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 ハロロドブシン 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

Takashi Tsukamoto (1), Takanori Sasaki (2), Kazuhiro Fujimoto (3), Takashi Kikukawa (4), Masakatsu Kamiya (2), Tomoyasu Aizawa (1), Keiichi Kawano (4), Makoto Demura (1) ((1) Faculty of Advanced Life Science, Hokkaido University.; (2) School of Science and Technology, Meiji University.; (3) Graduate School of Science, Kyoto University.; (4) Faculty of Science, Hokkaido University.)

Halorhodopsin from *N. pharaonis* (NpHR) acts an inward-directed, light-driven Cl^- pump, and forms a homo-trimer in the archaeal lipid bilayer. Recombinant NpHR expressed in *E. coli* cells is known to assemble into the trimer similar to archaeal lipid system. In the previous study, it was found that F150 residue, which was estimated to locate at the trimer interface, played the important role for trimerization.

The purpose of this study is to elucidate the mechanism of structural stabilization of NpHR trimer by F150. To achieve this purpose, we carried out the F150Y, F150W and F150A site-specific mutation work, measurement of visible CD spectra and size exclusion chromatography in the DDM solubilized system. From these experiments, it was suggested that the F150W mutant could dissociate to the monomer homogeneously. Only F150A mutant was capable of dimer formation. On the other hand, QM and MM methods were applied to the structure refinement and the calculation of interaction energy of F150 aromatic rings in the trimer. According to this result, it was suggested that multimeric conformation of protein is stabilized by $\text{CH}\cdots\pi$ interaction in the C_3 -symmetric geometry. In addition, the dispersion energy was the most dominant factor for $\text{CH}\cdots\pi$ interaction between three aromatic rings of F150. It was cleared that wild-type NpHR is the most and F150W mutant is the worst stable. In conclusion, we succeeded in revealing the mechanism of structural stabilization by combination of these methods.

2TP2-02 DMPC、DPPC 二重層に再構成したバクテリオロドプシンの可視領域における円二色性測定

2P-085 Circular dichroism measurement of bacteriorhodopsin reconstituted in DMPC and DPPC bilayer in visible region

Lumi Negishi (1), Taku Kitoh (1), Yasunori Yokoyama (1), Masashi Sonoyama (2), Shigeki Mitaku (1) ((1) Department of Applied Physics, Graduate School of Engineering, Nagoya University; (2) Department of Applied Chemistry and Chemical Biology, Graduate School of Engineering, Gunma University)

It is known that reconstituted bacteriorhodopsin in DMPC undergoes the melting of 2D-crystal coupled with the lipid bilayers gel-liquid crystal transition. The detailed study of phase equilibria in lipid bilayers has revealed that there are three types of phase transition; main transition, pretransition and subtransition. However, the relationship between the three types of transitions of lipid bilayers and the melting of 2-D crystal of bacteriorhodopsin has not been revealed yet. In this work, we have monitored the melting of a 2-D crystal of reconstituted bacteriorhodopsin by CD spectra in visible region. "Exciton coupling" of the CD spectra in the visible region indicates the crystalline lattice in bacteriorhodopsin, whereas the single positive peak represents the melted state of bacteriorhodopsin. For studying the temperature dependence of the melting of the 2-D crystal, we measured the CD at 580 nm. The CD at 580 nm abruptly changed from the negative to the positive values around the pretransition, indicating that the melting of 2D-crystalline array occurred. It is interesting that the melting of bacteriorhodopsin crystal is coupled with the transition from the gel to the ripple phases and not the ripple-liquid crystal transition. We found another abrupt change in the visible CD spectrum around the subtransition temperature. We will discuss the mechanism of the coupling with the change in the melting of bacteriorhodopsin crystal and the lipid bilayer transition.

2TP2-03 細胞溶解毒素の自発的な α ヘリックス- β バレル変換における α ヘリックスのアンフォールディングと β ストランドの整列化の解析

2P-079 The Spontaneous α -Helix to β -Barrel Transition of a Pore-Forming Protein: Timing of Helix Unfolding and β -Strand Alignment

Takehiro Sato (1), Tweten Rodney K. (2), Johnson Arthur E. (1) ((1) Texas A&M Health Science Center; (2) University of Oklahoma Health Sciences Center)

Perfringolysin O (PFO), a bacterial pore-forming toxin, is secreted as a soluble protein monomer that binds to a cholesterol-containing mammalian membrane and oligomerizes into a prepore complex of ~36 monomers. The prepore complex then spontaneously creates a β -barrel pore (diameter ~300 Å) by the cooperative insertion of two transmembrane β -hairpins (TMHs) per monomer. Since specific residues of neighboring monomers are located in close proximity when TMHs form a β -barrel, the juxtaposition of these residues can be detected by cysteine substitution and intermolecular disulfide formation. Thus, to determine the timing and nature of β -barrel formation, the close juxtaposition of TMHs from adjacent monomers was examined chemically.

We have prepared mono-cysteine mutants of PFO along the entire lengths of two

β -strands, β 1 and β 4, that hydrogen bond to each other in the β -barrel. Each β 1 mutant was mixed with each β 4 mutant to form a pore at 37°C and then oxidized. As expected, each β 4 mutant formed a disulfide crosslink to only one of the β 1 mutants, thereby showing that adjacent β -strands are precisely aligned in the β -barrel. We then examined whether β 1 and β 4 crosslink when PFO molecules form a prepore complex at low temperature on the membrane surface. Under these conditions, a Cys no longer crosslinks only a single Cys in the other strand, but also formed disulfides with nearby Cys substitutions. Thus, in the prepore complex, both TMH1 and TMH2 are fully extended. However, the β -strands are still in flux and moving relative to each other.

2TP2-04 KCNQ1 チャネル複合体ストイキオメトリーの密度依存的变化

2P-179 Density-dependent changes in stoichiometry of KCNQ1 complex

Koichi Nakajo (1), Maximilian Ulbrich (2), Yoshihiro Kubo (1), Isacoff Ehud (2) ((1) National Institute for Physiological Sciences; (2) University of California, Berkeley)

Many membrane proteins form multi-molecular complexes. Because the composition of a molecular complex may define the functional properties, it is important to determine the stoichiometry. KCNQ1 encodes a voltage-gated potassium channel α subunit, and like other voltage-gated potassium channels, four KCNQ1 subunits form one ion channel. KCNQ1 channel properties are drastically changed by co-expression of auxiliary subunit KCNE1. To determine how many KCNE1 subunits bind to KCNQ1, we expressed GFP-tagged KCNQ1 and KCNE1 in *Xenopus* oocytes and determined the subunit numbers by counting bleaching steps from single fluorescent spots with TIRF microscopy (Ulbrich and Isacoff, 2007, 2008). We first counted the bleaching steps from GFP-tagged KCNQ1 protein and observed 36 % spots showing four bleaching steps. This result confirms that KCNQ1 actually forms a tetramer. Next we counted the bleaching steps from GFP-tagged KCNE1 subunits with co-expressed mCherry-tagged KCNQ1. We observed at maximum four bleaching steps from KCNE1, suggesting that up to four KCNE1 subunit can bind to KCNQ1. Moreover, the number of KCNE1 subunits in each KCNQ1 complex seems to be dependent on the relative density of KCNE1 against KCNQ1; when KCNE1 density was low, only 4% of spots showed four bleaching steps, while 16% of spots showed four bleaching steps when KCNE1 density was relatively high. Our results indicate that the stoichiometry of KCNQ1-KCNE1 is not strictly determined but flexible and density-dependent.

2TP2-05 Trans-membrane delivery of semiconductor quantum dots in living cells

Vasudevanpillai Biju (1), Abdulaziz Anas (1), Tetsuya Okuda (1), Tamitake Itoh (1), Mitsuru Ishikawa (1) ((1) Health Technology Research Center, National Institute of Advanced Industrial Science and Technology (AIST), 2217-14 Hayashi-Cho, Takamatsu, Kagawa 761-0395)

Semiconductor quantum dots are promising materials for in vivo imaging and photodynamic therapy owing to their exceptional photostability and size-tunable luminescence color. Thus, efficient delivery of bioconjugated quantum dots in living cells and unraveling the mechanism underlying the intracellular delivery are of great significance. Herein we selected an insect neuropeptide, namely allatostatin (AST1, APSGAQRLYG FGL-NH₂), as a carrier-peptide for quantum dots, conjugated it with quantum dots, and investigated the intracellular delivery of the peptide-conjugated quantum dots in living A431 and 3T3 cells using flow cytometry and fluorescence microscopy. We show that multiple pathways such as clathrin-mediated endocytosis, receptor-mediated endocytosis, and transduction are necessary to account for the intracellular delivery of quantum dots, among which clathrin-mediated endocytosis is the most important. The intracellular delivery of quantum dots was suppressed considerably when the regulatory enzyme phosphoinositide 3-kinase was inhibited with wortmannin and blocked the formation of clathrin-coated vesicles, indicating clathrin-mediated endocytosis. In parallel, we characterized the clathrin-mediated endocytosis of quantum dots by colocalizing clathrin heavy-chain antibody with quantum dots. Also, we investigated the roles of receptor-mediated endocytosis and charge-based cell penetration on the intracellular delivery of quantum dots.

2TP3-01 集団ダイナミクスにおけるノイズ強度のシステムサイズ依存性はネットワーク構造が決定する

2P-254 Structure of networks determines the system size dependency of noise intensity in collective dynamics

Naoki Masuda (1), Yoji Kawamura (2), **Hiroshi Kori** (3) ((1) University of Tokyo; (2) JAMSTEC; (3) Ochanomizu University)

Purpose: We consider a collective dynamics in a network of noisy cells. Such a system describes synchronous dynamics of biological tissues, including the master clock of our circadian rhythms (coupled clock cells) and the heart pacemaker (coupled cardiac cells of the sinoatrial node). From the central limit theorem, one could expect that the reliability of collective dynamics increases with the number of cells and that the fluctuation of collective dynamics scales as