ligand N_3^- forms hydrogen-bond connection to the proton transfer pathway (K-pathway) through the hydroxyl group of heme a_3 and water, which is also observed in the CN-bound reduced structure but located at slightly different position. These results suggest that the proton transfer through the K-pathway is coupled to heme movement upon ligand binding to the heme-copper site.

2TP1-02 チトクロム酸化酵素結晶 F 型中間体の再酸化反応による生成

2P-074 Cytochrome *c* oxidase crystal in the F intermediate state produced by reoxidation

Kazumasa Muramoto (1), Rie Harada (1), Tomoko Maeda (1), Kyoko Shinzawa-Itoh (1), Eiki Yamashita (2), Tomitake Tsukihara (3), **Shinya Yoshikawa** (1) ((1) *Dept. of Life Sci., Univ. of Hyogo; Picobio. Inst., Univ. of Hyogo*; (2) *Inst. for Protein Res., Osaka Univ.*; (3) *Picobio. Inst., Univ. of Hyogo; Inst. for Protein Res., Osaka Univ.*)

Cytochrome *c* oxidase (CcO) reduces molecular oxygen to water using electrons from cytochrome *c* and protons from solvent. The oxygen reduction drives proton translocation across the membrane to generate proton motive force. In the oxygen reduction cycle, several intermediate species have been identified by spectroscopy. Oxygen molecule binds to the reduced heme a_3 -Cu_B site to form the oxy intermediate. Next intermediate is called P, in which oxygen-oxygen bond is already broken. In the next step, delivery of an electron and a proton to the catalytic site generates the F intermediate. The P and F intermediates are identified by their characteristic absorption spectra. In previous studies, we made bovine CcO crystal containing P and F intermediates by treatment of the resting oxidized crystal with hydrogen peroxide. X-ray structural analysis suggests that one oxygen atom binds to Fe_{a3} and another oxygen atom binds to Cu_B in the P and F intermediate. In this study, we made CcO crystal containing F intermediate by another way, where the reduced crystal is oxidized by reaction with oxygen molecule. The absorption spectrum indicates that the F intermediate is stable in the crystal during reoxidation. When the reoxidized crystal is treated with hydrogen peroxide, the absorption change was significantly smaller than that after hydrogen peroxide treatment of the resting oxidized crystal. These results suggest that the F intermediate is dominant species in the reoxidized crystal. X-ray structural analysis of the reoxidized crystal is under way.

2TP1-03 アミノ酸置換によるウシ心筋チトクロム *c* 酸化酵素の水素結合ネ

2P-076 ネットワークの機能解析

The functional analysis of hydrogen bond network in bovine heart cytochrome *c* oxidase by mutagenesis

Ryohta Aminaka (1), Kunitoshi Shimokata (2), Mai Itoh (1), Yukie Katayama (1), Tomitake Tsukihara (1), Shinya Yoshikawa (1), Hideo Shimada (1) ((1) *Graduate School of Life Science, University of Hyogo*; (2) *WORLD INTEC CO., LTD.*)

Cytochrome *c* oxidase (CcO) catalyzes reduction of molecular oxygen to water and proton pumping from the negative side of the mitochondrial and bacterial plasma membranes to the positive side. X-ray structural analysis of bovine and bacterial CcO reveals three hydrogen bond networks (D-, K-, and H-pathways), where D- and K-pathways are suggested to transfer the water-forming proton. This suggestion is supported by the mutagenesis results of the bacterial CcO. In the bovine CcO, the x-ray structural and mutagenesis analyses strongly support the H-pathway proton-pumping proposal. In contrast, mutations of the bacterial D-pathway such as Asn98Asp and Asn163Asp (bovine CcO numbering) decouple the proton pumping without impairing the oxidase activity. These results are taken to support the D-pathway proton-pumping proposal. We are studying the function of the bovine D-pathway by mutagenesis analysis employing the bovine/human hybrid CcO expressing HeLa cell system we have developed, where the hybrid enzyme are dominantly expressed over the endogenous human CcO. The Asn98Asp mutation had no effect on the enzymatic activity in contrast to the bacterial results. Furthermore, growth rate of HeLa cells dominantly expressing the Asn163Asp mutant was about 5-fold slower than that of the cells expressing the wild type and decoupled mutant enzymes, implying defect in the oxidase activity. These results suggest that the function of D-pathway is different between the bacterial and bovine CcO, though the structure of the pathway is essentially conserved.

2TP1-04 チトクロム *c* 酸化酵素におけるヒドロキシファルネシルエチル基

2P-075 の共鳴ラマンマーカースペクトル

Resonance Raman Marker Bands of Hydroxyfarnesylethyl Substituent of Cytochrome *c* Oxidase

Miyuki Sakaguchi (1), Kyoko Shinzawa-Itoh (1), Shinya Yoshikawa (1), Hiroshi Fujii (2), Takashi Ogura (1) ((1) *University of Hyogo*; (2) *Okazaki Institute for Integrative Bioscience*)

The redox change of heme *a* was proposed to drive proton pump in cytochrome *c* oxidase (CcO) [1]. X-ray crystallography showed that reduction of heme *a* induced conformational change of hydroxyfarnesylethyl(HFE) substituent, which opened a room near the HFE group, leading a collection of a water molecule from the negative side. Such a change of HFE group could be detected with resonance Raman (RR) spectroscopy. However, the vibrational modes associated with HFE group of CcO have not been identified. CcO in oxidized state gave a RR band at 1250-cm⁻¹ upon Soret excitation and it showed a downshift to 1247-cm⁻¹. In H₂O

minus D₂O Raman difference spectrum, we observed two positive peaks at 1241- and 1224-cm⁻¹ and two negative peaks at 1249- and 1232-cm⁻¹. These bands showed a unique behavior in their spectral pattern upon freezing at -20 °C, whereas any other porphyrin skeletal modes did not. Bis-imidazole complex of RR spectra of Fe(II)-2-vinyl-4-hydroxymethyl deuterioporphyrin gave quite similar difference pattern in the H₂O minus D₂O Raman difference spectrum as CcO did. On the other hand, bis-imidazole complex of Fe(II)-protoporphyrin did not give any difference pattern, indicating that the difference pattern for the former compound arose from the hydroxymethyl group. Based on the results, the Raman bands in the 1220-1250 cm⁻¹ region of CcO are assignable to vibrational modes involving the HFE moiety. [1] Tsukihara *et al.*, *Proc. Natl. Acad. Sci. U. S. A.* **100**, 15304-15309, 2003.

2TP1-05 チトクロム *c* 酸化酵素の CO 光解離後の構造緩和過程の追跡

2P-077 Structural Relaxation Process of Cytochrome *c* Oxidase Following Photolysis of Carbonmonoxide

Izumi Ishigami (1), Satoru Nakasima (2), Kyoko Shinzawa-Itoh (1), Shinya Yoshikawa (1), Takashi Ogura (1) ((1) *University of Hyogo, Grad.Sch. Life Sci.*; (2) *Picobiology Inst., Grad.Sch. Life Sci., University of Hyogo*)

Mitochondrial cytochrome *c* oxidase (CcO) reduces dioxygen to water, and transports protons from the negative to the positive spaces. CcO has 4 redox-active metal centers according to X-ray crystallography. His376 and His378, ligating to hemes a_3 and a , respectively, are the residues of helix X. Helix X exhibits conformational change upon redox-change and ligand binding. The dynamical process from ligated- to unligated-form of CcO has not been well understood.

In the present study, we tried to observe structural relaxation process from the carbonmonoxide (CO)-bound- to the equilibrium fully-reduced-states following photolysis of CO by using time-resolved resonance Raman spectroscopy. In the time-resolved experiments, we used 532.0 nm (708 μJ/pulse) and 435.7 nm (35.4 μJ/pulse), to photodissociate CO and excite Raman scattering, respectively. Concentration of CcO was 100 μM in 100 mM sodium phosphate buffer, pH 6.8, containing 0.2 % n-Decyl- β-D-maltoside. We found the changes in the porphyrin ν₉ mode, the ν_{Fe-His} mode, and bending mode of vinyl or formyl substituent of heme a_3 in the time-resolved spectra.

The porphyrin ν₉ mode at 232 cm⁻¹ of CO-bound heme a_3 reflects the planarity of porphyrin and exhibited intensity change within 100 ns after CO-photolysis, and stayed constant at larger delay times. The ν_{Fe-His} mode appeared at 220 cm⁻¹ at 10 ns after CO-photolysis, which was 6 cm⁻¹ higher than that of equilibrium reduced state. The frequency remained unchanged until 100 ns and then showed downshift exponentially with time to 214 cm⁻¹ with a rate constant of 1.3x10⁶ s⁻¹. The bending mode of vinyl or formyl substituent of heme a_3 exhibited upshift from 435 to 436 cm⁻¹ later than 5.3 ms after CO-photolysis.

The picture of protein dynamics based on the results will be presented.

2TP1-06 水生動物トラフグヘムオキシゲナーゼによるヘム分解の特徴

Characterization of the heme degradation by heme oxygenase from aquatic animal, *Takifugu rubripes*

Taiko Migita (1), Xuhong Zhang (2) ((1) *Yamaguchi University*; (2) *Yamagata University*)

Heme oxygenase (HO) catalyzes the α-meso-carbon specific degradation of heme into biliverdin IX_α, CO, and free iron through oxygen activation on the substrate heme itself in the presence of electrons. We report here the characterization of heme conversion by fish HO from *Takifugu rubripes* (puff fish), Pf HO, the first-obtained recombinant fish HO protein by use of an *E. Coli* expression system, where the expression vector was constructed with cDNA cloned for mRNA in the spleen of *T. rubripes*. The heme complex of Pf HO was characterized spectroscopically and the heme degradation mechanism was also investigated with UV-VIS and ESR spectroscopy. The homology of the amino acid sequence of Pf HO to that of rat HO1 (mammalian HO) is quite high (48.6 %) and the distal and the proximal helix parts of these proteins are almost identical. However, the pK_a of the heme-coordinated water molecule is larger than that of rHO1, that is consistent with higher content of the low spin alkaline form at pH 9.0 observed in the EPR spectrum. The g-values of the alkaline form of the ferric heme complex even show larger anisotropy, suggesting that the heme-pocket milieu is discriminating. Heme degradation in the presence of ascorbate occurred faster than that by rat HO1 and more amount of free-biliverdin was generated. The heme degradation also occurred in the NADPH/CPR coupled reactions. In this case, the produced biliverdin was also released from the heme pocket more easily than in rat HO1 reaction. The origins of these differences in Pf HO protein were addressed.

2TP2-01 ハロロドプシン NpHR の三量体構造は F150 残基の対称配置によ

2P-084 って安定化される

Stabilization of trimeric assembly of halorhodopsin, NpHR by C₃-symmetric geometry among F150 aromatic rings