The terminal oxidases of the aerobic respiratory chains in mitochondria and bacteria catalyze the oxygen reduction coupled to proton pumping, which generates the proton-motive force. The terminal oxidases containing heme and copper at the catalytic site form the heme-Cu oxidase (HCO) super family. Although conformations of the catalytic sites are similar in HCOs, the proton transfer pathways are not conserved and the energy transducing efficiencies are different. To identify structurally important elements and clarify common mechanism on the energy transduction, we aim to investigate *cbh₃*-type cytochrome *c* oxidase from *Vibrio cholera* by X-ray crystallographic analysis. In previous study, we have examined culture condition for *V. cholera* cells carrying His-tagged *cbh₃* oxidase expression plasmid and then performed purification by metal affinity chromatography. We have obtained *cbh₃* oxidase showing enough oxygen consumption activity, but SDS-PAGE showed the preparation still contained some impurity. In this study, to improve purity and yield, we examined the conditions of metal ion resin and solution composition on the chromatography. By using Ni ion resin, 5mg of *cbh₃* oxidase was obtained from 30g cells. Purity was significantly improved by increasing salt concentration in charge and wash solution on the chromatography. Currently, we collect many preparations by this method for crystallization.

2PT157 水素原子レベルでの酸化還元に伴う構造変化同定を目的としたウシ心筋チトクロム酸化酵素の X 線結晶構造解析

X-ray structural analysis of bovine heart cytochrome *c* oxidase to clarify redox coupled structural changes at H atom level

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Cytochrome *c* oxidase (CcO) catalyzes the molecular oxygen reduction coupling to the proton transfer across the inner membrane. To understand proton transfer mechanism in detail, it is necessary to determine orientations and protonation states of amino acid residues (imidazole and carboxyl group etc.) and waters in the proton pathways in both oxidized and reduced states. For this purpose, we are currently carrying out high resolution X-ray structural analysis of bovine CcO.

To reduce X-ray damage and increase data accuracy, many data from isomorphous crystals were collected. Low quality images whose R_{merge} or mosaicity were larger than 0.3 were removed from data set. At present, data of the oxidized state showed $I/\sigma(I)$ of 1.6 at 1.42 to 1.40 Å resolution range. As a result of structural analysis, many multi-conformations (Met residue etc.) were found. Electron density map of phospholipids, waters and peroxide at the O₂ binding site were improved. To determine H atom positions, residual density of $F_o - F_c$ map must be reduced. 1) about 2800 waters and 36 hydrocarbons were added, 2) 88 multi-conformations were introduced to amino acids, 3) individual anisotropic B-factor were introduced 50% amino acid residues. As a result, residual density of hydrophilic region was removed. R and R_{free} values were also reduced from 0.199 to 0.154 and 0.213 to 0.177, respectively. Structural refinement of reduced state carries out as well as oxidized state.

2PT158 有機薄膜電極上での P450 反応の電気化学制御と応用

Electrochemically-driven cytochrome P450 reactions at organic films and its bioprocess application

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Cytochrome P450 enzymes catalyze a vast array of oxidative biotransformations that are potentially useful for pharmaceutical synthesis. Generally, P450s require reducing power in the form of expensive cofactors such as NADPH and one or two additional redox proteins to transfer electrons to the P450 active site to limit their technological applications. Replacing the conventional electron supply system with an electrode is a promising approach to develop inexpensive P450-driven systems. Recently, we found that nanostructured gold electrode is useful for the purpose. In the present study, we prepared several organic films on nanostructured electrodes to investigate the effect of surface features of electrodes on the electrochemical P450 reactions. Nanostructured surfaces of gold electrodes were modified with some thiolates by dip treatment. Immobilizations of P450s on the thiolate films were examined. CYP modified electrodes were investigated with voltammetry of a normal three-electrode configuration. CYPs on hydrophobic electrode surfaces coated with aromatic thiolates showed clear non-turnover signal, indicating rapid direct electron transfer between P450 heme and electrode and gave the largest catalytic current. On the other hand, electrode surfaces with coating materials containing

hydrophilic functional groups were found to be mostly not suitable for the rapid electron transfer, and poorer P450-catalytic currents were obtained, indicating the importance of electrode surface nature for electrochemically-driven P450 reaction.

2PT159 ヒトヘモグロビン α 鎖の近位ヒスチジンのグリシンへの置換が構造と酸素結合機能に与える影響

Substitution effects of Gly for the proximal His of α subunits on the structure and oxygen binding function of human hemoglobin

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It has been proposed that the proximal histidine (His) in human adult hemoglobin (HbA) plays a pivotal role in the transmission of oxygenation-induced conformational changes in one subunit to another and in the resultant triggering of cooperativity [1]. We synthesized a mutant Hb in which the proximal His of α subunits was replaced by glycine (G), and examined its quaternary structure change upon ligand binding by means of near-UV circular dichroism (CD) and UV resonance Raman (UVR) spectroscopy as well as its oxygen binding function. Mutant Hb(α 87His→Gly) (α H87G) was produced in *E. coli*, and purified as previously described [2]. Oxygen equilibrium curve (OEC) was measured by an automatic OEC recording apparatus or by a tonometer method. The OEC of Hb(α H87G) showed an unusual shape having two components with a very high oxygen affinity and a distinctly low oxygen affinity without cooperativity. In near-UV region, a small positive CD band was observed for oxyHbA while a distinct negative CD for deoxyHbA. The negative CD of deoxyHbA at 287 nm, called "a T-state marker band", was observed in oxyHb(α H87G), indicating that the substitution of Gly for the proximal His failed to undergo the T-to-R quaternary structure transition. Changes of UVR spectrum upon ligand binding of Hb(α H87G) also suggested lack of quaternary structure transition.

[1] Perutz, M. F., *Nature*, **228**, 725-734 (1970).

[2] Nagai, M., Nagai, Y., Aki, Y., Imai, K., Wada, Y., Nagatomo, S., and Yamamoto, Y. *Biochemistry*, **47**, 517-525 (2008).

2PT160 細菌 *Vibrio alginolyticus* 由来呼吸鎖末端酸化酵素の遺伝子クローニングと同定

Gene cloning and identification of terminal oxidase of respiratory chain in *Vibrio alginolyticus*

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The terminal oxidase of the aerobic respiratory chain catalyzes reduction of molecular oxygen to water molecule. The terminal oxidases containing heme and copper at the catalytic site are classified into several types which form the heme-Cu oxidase superfamily. Although conformations of the catalytic sites are similar between different types, the proton transfer pathways are not conserved and the energy transducing efficiencies are different. In many bacteria, the cell is known to express multiple terminal oxidases in different types depending on growth environment. Genome sequence of *Vibrio alginolyticus* indicates that there are several gene homologs of terminal oxidases, one of which is *aa₃*-type homolog. Primary sequence of core subunit I shows about 47% homology to bovine mitochondrial *aa₃*-type and 46-47% homology to *Paracoccus denitrificans* or *Rhodobacter sphaeroides* *aa₃*-type, these are well studied ones. Structural modeling suggests that hemes *a* and *a₃* can be built into the subunit I. To identify the *aa₃*-type terminal oxidase in *V. alginolyticus*, we cloned possible *aa₃*-type genes and constructed His-tagged expression system in *V. alginolyticus* cell. Absorption spectrum of cultured cell suspension showed absorption peak at cytochrome *a* region, although its height was much lower than those of cytochromes *b* and *c* regions. Partially purified sample showed absorption peak at cytochrome *a* region in the reduced minus oxidized difference spectrum. These results indicate that the *aa₃*-type terminal oxidase is in *V. alginolyticus* cell.

2PT161 硬骨魚類の祖先型ヘモグロビン遺伝子の設計・合成および大腸菌内発現

Design and construction of ancestral Osteichthyes hemoglobin genes and its expression in *Escherichia coli*