Three-dimensional reconstruction from electron microscopic images is one of the powerful protein structure analytical methods. This technique includes single particle analysis and tomography. Each technique has good and bad points, respectively. We, here, adopt random conical tilting method (RCT), which overcomes difficulty of the projection angle decision in single particle analysis and reduce the electron damage to specimens comparing with electron tomography. Therefore the reconstruction gives us reasonable three-dimensional maps although the maps have missing information and so attempted semi-automated image processing to shorten time to analysis and reduce person's loads. Thus we designed the image processing programs for the RCT under Eos we have developed and integrated them using 'make'. To test the abilities of our tool, we analyzed negatively stained electron micrographs of low-density lipoprotein receptor-related protein 6 (LRP6). As an outline, we corrected CTF of EM images, picked up single particles, classified them by cluster analysis and averaged particles. Only after we picked single particles in a non-tilted image, we could successfully pick the same particles from the tilted images automatically. We are now integrating RCT reconstruction programs to construct the three-dimensional automatically. We will, here, compare three-dimensional images reconstructed automatically from images with tilted different angles such as 45° and 60°.

2P091 Particle pickup method using local-similarity classification for single particle analysis, and its applications

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Single particle analysis is an integrated method of data processing for the construction of three-dimensional (3D) biological macromolecular structures from electron micrographs. Most of the steps in the data processing, such as classification, alignment, and 3D-reconstruction of particle images, have been automated. The computational burden of data processing has also been alleviated using high-speed parallel processing techniques. However, fully automated generation of article images from micrographs is still prevented by the large variety of statistical characteristics caused by conditions of the target specimen and the various parameter settings. We report on the performance of various methods and software for enhanced automation of the acquisition of particle data, i.e. particle pickup, and on new techniques to improve performance of existing methods. Among the new methods, an approach based on classification of local similarity was found to be the most promising. While the basic strategy of the method is simple, we encounter some difficulties in implementation, such as treatment of false positives. We will present key techniques for the implementation of a new method to increase the reliability of particle recognition in micrographs, detail its algorithms, and demonstrate its performance using various real micrographs.

2P092 タンパク質に結合した水分子の振動を水溶液中で測定する 1F1420 Detection of a protein-bound water vibration in aqueous solution

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Internal water molecules are considered to play a crucial role in the functional processes of proteins, but detection of protein-bound functional waters is not easy. We have shown that light-induced difference Fourier-transform infrared (FTIR) spectroscopy is a powerful tool to monitor functional water molecules in photoreceptive proteins such as rhodopsins [1]. In the measurements, we prepare hydrated films of proteins, by which water content in the sample can be significantly reduced. Then, low-temperature or time-resolved difference FTIR spectra provide water O-H (O-D in D₂O) stretching vibrations, which is assigned by use of isotope water (¹⁸O). This is an established method, and it is generally accepted that protein dynamics in hydrated films are similar to those in aqueous solution. But then, how about protein-bound waters? Detection of protein-bound waters in water, not in hydrated films, is a great spectroscopic challenge.

Here we report this trial using attenuated total reflection (ATR) FTIR spectroscopy. In the measurements, membrane proteins in lipids are attached on the ATR cell, and buffer solution fills the cell. Then, IR evanescent wave monitors vibrations of the sample in aqueous solution. In this study, we tested *pharaonis* halorhodopsin (HR), a light-driven Cl⁻ pump. We successfully observed water signal under weak hydrogen bond in the difference IR spectra between Cl⁻ and Br⁻. Environment of the observed water molecules will be discussed.

[1] Kandori, *Biochim. Biophys. Acta* 1460, 177 (2000).

2P093 高速 AFM による FACT のリン酸化された天然変性領域の観察

Observation of the phosphorylated intrinsically disordered region of FACT with high-speed AFM

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Proteins in eukaryotes often have intrinsically disordered (ID) regions. The functional importance of ID regions in biological processes was recently recognized. However, the dynamics and functional behavior of the ID regions remain unknown at the molecular level. Recently we have visualized the non-phosphorylated ID regions of *Drosophila melanogaster* FACT (facilitates chromatin transcription) expressed in *E. coli* (A. Miyagi *et al.*, *Chem. Phys. Chem.*, 2008). We also revealed that the phosphorylated ID region of FACT expressed in insect cells strongly interacts with the structured high-mobility group (HMG)-box domain, and masks its nucleosomal DNA binding elements (Y. Tsunaka *et al.*, *J. Biol. Chem.*, 2009). On the other hand, it is assumed that the interaction between the non-phosphorylated ID region and the HMG domain is weaker than that for the phosphorylated ID region. Therefore, we observed the phosphorylated ID region with high-speed AFM, and compared the shape and length between the phosphorylated and non-phosphorylated ID regions.

We used the phosphorylated FACT expressed in Sf9 cell and the non-phosphorylated mutant protein whose actual phosphorylation sites of ID regions are entirely replaced by Ala (10SA). As a result, we confirmed that the length of the non-phosphorylated ID region observed was longer than that of the phosphorylated ID region. This result suggests that the interaction between the non-phosphorylated ID region and the HMG domain becomes weaker, and thus the ID region is released from the structural domain.

2P094 高速 AFM による天然変性タンパク質 PQBP-1 と FliK の直接観察

Direct visualization of intrinsically disordered proteins PQBP-1 and FliK using high-speed atomic force microscopy

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Functional proteins without unique 3D structures are highly abundant in nature. These intrinsically disordered proteins (IDPs) are involved in a number of crucial biological functions. In the study of the heterodimeric facilitates chromatin transcription protein, we previously reported the first direct visualization of the thin and flexible structures of the ID regions by high-speed atomic force microscopy (HS-AFM) (Miyagi *et al.*, *ChemPhysChem*, 2008). To further investigate IDPs, we here perform HS-AFM observations of the two IDPs, polyglutamine tract binding protein-1 (PQBP-1) and FliK. PQBP-1 is a nuclear protein that interacts with proteins containing expanded polyglutamine repeats, RNA polymerase II and a spliceosomal protein U5-15kD. HS-AFM movies reveal that PQBP-1 consists of a long unstructured chain and a small folded core, consistent with the NMR study (Takahashi *et al.*, *BBA*, 2009). We will report the mechanical properties of the unstructured chain and the effect of U5-15kD binding on the ID structure. FliK has been suggested to be a molecular ruler determining the length of the flagellar hook. The N-terminus of FliK is predicted to have an ordered structure while the C-terminal half is predicted to have an ID region. HS-AFM movies, however, show a structure in which two globular domains are connected by a flexible linker. The molecular length is ~25 nm, which is shorter than the total length of the hook and rod (~90 nm). We will discuss about the role of FliK in the regulation of hook length.

2P095 新生蛍光タンパク質の発色団形成反応の速度論的解析 Kinetic study on the de novo chromophore formation of fluorescent proteins

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Green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* is one of the most widely used proteins in biological and medical research. The widespread use results from the unique ability of GFP to form chromophore without the assistance of external enzymes or cofactors except for molecular oxygen. Rates of the chromophore formation have been determined as rates of fluorescence reacquisition after chemical reduction of the mature chromophore. However, it is unclear in what way reoxidation rates are related to *de novo* chromophore formation. We developed an efficient method to study the *de novo* chromophore formation. GFPs with immature chromophore were synthesized in the reconstituted *in vitro* translation system supplemented with an O₂ scavenging enzymes. When the reaction mixture was diluted into