**PS04.02.26 IDENTIFICATION OF $Ca^{2+}$ IONS BOUND TO PROTEINS USING AN X-RAY ANOMALOUS DISPERSION TECHNIQUE.** K. Hamada[1], Y. Hata[2], H. Miyatake[2], T. Fujii[2], F. Amada[3], K. Fukuyama[3], [1]Interdisciplinary Faculty of Science and Engineering, Shimane University, Matsue, 690 Japan, [2]Institute for Chemical Research, Kyoto University, Uji, Kyoto, 611 Japan, [3]Department of Biology, Osaka University, Toyonaka, Osaka, 560 Japan

Identification of $Ca^{2+}$ ions bound to moderately large proteins by an X-ray anomalous dispersion technique seems to be possible using a high-energy synchrotron source and a sensitive imaging plate. With three kinds of proteins, *Serratia* protease from *Serratia sp* E-15 (Hamada, K. et al., 1995, *J. Biochem.*, in press), alkaline protease from *Pseudomonas aeruginosa* IFO3080 (Miyatake, H. et al., 1995, *J. Biochem.* **118**, 474) and *Arthromyces ramosus* peroxidase (Kunishima, N. et al., 1995, *J. Mol. Biol.* **235**, 331), the identification of $Ca^{2+}$ ions in their crystals were carried out by the anomalous technique. The X-ray wavelengths used for data collection were 1.283 Å (synchrotron radiation) for *Serratia* protease, 1.488 Å (synchrotron radiation) for *Arthromyces ramosus* peroxidase and 1.542 Å (Cu-K$\alpha$) for alkaline protease. The determination of $Ca^{2+}$ binding sites was performed by Bijvoet difference Fourier maps calculated with coefficients of $\{F(+)-F(-)\}\exp i(\alpha_p-\pi/2)$. In the *Serratia* protease, each of five $Ca^{2+}$ ions was located between two neighbors of loops in the $\beta$-sheet coil formed by the repeated sequence of GGXGXDXBX (B: bulky hydrophobic residue, ideally leucine). In the alkaline protease whose tertiary structure is quite similar to that of *Serratia* protease, $Ca^{2+}$ ions were identified at seven sites, six of which were shared with the *Serratia* protease. These $Ca^{2+}$ ions in the both proteins are significantly important in stabilizing the $\beta$-sheet coil structure. In the peroxidase, the two $Ca^{2+}$ ions were identified at the expected positions. The $Ca^{2+}$ ions contribute to stabilization of the tertiary structure of the peroxidase.

**PS04.02.27 STRUCTURE DETERMINATION OF NiFe HYDROGENASE.** Yoshiki Higuchi, Noritake Yasuoka*, Division of Chemistry, Graduate School of Science, Kyoto University, Sakyo-ku, Kyoto 606-01, Japan, *Department of Life Science, Himeji Institute of Technology, 1479-1 Kanaji, Kamigori, Hyogo 678-12, Japan

NiFe hydrogenase from sulfate-reducing bacterium, Desulfovibrio vulgaris Miyazaki F is a membrane protein composed of heterodimer of 62.5 and 28.8 kDa. It has two $Fe_4S_4$, one $Fe_3S_4$ clusters, and one nickel atom as active centers. The single crystals of hydrogenase was prepared from 40% of 2-methyl-2,4,pentanediol solution ( space group is $P\,2_12_12_1$, $a$ =101.0 $b$ =126.5 $c$ =66.5Å). The structure determination has been carried out by the multiple isomorphous replacement (MIR) method combined with the multiwavelength anomalous diffraction (MAD) effect from native crystal. Six derivatives were successfully prepared and showed effective changes in their diffraction intensity. Native anomalous diffraction data were collected at five wavelengths (1.040, 1.489, 1.730, 1.743 and 1.750Å) from one crystal. Native phases were obtained from MIR and MAD data set using the MLPHARE in CCP4 program package. The initial electron density map at 3.0Å was not in good quality but had many ghost peaks around heavy atom binding sites. The quality of electron density map was slightly improved by iterative solvent flattening and histogram mapping. The main chain tracing was carried out at 3.5 Å map, and new molecular envelope was recalculated from this main chain backbone model. The phases were further improved by solvent flattening using this envelope. The new electron density map calculated at 3.0Å was clear enough to locate the side chain atoms and even carbonyl oxygen atoms. The structure of hydrogenase is now in under refinement using the program XPLOR. The R-value was reduced to 0.30 from the initial model (0.510) by only positional refinement. The structure comparison with NiFe hydrogenase from the different strain, *Desulfovibrio gigas* will be discussed.

**PS04.02.28 SITE-DIRECTED MUTAGENESIS OF HUMAN CARBONIC ANHYDRASE I: STRUCTURE AND FUNCTION.** K.K. Kannan, A.K. Mohanty, M.V. Hosur, M.B. Satyamurty, A.V.S.S. Narayan Rao, S.K. Mahajan, Bhabha Atomic Research Centre, Bombay-400 085, India

Human carbonic anhydrase, a zinc metalloenzyme, catalyses the reversible hydration of carbon dioxide. The proposed catalytic mechanism comprises a proton shuttle through a hydrogen bond network among residues T199, E106 (Kannan et al., FEBS LETTS, 73: 115-119, 1977). To verify this proposal we have undertaken site-directed mutagenesis studies of human carbonic anhydrase I (HCAI). Total RNA was prepared from an HEL cell line and used to prepare cDNA using AMV reverse transcriptase and an hCAI specific primer. The cDNA was amplified by PCR and cloned into the expression vector pET-3a. The gene was induced by IPTG, and the protein purified by affinity chromatography was found to be as active as the RBC HCAI. DNA sequencicg revealed that the cloned gene differed from the published RBC hCAI sequence (Barlow, J.H., Nucl. Acid. Res, 15:2386, 1987) with two start codons (ATG) and five other base changes. At the protein level there are two N-terminus methionines and two mutations V31I and V218A. Site-directed mutagenesis (T199V) of this gene was done by a PCR technique using mutated oligonucleotides and confirmed by gene sequencing. Specific activity of the purified mutant protein was assayed and found to be 2% of the wild type. The mutant protein is being crystallized. The recombinant protein crystallized in thick walled glass capillary tubes (Kannan et al., JMB, 63:601-604,1972) are of a different morphology compared to the RBC HCAI. The structure of the recombinant protein and its comparison to the RBC HCAI will be reported.

**PS04.02.29 MECHANISM OF FE(III)-ZN(II) PURPLE ACID PHOSPHATASE BASED ON CRYSTAL STRUCTURES.** T. Klabunde[a], N. Strätер[a], H. Witzel[b], B. Krebs[a], [a]Anorganisch-Chemisches Institut, Westfälische WilhelmsUniversität, Wilhelm-Klemm-Strasse 8, D48149 Münster, Germany, [b]Institut für Biochemie, Westfälische, Wilhelms-Universität, Wilhelm-Klemm-Strasse 2, D48149 Münster, Germany

Purple acid phosphatases (PAPs) containing a dinuclear Fe(III) -Me(II) center (where Me can be Fe or Zn) in their active sites catalyse the hydrolysis of activated phosphoric acid esters and anhydrides, like ATP, at a pH range from 4 to 7. All mammalian PAPs characterized so far are monomeric proteins with a molecular mass of approximately 35 kDa containing an Fe(III)-Fe(II) center in the active site. In contrast, the most intensively studied plant enzyme from kidney bean (kbPAP) is a homodimeric Fe(III)-Zn(II) metalloprotein of molecular mass 111 kDa.

X-ray structures of kbPAP complexed with phosphate, the product of the reaction, and with tungstate, a strong inhibitor of the phosphatase activity, were determined at 2.7 and 3.0 Å resolution, respectively. Furthermore the resolution of the unliganded enzyme, recently solved at 2.9 Å [1] could be extended to 2.65 Å with completely new data. In the inhibitor complex as well as in the product complex, the oxoanion binds in a bidentate bridging mode to the two metal ions, replacing two of the presumed solvent ligands present in the unliganded enzyme form. All three struc-

tures support a mechanism of phosphate ester hydrolysis involving interaction of the substrate with Zn(II) followed by a nucleophilic attack on the phosphorus by an Fe(III)-coordinated hydroxide ion. The negative charge evolving at the pentacoordinated transition state is probably stabilized by interactions with the divalent zinc and the imidazole groups of His-202, His-295 and His-296, the later protonating the leaving alcohol group.

[1] N. Sträter, T. Klabunde, P. Tucker, H. Witzel & B. Krebs, *Science* **268**,1489

**PS04.02.30 CRYSTAL STRUCTURE OF A EUKARYOTIC (PEA SEEDLING) COPPER-CONTAINING AMINE OXIDASE AT 2.3Å RESOLUTION.** Vinay Kumar, Hans C. Freeman and J. Mitchell Guss (University of Sydney, NSW 2006, Australia); David M. Dooley and Michele A. McGuirl (Montana State University, Bozeman, MT 50717, USA.)

We report the first structure analysis of a eukaryotic amine oxidase, pea seedling amine oxidase (PSAO), at 2.3Å resolution. The structure was solved using phases derived from a single heavy-atom (phosphotungstic acid, $H_3PW_{12}O_{40}$) derivative. The positions of the tungsten atoms in the $W_{12}$ cluster were obtained by molecular replacement using the prokaryotic amine oxidase from *E. coli* (ECAO) [Parsons, M.R. *et al.* (1995). *Structure*, **3**, 1171-1184] as a search model. However, the methodology avoided bias from the search model and resulted in an essentially independent view of a eukaryotic amine oxidase molecule.

Copper-containing amine oxidases are a widely distributed class of enzyme whose function is to catalyze the oxidative deamination of biogenic amines to the corresponding aldehyde. The redox reaction is facilitated by an organic cofactor, topa quinone (TPQ), which is formed by the post-translational modification of an invariant Tyr residue.

The PSAO molecule is a homodimer with dimensions 100x63x42 $Å^3$. The copper(II) atom at the active site of each subunit is coordinated by three histidine side chains and two water molecules in an approximately square-pyramidal arrangement. All the atoms of the topa quinone (TPQ) cofactor are unambiguously defined. The closest contact to the copper atom is ~6Å. A second metal atom revealed by the structure analysis is tentatively identified as manganese(II).

The molecular structure of PSAO is similar to that of the prokaryotic ECAO. A detailed comparison of the two structures suggests that the TPQ side chain is sufficiently flexible to move between uncoordinated and coordinated positions with respect to the copper atom. Such flexibility may be associated with the different spatial requirements for TPQ biogenesis and amine oxidation.

**PS04.02.31 CRYSTAL STRUCTURE OF A ZINC METALLOENDOPROTEASE FROM *STREPTOMYCES CAESPITOSUS* AT 1.6Å RESOLUTION.** G.Kurisu, A.Sugimoto, Y.Kai and S.Harada†, Department of Applied Chemistry, Faculty of Engineering, Osaka University, Suita, Osaka 565, Japan, †Faculty of Pharmaceutical Sciences, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan

A zinc protease from *Streptomyces caespitosus* (ScNP), which is specific for peptide bonds on the amino side of aromatic residues, consists of 132 amino acid residues with one disulfide bond. While ScNP has the zinc-binding sequence His83-Glu-Xaa-Xaa-His87, it does not share overall significant similarity to the sequences of other zinc proteases (S. Harada, T. Kinoshita, N. Kasai, S. Tsunasawa and F. Sakiyama, (1995). *Eur. J. Biochem.* **233**, 683-686).We crystallized ScNP and determined its three-dimensional structure at 1.6 Å resolution. The structure analysis was performed by the MIR method. The crystallographic R-factor of the structure refined by XPLOR and PROLSQ was 0.16. ScNP consists of a highly twisted five-stranded β-sheet, four α-helices, one catalytically essential zinc ion and one calcium ion as shown in Figure 1.

This structure is topologically similar to those of the catalytic domains of other zinc proteases such as atacin, thermolysin, serratia, snake venom and collagenase despite a lack of sequence homology. The zinc atom of ScNP is tetrahedrally ligated by His83 and His87 in the zinc-binding sequence, Asp93 and a water. ScNP is the first zinc endoproteases in which an aspartate ligates to the zinc, and thus represents a novel organization of zinc ligands.

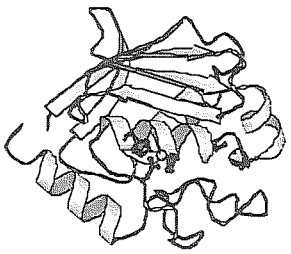

Figure. 1 Schematic drawing of ScNP[1)]

1)Kraulis, P.J. *J.appl.Crystallogr.* **24**, 964-950 (1991)

**PS04.02.32 BACILLUS CEREUS NEUTRAL PROTEASE G197D AND E144S MUTANT STRUCTURES.** S.A. Litster and P.W. Codding, Department of Chemistry and D.R. Wetmore, R.S. Roche, Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada, T2N 1N4

To gain an understanding of the role of calcium binding in the thermal stability of thermolysin-like neutral proteases, mutants of Bacillus cereus neutral protease (CNP) were developed and the structures determined. Here we present the crystal structures of the G197D and E144S mutants of CNP, at 3.0Å and 2.8Å resolution, respectively. A comparison of the structures and how they relate to the thermal stability and hydrolyase activity of the enzyme will be made. The G197D structure is novel in that it contains only three calcium ions, with the missing calcium ion being Ca(II); the ion thought to bind cooperatively along with Ca(I) to form the double calcium binding site in native CNP and thermolysin. The second structure, that of the inactive E144S mutant, the Glu to Ser mutation reduces the protease activity of the enzyme to 0.16% that of wild type and represents the first crystal structure of an active site mutant of a neutral protease. The mutant structure reveals a modified environment around the catalytic zinc ion and suggests a major role for bound water molecules. The mutants crystallize in the hexagonal space group P6(sub5)22 which is isomorphous with wild type crystals.

CNP: Pauptit R.A., Karlsson R., Picot D., Jenkins J.A., Niklaus-Reimer A., Jansonius, N., *J. Mol. Biol.* (1988), 199, 525-537.