1P168 クライオ電子顕微鏡法による車軸藻ミオシンの高速移動分子メカニズムの解明
Elucidation of the molecule mechanism of Chara myosin's fast movement by electron cryomicroscopy

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Chara corallina class XI myosin is a motor protein, which is involved in cytoplasmic streaming of *Chara corallina*. The myosin has the fastest actin sliding velocity (~70μm/s) among all myosin classes known so far. To unravel the mechanism how Chara myosin moves so fast, the kinetic properties of the myosin have been analysed. These studies elucidated that Chara myosin has high ATPase activity. In addition, in its ATPase cycle, the ATP binding rate is extremely fast and the ADP release rate is dramatically accelerated by binding to actin(1000-fold). The sequence analysis and electron microscopy show that Chara myosin consists of a motor domain (motor domain : MD) that belongs to class XI, a neck region with six tandem IQ motifs that serve as binding sites for six light chains, an α-helical coiled-coil domain for dimerization, and a globular tail domain to bind to cargo. Nonetheless, its detailed structural features are not clear enough to understand the molecular mechanism of its kinetics.

Thus, we have analyzed the structure of Chara myosin by electron cryomicroscopy and 3D reconstruction. Then we obtained 3D maps of the myosin with/without ATP to find the nucleotide-binding cleft is open without nucleotide in binding actin filaments. We are now trying to acquire high resolution 3D-structures of Chara myosin MD without nucleotides and Chara myosin tightly bound to actin. We will here report the recent progress to clarify the relationship between structure and kinetic feature of Chara myosin.

1P169 タンパク質標識に用いる金ナノ粒子の作製法の開発
Development of a new method to synthesize functionalized gold nanoparticles for labeling a protein.

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Analysis of the detailed structure of protein complexes is very important to understand their functions. Especially, to identify a position of a sub-unit in a protein complex is desired. A local labeling in a protein using gold nanoparticles (Au NPs) is attractive because it is applicable to structural analysis by using transmission electron microscopy (TEM). The purpose of this study is to develop a new simple method to functionalize an Au NP surface with nickel-nitrilotriacetic acid (Ni-NTA) that selectively binds a polyhistidine-tag (His-tag). In this method, the Ni-NTA Au NPs were made by only the three steps. Firstly, Au NPs were synthesized by means of reduction of HAuCl₄ using trisodium citrate and tannin acid. Secondly, the Au NPs were functionalized with NTA group by simply mixing with C2-NTA that binds specifically to the Au NP surface with a reactive disulfide group. Finally, the mixed solution was passed through an ion-exchange column that had been previously charged with nickel ion to form Ni-NTA Au NPs. The Au NPs were highly homogeneous. The mean diameter was 4.7±0.5nm. The Au NPs mixed with kinesins that have the His-tag were observed with TEM to estimate affinity between them. TEM images show that the Au NPs are surrounded by the kinesins, which indicates that the Ni-NTA Au NPs preferentially bind the His-tag in the protein. In conclusion, the Ni-NTA Au NPs synthesized by our new method are useful for elucidating the position of the sub-unit containing the His-tag in a protein complex by using TEM.

1P170 金ナノ粒子を用いた細胞質ダイニン複合体の電子顕微鏡観察
Electron Microscopic observation of cytoplasmic dynein using gold nanoparticles.

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Cytoplasmic dynein walks along microtubule toward its minus end utilizing the energy from ATP hydrolysis, and has many basic and important roles in eukaryotic cells including mitosis, vesicle transport and cell migration. Cytoplasmic dynein is a huge protein complex having two domains, the head domain, and the tail domain. So far, many studies have investigated about the head domain, because it is a motor domain consisting of six AAA modules and a stalk, and the conformational change depending on the nucleotide

binding states was elucidated. On the other hand, the tail domain consists of N-terminal third of heavy chains, intermediate chains (IC), light intermediate chains (LIC), and light chains (LC), and is implicated in cargo binding. However, the detailed molecular structure of the tail domain remains to be unveiled. In this study, we used nitrilotriacetic acid (NTA) conjugated gold nanoparticles to label polyhistidine tag (His-tag) of recombinant proteins and observed them in a electron microscopy (EM) by negative staining. We identified the C-terminal positions of the IC and LIC in the tail domain. Based on our observations, we will propose a new model of the architecture of the dynein tail domain and discuss about its functions.

1P171 多機能 GFP タグで見えてきた哺乳類細胞におけるダイニン-ダイナクチンのダイナミックな動態
Dynamic behavior of dynein-dynactin in living mammalian cells visualized by multifunctional GFP tag

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Cytoplasmic dynein is a minus-directed microtubule (MT) molecular motor and plays an important role in various cellular functions. We have recently demonstrated cell cycle-dependent dynamic behavior of dynein in living mammalian cells, by visualization with a novel multifunctional GFP (mfGFP) tag (Kobayashi & Murayama, 2009). Dynactin is a large multisubunit protein complex that is essential for most types of dynein-mediated transport. However, it remains unclear how dynein-dynactin interaction is regulated. To investigate spatiotemporal patterns of dynein-dynactin interaction, we visualized dynactin in mammalian cells and compared its behavior with that of dynein. Dynactin subunit p50 was fused with mfGFP and stably expressed in HeLa cells. Isolation of p50-mfGFP co-purified with other dynactin subunits. In combination with nanogold labeling technology, location of mfGFP was mapped on the dynactin structure under negative staining electron microscopy. Thus, p50-mfGFP was successfully incorporated into dynactin complex. On live-cell imaging of interphase cells, dynactin was observed as discrete foci in the cytoplasm which were moving along with MTs. In mitosis, dynactin was localized at spindle MTs where it was frequently moving toward MT plus ends. It also existed at kinetochores and near cell cortex. Dual color imaging demonstrated that dynactin was colocalized with dynein in most cases. These findings suggest that dynein-dynactin interaction may stably occur throughout the cell cycle.

1P172 ミオシンフィラメント双極トラックに沿ったアクチンフィラメント滑り運動の活性化エネルギー
Thermal activation energy for bidirectional movement of actin filament along bipolar tracks of myosin filaments

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In vitro motility assays using bipolar myosin thick filaments demonstrated that actin filaments were capable of moving in both directions along the myosin filament tracks. The movements, however, were slower in the direction away from the central bare zone than towards the zone. To understand the mechanism underlying these different motile activities depending on the direction of the movements, we have examined the effects of temperature on the velocities of the movements in the both directions along the reconstituted myosin filaments. Activation energies of the movements were determined by the Arrhenius plot at high and low concentrations of ATP. As a result, the thermal activation energy of the movement away from the central bare zone was significantly higher than that of the movement toward the zone. Given that the backward movement in the direction away from the central zone would cause the myosin heads to be constrained and increase the stiffness of the cross-bridge, these results may provide evidence that elastic energy required for the cross-bridge transition is supplied by thermal fluctuations.

1P173 1分子イメージングによるトランス-トランスレーションにおけるSmpBのC末端の機能解析
Functional analysis of the C-terminus region of SmpB in trans-translation by single-molecule imaging

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In Eubacteria, the stalled ribosomes are rescued by trans-translation mecha-

nism, in which multiple components such as tmRNA, SmpB and EF-Tu are facilitated in the process. Our previous results show that GTP hydrolysis stimulated by SmpB is necessary for tmRNA A-site entry and subsequent tagging of specific amino acids. In these experiments C-terminus truncation of SmpB has negative effect on GTP hydrolysis of EF-Tu and tagging ability of tmRNA, suggesting that SmpB coordinates with each process. To elucidate the mechanism precisely, it is important to understand the interaction of each component, especially the order of binding and unbinding or the dwell time of each event. Here we apply single molecule fluorescence imaging *in vitro* to elucidate the trans-translation mechanism.

Ribosomes were specially immobilized onto glass surfaces and the GTP hydrolysis reaction in trans-translation occurs in adjustable PURE system using recombinant factors. The interaction of tmRNA and ribosome was monitored by fluorescence dye labeled tmRNA or TLD, tRNA-like domain of tmRNA. Immobilized ribosome subunits were also labeled by fluorescence dye. The association of tmRNA and ribosome was measured by colocalization of tmRNA and ribosome. With EF-Tu, interaction of tmRNA and ribosome was observed. And with the loss-of-function mutations EF-Tu (G222D), tmRNA did not interact with ribosome.

1P174 新規偏光顕微鏡による単一分子の3次元配向測定 Three-dimensional orientation measurements of single molecules by newly developed polarized fluorescence microscopy

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Single molecule fluorescence techniques have become increasingly useful for observing the dynamic properties of single molecules. One such property is a protein's orientation, which is necessary to measure the protein's three-dimensional motility. We have developed new microscopy for determining such orientations based on the principals of polarization analysis proposed by Fourkas. This method requires only that one collect fluorescence counts from a single molecule at three different polarizations followed by a simple mathematical calculation to yield the three-dimensional orientation. In this method, a relatively small number of photons are sufficient for reliable orientation measurements. This fact should also decrease the time scale needed to determine the orientation of a given fluorophore. Here, we demonstrate the axial rotation of actin filaments sliding over myosin molecules fixed onto a glass surface by measuring the polarization of individual rhodamine phalloidin fluorophores sparsely bound to the filaments. This new microscopy will have particular applicability for various biophysical studies interested in a wide range of dynamic processes that depend on single molecule orientation dynamics.

1P175 プリズムによる3次元光学トラッキング技術の応用 / 1分子を 1B1255 対象にした光ピンセット操作と立体視表示 Application of 3-D prismatic optical tracking: single-molecule optical tweezers approach and stereoscopic 3-D view

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We have developed a new method for tracking single particles in three dimensions under a conventional microscope by dividing light in two components using a wedge prism. This method, 'three-dimensional prismatic optical tracking' termed *tPOT*, is here combined with optical tweezers to quantify molecular scale 3-D force in motor proteins. Precise calibration of trapping force to bead displacement with sub nanometer scale enables us to determine the force exerted not only in *xy*-plane direction but also along optical axis (*z*-axis). With this experimental set-up, we applied force to rotary motor F₁-ATPase that showed rotation on a glass surface. Our research group previously used the procedure in which the objective was displaced to impose the load on single molecules held between the trapped bead and the glass, which frequently caused the measurement error because of drifting motions of the specimen. This method has been modified as follows; 1) the trap center is displaced with time by moving the focusing lens along *z*-axis; 2) spring constant of the trap is increased via the adjustment of the laser power. In either case positions of the stage and the objective were fixed and thus repetitive measurements were reliably performed. We also demonstrate an application of *tPOT* to a variety of microscopy techniques taking advantage of two components of light as a parallax view. Stereoscopic 3-D view on a monitor is reconstructed by two eyes as a real-time image captured under a conventional microscope with high-magnification.

1P176 新規蛍光性 ATP アナログと ATP 駆動型分子モーターとの相互作用

Interaction of novel fluorescent non-nucleotide ATP analogue with ATP-driven molecular motors

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Previously numerous ATP analogues have been synthesized for analysis of ATPases, such as myosins or kinesins. Ribose-modified ATP analogues are known to be 2' and 3' isomers mixture. To avoid mixture of isomers, we designed and synthesized non-nucleotide fluorescent ATP-analogue, *N*-methylanthraniloyl amino ethyl triphosphate (MANTTP) which similar structure to the non-nucleotide ATP analogue 2-[(4-azido-2-nitrophenyl) amino] ethyl triphosphate (NANTP). It is known that NANTP are good substrate for skeletal myosin and induces actin gliding *in vitro* motility assay. Excitation and emission maximums in the fluorescence spectrum MANTTP were 374nm and 430nm, respectively. MANTTP was hydrolyzed by conventional kinesin and skeletal myosin, and induced dissociation of acto-myosin. The MANTTPase of myosin was significantly activated by actin. The ADP form of MANTTP showed the formation of skeletal muscle myosin-MANTDP-BeFn complexes which mimic the transient state in ATPase cycle. The fluorescent intensity of MANTTP itself does not change on binding to myosin ATP binding site, however, MANTTP showed significant FRET between intrinsic tryptophan residue of skeletal muscle myosin and MANTTP. The binding of MANTTP to myosin can be observed as fluorescence increase on the stopped flow system. The second-order rate constant for MANTTP first binding to myosin is $0.30 \times 10^{-6} \text{ M}^{-1} \text{ s}^{-1}$. It was shown that the novel fluorescent ATP analogue is applicable to the kinetic studies on ATPases.

1P177 Observation of conformational change of β subunit in $\alpha_3\beta_3$ subcomplex of F₁-ATPase by high-speed AFM

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F₁-ATPase is a rotary motor enzyme driven by ATP hydrolysis. The rotational motion of the central shaft γ subunit is considered to be driven by conformational change of the three catalytic β subunits. The domain motion of the each β subunit with three steps, which synchronizes with the substep rotation of the γ subunit has been observed using advanced TIRF[1]. A recent study also showed that F₁ mutant with a largely truncated γ could rotate in the correct direction [2], implying the possibility that the cooperative conformational change of the β subunit occurs even in the $\alpha_3\beta_3$ subcomplex without γ . Here we applied high-speed AFM to directly observe the conformational change of the β subunit in $\alpha_3\beta_3$ complex.

High-speed AFM images clearly resolve each subunit of the $\alpha_3\beta_3$ complex fixed on a mica substrate. The subunits appear to be arranged in six-fold symmetry and the whole shape does not change without ATP. At lower ATP concentrations (1-3 μM), AFM movies shows the conformational change in the three alternate subunits in the $\alpha_3\beta_3$ complex which are likely to be the catalytic β subunits. Furthermore, the conformational change of the three subunits takes place in counterclockwise rotation. These results indicate that the conformational change of the three β subunits occurs cooperatively and unidirectionally even without the γ subunit.

[1] T. Masaie et al. (2009) *Nat. Struct. Mol. Biol.* **15**, 1326

[2] S. Furuike et al. (2008) *Science* **319**, 955.

1P178 高速原子間力顕微鏡でタンパク質の化学-力学変換を可視化するための PEG-ATP の合成

Synthesis of PEG-labeled ATP to directly visualize the mechanochemical coupling in proteins by high-speed atomic force microscopy

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We have developed and improved a high-speed atomic force microscopy (HS-AFM) that enables us to directly visualize activity of biological molecules in buffer conditions with sub-nanometer resolution at rate of several frames per second (Ando et al., PNAS, 2001; Ando et al., *Pflugers Archiv. Eur. J. Physiol.*, 2008). Thus far, HS-AFM imaging has elucidated several dynamic processes performed by proteins including bacteriorhodopsin, myosin-V and chaperonin GroEL-GroES. However, because this microscopy cannot visualize small molecules such as nucleotides, the mechanochemical coupling in proteins has not been structurally studied yet. To realize this study, we here synthesized PEG-labeled ATP in which EDA-ATP was covalently modified by various lengths NHS-PEG. The purity of PEG-labeled ATP was more than 95%. To test the function of PEG-labeled ATP, we examined actin-gliding assay of myosin II with TIRF microscopy. The average translocation velocity of actin filaments obtained by PEG5000-labeled ATP was similar to that obtained