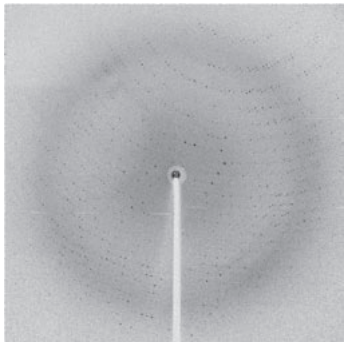
Clostridium acetobutylicum, an obligatory anaerobe. Rpr consists of 181 amino acids with a molecular weight of 22,500. It is rubrerythrin-like protein, and conserves rubredoxin-type [$Fe(SCys)_4$] site in the N-terminus. Rpr rapidly reduces hydrogen peroxide using NADH as an electron donor, and slowly reduces t-butyl hydroperoxide and dioxygen. $2H^+ + 2Rpr_{red}(1Rpr_{dimer\ red}) + H_2O_2 = 2Rpr_{ox}(1Rpr_{dimer\ ox}) + 2H_2O$ In order to reveal the reaction mechanism of the reduction of hydrogen peroxide by Rpr, we have started the crystallographic study. Purified Rpr was crystallized by the sitting-drop vapor diffusion method using poly(ethylenglycol)(PEG) 4000 as a precipitant (final composition of the droplet: 12 mg/mL in 50 mM K-Pi pH 7.0 buffer, Outer droplet: 9% PEG 4000, 0.1 M Zn acetate in 0.1 M MES pH 6.0 buffer). Crystals diffracted to 2.3 angstrom resolution at the beamline BL38B1 (SPring-8), and belong to the space group $P2_12_12$ (a = 81.6 Å, b = 117.4 Å, c = 141.5 Å). Structure analysis is now in progress.



Keywords: X-ray crystallography of proteins, metalloproteins, redox enzymes

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Roles of heme-6-propionate side chain in monooxygenase cytochrome P450cam

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Cytochrome P450cam (P450cam) responsible for *d*-camphor hydroxylation binds a protoheme IX as a prosthetic group in which two heme-propionate side chains interact with Arg112 and Arg299, respectively. To understand the structural and functional roles of the heme-6-propionate side chain in P450cam, we prepared the reconstituted P450cam with one-legged heme where the 6-propionate side chain was replaced with methyl group. The crystal structure of the reconstituted P450cam at a resolution of 1.55 angstrom is highly superimposable with that of the wild type protein, and a chloride anion is located in the position of the 6-propionate (Figure 1). The reactivity of the reconstituted P450cam toward the substrate hydroxylation was almost comparable with that observed for the wild type protein, whereas we found that the removal of the 6-propionate side chain accelerated the unfavorable conversion of the P450 active form into the inactive P420 species. In this presentation, we discuss the exact roles of the 6-propionate side chain in P450cam.

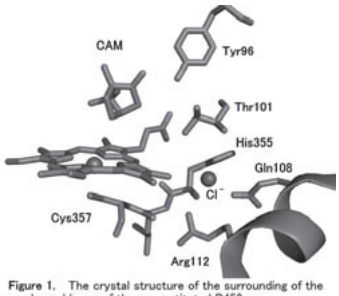

Figure 1. The crystal structure of the surrounding of the one-legged heme of the reconstituted P450cam.

[1] K. Harada, K. Sakurai, H. Shimada, T. Hayashi *et al.* (2007) *J. Am. Chem. Soc.*, **130**, 432.

Keywords: heme proteins, monooxygenases, crystallography of biological macromolecules

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The crystal structure of heme oxygenase catalytic intermediate unravel the enzyme mechanism

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Heme oxygenase (HO) catalyzes O_2-dependent regiospecific conversion of heme to biliverdin, CO, and free iron by three consecutive monooxygenase steps. In order to understand the reaction mechanism of HO, we have solved the structures of catalytic intermediate of HO catalysis using HmuO, a heme oxygenase from *Corynebacterium diphtheriae*. In the substrate-free structure, the proximal and distal helices, which sandwich the heme group, move farther apart with changes in their conformations resulting in opening of the heme pocket so as to fascilitate heme binding. Crystals of the hydroperoxo intermediate, have been prepared using cryogenic reduction technique, in which the oxy crystals were irradiated by synchrtron radiation at 100 K. The structure of the ferric hydroperoxo heme-HmuO complex is very similar to that of the oxy form but has slightly longer Fe-O and O-O bond distances. Flash-annealing of the irradiated oxy crystals has yielded the hydroxyheme intermediate, the structure of which has been solved and refined at high resolution. In the structure, the distal helix moves away from hydroxyheme. Possible reaction mechanism, based on the crystal structures of catalytic intermediates will be discussed at the meeting.

Keywords: heme enzyme structure and function, reactive intermediate, cryo crystallography

P4

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X-ray crystal structural analysis of cyanide binding cytochrome *c* oxidase

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For elucidation of the reaction mechanism of the cyotochrome *c* oxidase(CcO), it is desirable to determine the binding mechanism of cyanide to the oxygen reduction site of the enzyme. Here, we analyzed the structure of the cyanide derivative of the fully oxidized CcO. Cyanide induces extremely small change in the α-band spectrum of the enzyme. Thus, it is impossible to trace cyanide-binding to the enzyme in the crystals by measuring the absorption spectrum of the crystals, because accurate measurement of Soret-band of the enzyme in crystals is impossible. However, we found that cyanide once bound to CcO was not removal by repeat dialysis,

since the dissociation of cyanide is essentially irreversible. The occupancy of cyanide at the O_2 reduction site in the CcO crystal was estimated by the Soret-band spectrum of the enzyme solution prepared by dissolving the cyanide-treated crystals after washing the cyanide-treated crystals with the cyanide-free medium. No significant cyanide-binding to the enzyme in the crystals was detectable in the medium including 5－10 mM cyanide for about a week at 4℃. However, the enzyme in crystals was saturated with cyanide in one week by exchanging the freshly prepared medium of the crystals including cyanide every day. Cyanide-bound enzyme crystals, prepared by this method, gave X-ray diffraction up to 1.8 Å resolution under 100K at SPring-8, BL44XU. The result of the data analysis suggested two possibilities of the cyanide-binding geometry that cyanide is slightly bending(N-Fe-C=90°, Fe-C-N=150°) or tilting(N-Fe-C=80°, Fe-C-N=180°) versus heme plane.

Keywords: cytochrome oxidase, heme enzyme structure and function, protein-inhibitor complex

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Delay time-resolved X-ray crystallographic analysis of reaction mechanism of nitrile hydratase

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Nitrile hydratase (NHase) catalyzes the hydration of various nitriles to the corresponding amides. NHase has been attracted by the application to industrial production of acrylamide as well as by its unusual structure of the metallocenter. NHase from *Rhodococcus* sp. N771 has a non-heme iron catalytic center with two post-translationally modified cysteine ligands, αCys112-SO_2H and α Cys114-SOH. Because of the unique catalytic center, the catalytic mechanism remains unclear. Recently, we found that NHase catalyzes the conversion of tert-butylisonitrile (t-BuNC), behaving as a strong competitive inhibitor for NHase, to tert-butylamine. t-BuNC is likely to be a good substrate for studying the reaction mechanism of NHase. To understand the stoichiometry of the novel catalytic activity, the reaction was pursued by ATR-FTIR. By trapped with reduced hemoglobin, the product was identified as a carbon monoxide (CO). Thus, NHase hydrolyzes isonitriles to the corresponding amine and CO. Time-resolved crystal structure analysis was performed using crystals of the nitrosylated inactive NHase soaked with t-BuNC. The catalytic reaction was initiated by photo-induced denitrosylation. The reaction was stopped by flash-cooling with nitrogen gas and then the X-ray diffraction data sets were collected at elapsed times. At 0 min, a t-BuNC molecule was located in the reaction cavity, but had no direct interaction with NHase. At 120 min, the carbon atom of －NC group was coordinated to the iron at a distance of 2.07 Å. At 440min, new electron density appeared neighborhood－NC group at a distance of 1.26 Å. The structure is likely to represent the enzyme-reaction intermediate complex. Based on the results obtained, the catalytic mechanism of NHase will be discussed.

Keywords: time-resolved crystallography, enzyme catalytic reaction mechanism, nonheme iron enzyme model

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X-ray structure of carbon monoxide at copper site of the dinuclear site of cytochrome *c* oxidase

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The copper site (Cu_B) in the O_2 reduction site of cytochrome *c* oxidase is silent to most of spectroscopic techniques and thus the role of Cu_B in the O_2 reduction mechanism is poorly understood. The fully reduced carbon monoxide (CO) derivative of bovine heart cytochrome *c* oxidase photolyzed below 140 K shows C-O stretch band at 2062 cm^{-1}, assignable to CO bound at Cu_B. However, the infrared result provides no direct geometric information for the bound CO. Electron density map of the fully reduced-CO bound form of bovine heart cytochrome *c* oxidase under light conditions at 100 K at 1.8 Å resolution shows an electron density peak assignable to CO near Cu_B atom. The *Fo-Fc* map strongly suggests a side-on binding of CO to Cu_B, although the possibility of an end-on binding can not be excluded at this resolution. The distances between Cu_B and the two atoms of CO are 2.5 Å and 2.4 Å, suggesting a fairly weak metal/ligand interaction. The weak interaction is likely to contribute the stability of the oxygenated form of the enzyme ($Fe a_3$-O_2), which is prerequisite for the four electron reduction of O_2 at $Fe a_3$.

Keywords: cytochrome oxidase, heme enzyme structure and function, protein-inhibitor complex

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X-ray structural analysis of Zinc/Cadmium inhibitory site in bovine heart cytochrome *c* oxidase

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Cytochrome *c* oxidase (CcO) is a supramolecular protein complex (MW 210 kDa) in the mitochondrial innermembrane which catalyzes the oxygen reduction coupled to the electron and proton transfer. The energy from oxygen reduction is transduced to an electrochemical potential energy of proton, which is used for ATP synthesis. Previous biochemical studies indicated that Zinc (Zn) inhibited proton transfer by binding either inside or outside surface of CcO. Similar inhibition is also caused by Cadmium (Cd). To identify the Zn/Cd inhibitory sites, we have carried out the x-ray structural analyses of bovine heart CcO-Zn/Cd complex. The crystals of CcO was soaked in the Zn/Cd solution and frozen by a cryostream. X-ray diffraction was measured by using a synchrotron radiation at SPring8 BL44XU. Anomalous difference Fourier maps obtained from the crystal of dimeric CcO revealed several Zn/Cd-binding sites. The highest affinity Zn/Cd-binding site (Zn2/Cd1 site) is located at the inside surface of the subunit III. The second highest affinity site (Zn3/Cd2 site) on the