of the export process. However, the efficiency of finding the basal body with the FliH/FliI complex attached is still low. The search for optimum mixing ratio and solution conditions for structural analysis by electron tomography of the basal body complex at different stages of the export process is in progress.

3P-071 重水素標識を利用した中性子小角散乱法によるプロテアソーム構成タンパク PA28 および α 7 サブユニットの溶液構造解析

Solution structural analyses of PA28 and α 7 subunit of the 20S proteasome by SANS in conjunction with deuterium labeling techniques

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Small-angle neutron scattering (SANS) is a very powerful tool to obtain the structural information of large protein complexes, since it allows discrimination between protonated and deuterated subunits. The objective of this study is to understand the assembly mechanisms of proteasome complexes by SANS analyses of PA28 and α 7 subunit of the 20S proteasome. Although the crystal structure of the homo oligomer of α subunits of PA28 (PA28 α) has been resolved, they actually form a hetero-oligomer together with homologous β subunits in vivo and its packing structure remains to be elucidated. On the other hand, the α 7 subunit of the 20S proteasome forms a self-assembling ring-like structure, which, however, remains to be characterized in detail. These proteins were produced in *E. coli* and subjected to SANS measurements in solution. Our simulation of the SANS profiles based on the previously reported crystallographic data revealed that PA28 assumes a 14mer in which the heptameric rings are dimerized through their interfaces, which are also used for the interaction with the 20S proteasome. Furthermore, the subunit arrangement in the hetero-oligomer was characterized by a contrast variation method of SANS using deuterated α subunits assembled with protonated β subunits. The SANS analysis also revealed that the α 7 subunits of proteasome form a homo-heptameric rings that are stacked back-to-back. Subunit exchange process of this α 7 oligomer in solution will be discussed on the basis of SANS data obtained by the deuteration technique.

3P-072 バルク脂質膜表面積を再現する膜タンパク質系の分子動力学シミュレーション法

Development of the method reproducing the bulk lipid surface-area in the molecular dynamics simulation of the protein-membrane complex

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In the all-atom molecular dynamics (MD) simulations of lipid bilayers or membrane proteins in the bilayers, a surface area per lipid is one of the most important parameters. In the simulations of lipid bilayers, the surface area is taken from the experimental value of the bulk membrane system and fixed to the value during the simulation in most cases. However, this approach, which is called as NPAT ensemble, cannot be directly applicable to the simulations of membrane proteins, because no experimental values for the surface area around the proteins are measured.

In this study, we propose a new method to reproduce a structural feature of bulk membrane, especially a surface area of lipid, in the MD simulation of membrane proteins. Our method is based on the combination of NPT and NPAT ensembles, where the lipid surface area was calculated by the Voronoi tessellation. We performed all-atom model MD simulations of the SecY protein-conducting channel and Ca²⁺-ATPase in phospholipids and analyzed the lipid surface area. From the simulation, it was found that when we used the usual modeling and equilibration method, the lipid surface area was close to that of the bulk lipid but slightly greater than the experimental value. In contrast, our proposed method could reproduce the exact surface area of bulk lipids. These results suggest that by using our method we can perform a more reliable simulation of the membrane protein system.

3P-073 逐次モンテカルロ法による FRET 時系列解析

Analysis of single-molecule FRET trajectories by sequential Monte Carlo method

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Förster resonance energy transfer (FRET) is a powerful method for determining various mechanistic properties of biomolecules. However, except for the simple cases of well-separated, low-noise, and limited to only a few states, it is usually

difficult to determine the configurational states and the transition rates between them from FRET trajectories.

The general state space hidden Markov models have opened a possibility to analyze such a complex time series. With the help of the recent advances in computer power and sampling techniques, it has become possible to determine the hidden states of the high-dimensional models even from low-dimensional information. In this study, we propose the use of the sequential Monte Carlo method to identify the conformational changes of protein based on FRET trajectories. We applied the method to the high-resolution single-molecule FRET trajectories (J. A. Hanson et al., *PNAS* 2007) of the domain movements in adenylate kinase.

3P-074 *Paracoccus denitrificans* チトクロム c 酸化酵素の大腸菌由来無細胞系での合成

Synthesis of *Paracoccus denitrificans* cytochrome c oxidase in the *E. coli* cell-free system

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The proton pumping mechanisms of bovine heart and *P. denitrificans* cytochrome c oxidase (CcO) have been advocated from the fine X-ray structural analyses and structure-based mutagenesis studies. However, to clarify the reaction mechanism, changes in protonation state and polarity of the amino acid functional groups at the proton pumping pathway should be examined during the proton pumping process by time-resolved infrared spectral measurements. The spectral data could be assignable by site-specific stable isotope labeling of the functional amino acid residues, which is currently achievable only by the cell-free protein synthesis. We have already succeeded in the synthesis of *P. denitrificans* CcO by an *E. coli* cell-free system. Here, we report kinetic constant (kcat) of the synthesized protein, which were 140 sec⁻¹ comparable to 200 sec⁻¹ of the authentic enzyme. This result is consistent with normal Soret/vis spectrum of the synthesized protein. In our system, efficiency of functionalization for the synthesized protein is still less than 1 %. In an attempt to increase the efficiency, *E. coli* cell membrane fraction, where synthesized proteins are assembled in our cell-free system, were enriched with an assembly factor Surf1 to facilitate the CcO assembly. However, noticeable increase in the efficiency was not obtained similarly to the previous result of enrichment of YidC that accelerates membrane insertion, suggesting that the efficiency is limited by neither the assembly- nor membrane insertion-step.

3P-075 基質カンファー結合によるチトクロム P450cam の構造変化

Substrate *d*-camphor binding induces structural change of cytochrome P450cam

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Cytochrome P450cam (P450cam) is a thiolate-heme containing monooxygenase that catalyzes the regino- and stereo-selective hydroxylation of *d*-camphor to produce 5-*exo*-hydroxycamphor. The reaction is initiated by the binding of camphor to the resting state of the enzyme. *d*-Camphor binding excludes a cluster of waters at the active site, raising the redox potential of heme by an extent that allows efficient reduction by the electron transfer system. This binding was reported to involve no significant structural changes in the protein. Here, two ferric P450cam structures partially complexed with *d*-camphor (occupancies were 65 and 80 %, respectively) were determined by X-ray crystallography at 1.30–1.35 Å resolutions, revealing the structures of the substrate-free and -bound forms. In the substrate-free state, the active site hydroxyl group of Tyr96 forms a hydrogen bond (H-bond) with Thr101 side chain. Upon substrate binding, the hydroxyl group of Tyr96 forms a H-bond with the carbonyl group of *d*-camphor, while the Thr101 side chain rotates around its C α -C β bond by breaking the H-bond with Tyr96 and forms a H-bond by donating the hydrogen with the peripheral heme-6-propionate. This bonding contributes to the redox potential change together with spin state change of heme iron from low to high occurring associated with water exclusion from the active site.

3P-076 チトクロム P450cam の活性部位からの水クラスター排出機構

Mechanism of the water exclusion from the active site of cytochrome P450cam

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A cluster of waters (6 molecules) located at the active site of P450cam is not accessible from the protein surface. However, this water cluster is expelled from the site upon substrate camphor binding, which changes the spin state of heme iron from low to high. Thus, the water exclusion from the active site is essential to the catalytic reaction. We have proposed that Asp297-Glu322-heme-7-propionate hydrogen bond networks constitute a gate controlling water passage to the bulk water. Mutation of Asp297 to bulkier Leu lowered the camphor binding affinity

by over 10,000-fold, suggesting significant stabilization of the bound waters. X-ray structure of the Leu-mutant at 1.6 Å resolution revealed that Leu side chain blocks water passage through the gate in either substrate-free or -bound forms. These results support our proposal. Here, Asp297 was mutated to less bulky Ala to produce a space corresponding to that would be generated by conformational change of Asp297 side chain occurring during the water exclusion. This mutation did not change significantly the camphor binding affinity, indicating normal water exclusion. X-ray structure of the Ala-mutant at 1.6 Å resolution demonstrated that Phe87, contacting with Asp297 and Tyr96 in the wild type protein, changes its side chain conformation in a way to generate a space for camphor passage. Bound camphor forms a hydrogen bond with Tyr96. The present results suggest that conformational change of Phe87 to allow camphor passage induces that of Asp297, which facilitates the gate opening.

3P-077 インドールアミン 2、3 ジオキシゲナーゼの酸素同位体感受性共鳴ラマンバンドの検出

Detection of Two Oxygen-Isotope Sensitive Resonance Raman Bands for Human Indoleamine 2,3-Dioxygenase

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Indoleamine 2,3-dioxygenase (IDO), found in mammals, catalyzes direct incorporation of two oxygen atoms into L-tryptophan in kynurenine pathway in which this reaction is the initial and the rate-limiting step. IDO is a soluble enzyme containing a heme B as its reaction center. Sugimoto *et al.* discussed the mechanism of dioxygenase in terms of the crystal structure of recombinant human IDO (rhIDO) (1). No structural information about the catalytic intermediate of any IDO has been reported. Resonance Raman (RR) spectroscopy is one of the most powerful tools to study the structure of the active site of heme containing enzymes. The RR spectra of rhIDO with and without substrate have been reported (2) yet in the static forms. Here, we report the RR spectra of rhIDO in oxygenated and oxo forms. The RR spectrum of oxygenated rhIDO exhibits the oxidation state marker band (ν_4) at 1375 cm^{-1} which is higher compared to that of the ferric form at 1371 cm^{-1} . The Fe-O stretching mode for the oxygenated rhIDO is found at 569 cm^{-1} in $^{16}\text{O}_2$ -bound form, which has been confirmed by an isotopic shift to 541 cm^{-1} in $^{18}\text{O}_2$ -bound form at pH 8.0. When 2 mM L-tryptophan is added to 10 μM of oxygenated rhIDO, the ν_4 band is found at 1375 cm^{-1} , whereas the Fe-O stretching mode is found at 798 cm^{-1} for $^{16}\text{O}_2$ -rhIDO, shifting to at 762 cm^{-1} for $^{18}\text{O}_2$ -rhIDO. According to the region of the oxygen-isotope sensitive RR band, the state of the heme can be concluded to an oxo form. This species has absorption maxima at 410, 544, 577 and 593 nm compared to those found in oxygenated rhIDO at 413, 542 and 577 nm.

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3P-078 結晶中の完全酸化型ウシ心筋チトクロム酸化酵素の酸素還元中心の解析

Characterization of the O_2 reduction site of crystalline fully oxidized bovine heart cytochrome *c* oxidase.

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It has been well established that bovine heart cytochrome *c* oxidase shows two types the fully oxidized forms under nonturnover conditions, namely, fast and slow forms, characterized by cyanide binding rate and the Soret band position and that the fast form is enzymatically active. The physiological significance of the slow form is still not clear. Bovine heart cytochrome *c* oxidase purified with our procedure for preparing crystals for X-ray diffraction experiments shows the Soret band at 423 nm and reacts with cyanide showing the half life time of the order of one second, indicating clearly that our enzyme preparation is in the fast form. However, the crystalline enzyme showed two peaks at 428 nm and 411 nm. Both band positions are clearly different from those of the slow and fast forms. The Soret band splitting are likely to be induced by strong protein-protein contacts in the crystal lattice which stabilize part of the multiple conformers of the enzyme in the solution or by the anisotropy in relative positions of the two hemes in the crystals.

In order to examine the possibilities, the crystals were dissolved in a neutral buffer containing decylmaltoside, to give its Soret band at 417 nm which is close to the Soret band position of the slow form. The Soret band gradually returned towards the position of the Soret band before crystallization. The results suggest that the dissolved enzyme preparation is not in the slow form. Or, the species with its Soret band at 417 nm seems to be an enzyme species which transiently appears. Extensive examinations of factors affecting the absorption spectral changes after dissolving the crystals are under way, in order for characterization of the O_2 reduction site of the bovine cytochrome *c* oxidase in the crystal lattice.

3P-079 化学修飾ヘモグロビン酸素結合機能の蛋白濃度依存性

Tetramer-dimer equilibrium of chemically modified hemoglobin as studied by protein concentration-dependences of the oxygen equilibrium curve

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Vertebrate hemoglobin is in an equilibrium between the $\alpha_2\beta_2$ tetramer and the $\alpha\beta$ dimers. This equilibrium is shifted toward dimers upon dilution of the Hb solution and also upon oxygenation when the several intersubunit salt-bridges formed in deoxyHb are ruptured. The chemical modification of the sulfhydryl group of cystein-93(F9) β with *N*-ethylmaleimide (NEM) causes the breakage of the salt-bridge in deoxyHb between the C-terminal histidine imidazole group and the γ -carboxyl group of aspartate-94(FG4) of the same β chain. This breakage will, in turn, reduce the stability of another salt-bridge between the β chain C-terminal carboxyl group and the ϵ -amino group of lysine-40(C5) of the α chain of the $\alpha_1\beta_2$ pair. Thus, it is expected that this modification will enhance the dissociation of Hb tetramer into dimers. In the present study, we measured oxygen equilibrium curves for native Hb and NEM-treated Hb in a wide range of protein concentration (1 to 1000 μM on a heme basis) and analyzed them to estimate the tetramer-to-dimer dissociation constants. The results indicated that the modification with NEM does not enhance the dissociation into dimers, contrary to the expectation.

3P-080 シトクロム *c* におけるシトクロム *c* 酸化酵素との電子伝達反応に重要なアミノ酸残基の検討

Identification of amino acid residues responsible for the electron transfer reaction with cytochrome *c* oxidase in cytochrome *c*.

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Cytochrome *c* (Cyt *c*), one of the heme proteins in the mitochondrial respiratory chain, transfers electrons between cytochrome *b*₅ and cytochrome *c* oxidase (CcO). While the electron transfer (ET) to CcO is the crucial step for the four-electron reduction of molecular oxygen to water molecules, the detailed regulation mechanism for the ET reaction has not yet been fully understood. Recently, we have measured the ^1H - ^{15}N HSQC spectra for Cyt *c* in the absence and presence of CcO, showing that some Lys and Ile residues are involved in the binding site of Cyt *c* with CcO and suggesting that the specific electrostatic and hydrophobic interactions play essential roles in the formation of the ET complex. To get further insights into the interactions between Cyt *c* and CcO, we focused on some Lys and Ile residues located on the proposed binding site and prepared four Lys mutants (K7,8L, K13L, K79L, K86,87L) and three Ile mutants (I9A, I11A, I81A). Michaelis-Menten analysis for the ET reactions from Cyt *c* to CcO revealed that all of the Lys mutants have large K_M (1.9 - 10.9 μM), compared to wild-type Cyt *c* (0.92 μM) and the negative control mutant K39L (0.57 μM), while the mutations of Ile resulted in the minor perturbation of K_M . The prominent increases in K_M for K13L (10.9 μM) and K86,87L (5.6 μM) clearly indicate that these Lys residues are key residues for the ET complex formation and the electrostatic interactions mediated by these Lys residues would fix the relative positions of heme and the electron entry site of CcO to facilitate the ET reaction from Cyt *c* to CcO.

3P-081 コンピュータシミュレーションによる ABC トランスポーター・Sav1866 の薬剤排出機構の解明

Computer simulation reveals the drug transport mechanism of ABC transporter Sav1866

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The ATP-binding cassette (ABC) transporters are integral membrane proteins which couple the energy of ATP hydrolysis with the translocation of chemically diverse substrates across the lipid bilayers of cellular membranes. ABC transporters are generally classed as either exporters or importers. For example, human ABC exporters, such as ABCB1 and ABCC1, contribute to multidrug resistance of cancer cells through the extrusion of cytotoxic compounds. Despite the various studies, however, a detailed transport system has remained elusive. Sav1866, a *S. aureus* homolog of human ABCB1, is one of the structure-determined ABC transporters. In this study, we applied atomic-level MD simulation and anisotropic network model (ANM) calculation to Sav1866 in order to understand its drug transport mechanism. From the results of ANM calculation, we found that Sav1866 potentially has some characteristic motions including an opening/closing motion in the transmembrane domain that could form a pathway for transporting a trapped drug. The principal component analysis for the MD trajectories also showed that such a motion is a dominant one in the ATP-