates a Fe^{IV}-state. From compound II we were able to generate compound III (an oxy-complex). This intermediate was reduced by the synchrotron radiation giving an equivalent of compound 0 (Fe^{III}-peroxide) for which we have solved the structure. The different states were confirmed by microspectrophotometry.

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Keywords: metalloproteins, crystallography, spectroscopy

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Crystal Structure of PA0740, a Novel Zinc Hydrolase of *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa is an opportunistic pathogen causing acute and chronic infections. During infection, *P. aeruginosa* expresses a range of virulence factors as well as proteins needed for biofilm formation. Expression of most of these proteins is primarily regulated by a sophisticated acyl-homoserinelactone (AHL) based quorum sensing system. By means of transposon mutagenesis we searched for further virulence factors of *P. aeruginosa*. During these efforts a strain in which the gene coding for the 73 kDa protein PA0740 had been knocked out, showed an increased production of AHLs. Therefore PA0740 presumably has an AHL degrading activity and may hence regulate *P. aeruginosa* quorum sensing. To further investigate the function of PA0740, we solved the crystal structure at 2.7 Å resolution. PA0740 is a symmetric dimer, exhibiting an unusual α-helical dimer interface in which the monomers are intricately intertwined. Each monomer contains an N-terminal β-sandwich domain reminiscent of class B β-lactamases. Molecular modelling indicates that 3-Oxo-C12-HSL, a putative substrate, could comfortably bind to the active site, resulting in its hydrolysis. The central domain of PA0740 is involved in dimerization, while the C-terminal domain is structurally similar to sterol carrier protein-2.

Keywords: *P. aeruginosa*, quorum sensing, zinc hydrolase

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Crystal Structure of Carboxypeptidase 1 from *Thermus thermophilus*

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Carboxypepidase 1 from *Thermus thermophilus* (*Tth*CP1) is a metallopeptidase which hydrolyzes a peptide bond from the C-terminus of peptides and proteins and requires a divalent metal ion such as Zn^{2+} or Co^{2+} for its activity. The metal ion binding motif of TthCP1 differs from those of classical metalloproteases and a distinctive catalytic mechanism has been proposed. In this research, we have solved the crystal structure of *Tth*CP1 to analyze the structural basis of its catalytic mechanism and heat stability, and also characterized its substrate specificity.

*Tth*CP1 was crystallized using PEG8000 as the precipitant by sitting drop vapor diffusion method. A native dataset was obtained to a resolution of 2.6 Å Diffraction data were collected using an ADSC Quantum 210 detector system at beamline PF-AR NW12 at Photon Factory (Tsukuba, Japan) [1]. The crystal structure was determined by molecular replacement using the atomic coordinates of carboxypeptidase from *Pyrococcus furiosus* (*Pfu*CP, PDB code: 1KA2 [2]). The structure, substrate specificity and thermostability of *Tth*CP1 will be presented and compared with those of *Pfu*CP [2, 3].

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Keywords: carboxypeptidase, structure-function protease, thermostable enzyme

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Mimicking Evolution from Inactive *Bacillus subtilis* SOD-like Protein to Active Mutants

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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