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

inside surface is located at the D-pathway entrance. The zinc binding affinity for the second site suggests that the zinc site is tightly coupled with the proton-pumping site. Recently, we analyzed Zn/Cd-binding to monomeric CcO which gives crystal packing different from that in the dimeric CcO crystal. The x-ray structural analysis showed Zn-binding to the Zn2, Zn3 and additional sites including the site near the K-pathway entrance. Several Zn-binding sites have been found on the outside surface. However none of them is located on the subunit I surface from which pumping protons exit.

Keywords: cytochrome oxidase, metal-biomolecule interactions, proton transfer

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Structural analysis for lipid/protein interactions in bovine heart cytochrome *c* oxidase

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All 13 lipids, including two cardiolipins, one phosphatidylcholine, three phosphatidylethanolamines, four phosphatidylglycerols and three triglycerides were identified in a crystalline bovine heart cytochrome *c* oxidase (CcO) preparation. The chain lengths and unsaturated bond positions of the fatty acid moieties determined by mass spectrometry suggest that each lipid head group identifies its specific binding site within CcO. Binding of dicyclohexylcarbodiimide to the O_2-transfer pathway of CcO causes two palmitate tails of phosphatidylglycerols to block the pathway, suggesting that the palmitates control the O_2 transfer. The phosphatidylglycerol with vaccenate (*cis*-Δ^{11}-octadecenoate) was found in CcO of *Paracoccus denitrificans*, a possible ancestor of mitochondrion. This indicates that the vaccenate is conserved in bovine CcO in spite of the abundance of oleate (*cis*-Δ^{9}-octadecenoate). The X-ray structure indicates that the protein moiety selects *cis*-vaccenate against *trans*-vaccenate for the O_2-transfer pathway. These results suggest that vaccenate plays a critical role in the O_2-transfer mechanism and that the lipid binding specificity is determined by both the head group and the fatty acid tail.

Keywords: cytochrome oxidase, mass spectrometry, protein-lipid interactions

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Inter- and intra-molecular complex structures of Cu-containing nitrite reductase with cytochrome *c*

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Copper-containing nitrite reductase (CuNIR) is known as a key enzyme in biological denitrification, catalyzing one-electron-reduction of nitrite to the gaseous nitric oxide. CuNIRs are homotrimers with two distinct Cu sites per ca. 35-kDa monomeric unit. The type 1 Cu site (T1Cu) buried within each monomer relays an electron from the redox partner protein to the type 2 Cu site (T2Cu) where NO_2^- is reduced to NO. Despite much effort by several groups, a crystal structure of the protein-protein complex state between a CuNIR and its redox partner(s) has not been determined. This difficulty is probably a reflection of the low free energy for complex formation and short lifetime that is conducive to rapid electron transfer in such complexes. As a first topic, we report the X-ray crystal structure analysis at a resolution 1.7 Å of a binary protein-protein complex between blue CuNIR and its redox partner protein cytochrome c_{551} from *Alcaligenes xylosoxidans* GIFU1051. The CuNIR-Cyt c_{551} interface is largely hydrophobic, covering ca. 500 Å^2 of surface on each molecule. The closest distance from heme-edge to T1Cu is 10.5 Å. Second is the X-ray crystal structure analysis of the novel CuNIR from the versatile marine Antarctica bacterium *Pseudoalteromonas haloplanktis* TAC125. This enzyme consists of two distinct functional domains, belonging to the new type of CuNIR. The N-terminal domain contains two copper atoms, T1Cu and T2Cu, and is homologous of the well-known CuNIRs. The *c*-type heme attached C-terminal domain is combined with the N-terminal domain by a linker region. Using both high-resolution X-ray data, structural and mechanistic insights into the multiple electron transfer reaction from heme *c* to T1Cu, following to the T2Cu for the reduction of NO_2^- to NO, are given.

Keywords: electron transfer, metalloproteins, protein complex structure

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Crystal structure of cytochrome P450 105A1 in complex with 1 α ,25-dihydroxyvitamin D3

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The bacterial enzyme cytochrome P450SU-1 (CYP105A1) from Streptomyces griseolus has been known for its ability to convert vitamin D3 into its active form in two steps. We determined the crystal structures of hyperactive mutant (R84A) of P450SU-1 (CYP105A1) in complex with and without the final reaction product 1alpha,25-dihydroxyvitamin D3. The product is bound between B', G, and I helices within the distal pocket at the distance of 11 angstroms from the heme iron. The loop after K helix shows remarkable conformational difference upon product binding, resulting in the different shape of active site pocket. Nonetheless, orientation of 1alpha,25(OH)2D3 is similar to that of VD3 in human CYP2R1, suggesting a common substrate-binding mode for 25-hydroxylation. R84A shows a 32-fold increase in 25-hydroxylation activity compared with the wild type enzyme. A plausible explanation for this effect of the R84A mutant is that the loss of the interaction with the Arg84 side chain the in B' helix opens