

PS04.02.26 IDENTIFICATION OF Ca²⁺ IONS BOUND TO PROTEINS USING AN X-RAY ANOMALOUS DISPERSION TECHNIQUE. K. Hamada¹, Y. Hata², H. Miyatake², T. Fujii², F. Amada³, K. Fukuyama³, ¹Interdisciplinary Faculty of Science and Engineering, Shimane University, Matsue, 690 Japan, ²Institute for Chemical Research, Kyoto University, Uji, Kyoto, 611 Japan, ³Department of Biology, Osaka University, Toyonaka, Osaka, 560 Japan

Identification of Ca²⁺ 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 Ca²⁺ 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-Kα) for alkaline protease. The determination of Ca²⁺ binding sites was performed by Bijvoet difference Fourier maps calculated with coefficients of {F(+)-F(-)}exp(i(α_p-π/2)). In the *Serratia* protease, each of five Ca²⁺ ions was located between two neighbors of loops in the β-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, Ca²⁺ ions were identified at seven sites, six of which were shared with the *Serratia* protease. These Ca²⁺ ions in the both proteins are significantly important in stabilizing the β-sheet coil structure. In the peroxidase, the two Ca²⁺ ions were identified at the expected positions. The Ca²⁺ 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 Fe₄S₄, one Fe₃S₄ 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* 2₁2₁2₁, *a* = 101.0 *b* = 126.5 *c* = 66.5 Å). 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 1.750 Å) 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 3.0 Å 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 3.0 Å 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 sequencing 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. Sträter^a, H. Witzel^b, B. Krebs^a, ^aAnorganisch-Chemisches Institut, Westfälische Wilhelms-Universität, Wilhelm-Klemm-Strasse 8, D48149 Münster, Germany, ^bInstitut 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-