stator that conducts protons, and the C ring, which functions as the rotor. The mechanism of energy coupling between proton influx and torque generation remains unknown. To understand the energy coupling mechanism, the proton influx coupled with flagellar motor rotation should be measured with high spatial and temporal resolution. We have developed an intracellular pH imaging system that can measure local pH near the motor using a pH-sensitive green fluorescent protein, pHluorin, as a ratiometric fluorescent probe (Miesenböck et al., 1998). This system can measure the intracellular pH distribution at a pH resolution of 0.02 at every 3 ms. We carried out single-cell pH imaging upon shifting external pH from 7.0 to 6.0 in the presence of a membrane-permeant weak acid, benzoate, and observed an intracellular pH shift from 7.3 to 6.0 within 3 sec. We then measured intracellular pH and flagellar motor rotation simultaneously. Upon a downward pH shift, the direction of motor rotation switched from counterclockwise to clockwise within 10 ms. We also fused pHluorin to flagellar motor proteins to measure the pH in the cytoplasm around the motor. Simultaneous measurements of local pH and motor rotation are under way.

3P-124 自己調節するキネシン運動の可視化

Visualization of self-regulated kinesin motility.

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Kinesin is a motor protein that transports various cargoes and organelle along microtubules in cells. It is composed of a motor domain, which hydrolyzes ATP; a tail domain binding, which binds to the cargo; and a long coiled-coil region, which connects the motor and tail domains.

Because its primary responsibility is transport, it is important to understand the mechanism for kinesin motility regulation. We therefore observed the self-regulated behavior of reconstituted kinesin at the single molecule level using a recombinant motor domain and a recombinant tail domain *in vitro*. Furthermore, we directly observed the role of the tail domain in living cells.

We found that the tail domain did not affect motility velocity but did affect the binding of the motor domain to the microtubule both *in vitro* and in the cell. We propose a simple model for the mechanism of the self-regulated kinesin motility in accordance with these results.

3P-125 アクチンへの G146V 変異は骨格筋ミオシンとの相互作用中の力発生を阻害する。

G146V mutation in actin inhibits force generation during interaction with skeletal myosin.

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To unveil the possible roles of structural changes between the large and small domains of actin in myosin movements, we have been characterizing mutant actin in which Gly146 located between the two domains was replaced by Val. Last year, we reported that gliding velocity of G146V filaments on skeletal (sk) HMM surfaces was greatly decreased (by 80%). Force production in multiple motors assay was also inhibited to a similar extent, but parallel decrease of actin activated sk S1 ATPase activity was not observed. In contrast, those filaments moved and produced force normally on myosin V.

Thus, we are currently performing experiments to understand the molecular mechanism of this myosin class-specific inhibition of movement by G146V actin. Stopped-flow analyses showed that ATP induced dissociation rates of acto-sk S1 with or without bound ADP did not differ between G146V and WT filaments, indicating that prolonged strongly-bound state is not the cause of the slow movement of G146V filaments on sk HMM. Taken together, it was suggested that force generation itself is impaired. We are developing a FRET-based assay to probe the structural changes of actin involving G146. We prepared control actin carrying two engineered Cys residues at Thr41 and Ala235 in the small and large domain, respectively, but lacking the endogenous reactive Cys374, and labeled the two Cys residues with two fluorophores for intramolecular FRET. Single molecular FRET analysis of this actin in filaments showed that actin subunits take multiple states. We plan to analyze this FRET signal with and without the G146V mutation in the presence or absence of myosin. Also, to understand why movement of G146V filaments on myosin V was normal, we are characterizing mutant myosin II carrying a chimeric substitution with a myosin V sequence.

3P-126 2mM ATP における DNSO 下での F1-ATPase のステップ運動 Stepping rotation of F1-ATPase at 2 mM ATP in the presence of DMSO (dimethylsulfoxide).

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F1-ATPase is the catalytic moiety of FoF1-ATP synthase which catalyzes ATP synthesis from ADP and inorganic phosphate using the electrochemical potential difference of proton across the mitochondrial inner membranes, chloroplast thylakoid membranes and bacterial plasma membranes. Previously, Feldman and Sigman reported that enzyme-bound ATP was synthesized by chloroplast F1-ATPase from medium Pi and enzyme-bound ADP. Yohda and Yoshida also reported that TF1 synthesizes enzyme-bound ATP in the presence of DMSO, suggesting that DMSO increases the affinity for Pi. In this report we examined the effect of DMSO on the hydrolysis of ATP and ATP-driven rotation by $\alpha 3 \beta 3 \gamma$ subcomplex of thermophilic F1-ATPase. We found that the ATPase activity was severely attenuated and clear stepwise rotation was observed at 2mM ATP in the presence of 30% DMSO. We are now trying to identify the position of DMSO induced pause and investigating which catalytic step is slowed down by DMSO.

3P-127 F1-ATPase の β -サブユニット(Y341W)単体へのヌクレオチド結合の温度依存性

Temperature dependence of nucleotide binding to β -subunit of F1-ATPase revealed by fluorescence measurement.

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In the $\alpha 3 \beta 3 \gamma$ sub-complex of F1-ATPase, the central γ subunit rotates 120 degree against $\alpha 3 \beta 3$ ring while hydrolyzing one ATP molecule; thus it is called as a molecular motor. Recently, the rotary mechanism of this molecular motor has been elucidated in detail. But thermodynamic aspect of the process is still unclear. As a first step of investigation, we examined the temperature dependence of nucleotide binding to the $\alpha 3 \beta 3 \gamma$ complex by use of the genetically engineered Trp at the nucleotide binding site (β Y341W). The fluorescence intensity of the Trp decreases upon nucleotide binding and we could monitor the nucleotide binding at a temperature range of 277 to 323 K. The van't Hoff plot of ADP binding gave a straight line and ΔH_0 and ΔS_0 were estimated to be some -20 kJ/mol and 20 J/mol/K, respectively. We are now trying to carry out a similar analysis for the isolated β subunit. By comparing the thermodynamic data for $\alpha 3 \beta 3 \gamma$ complex and isolated β subunit, we will discuss the effect of complex formation, subunit-subunit interaction, and conformational change induced by nucleotide binding.

3P-128 レーザー照射による繊維状タンパク質のバイアスブラウン運動

Biased brownian motion of fibrous proteins under laser irradiation.

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Most types of biological motion were driven by motor proteins moving along fibrous rail proteins using the energy of ATP. The rail proteins have a unique structural polarity which determines the direction of moving. In connection with the mechanism of mechano-chemical energy transduction, one of the potent models is a thermal ratchet model which illustrates that the temperature differences between motor and rail proteins convert the isotropic Brownian motion into directional movement. Recently, we have demonstrated that the infrared laser irradiation to the nanogold-attached actin filament could modulate the one-dimensional Brownian motion from isotropic to be anisotropic. In order to obtain the further evidence that the temperature differences between filamentous proteins with structural polarity and their surrounding molecules could drive the filament into the biased Brownian motion, we have tried to examine two biological filaments; microtubules as another rail protein and bacterial flagella as a non-rail protein with structural polarity.

For the purpose to obtain higher yield for thermal energy from laser light, we have introduced the semi-conductor probe with fluorescent emission, "quantum-dot": One of the famous tools for the field of cell biology. In addition with the improvement in thermal probe, the equipment for the filament observation was also improved. Inverted dark field microscope with visible laser light was contrived in order to obtain a clear image of filaments and to alleviate the spiritual stress of experimenter.

3P-129 アクチンフィラメントへのミオシンの協同的結合はアクチン-ミオシン-ADP-Pi 状態で起こる

Cooperative binding of myosin to actin filaments occurs in the actin-myosin-ADP-Pi state

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Many reports have been made about cooperative interaction between myosin and actin filaments. These reports suggest that the cooperative binding involves with force generation of the actomyosin motor, but the mechanism remains unknown. In this study, we examined the cooperative interaction in more detail using fluorescence microscopy. To analyze the cooperative binding, we prepared GFP-

fused wild type HMM (HMM(wt)-GFP) and YFP-fused G680V mutant HMM (HMM(G680V)-YFP). HMM(wt)-GFP formed fluorescent clusters along actin filaments when HMMs added at substoichiometric amounts. Quantitative analysis of the fluorescence intensity of the clusters revealed the presence of two distinct types of cooperative binding, a strong and a weak forms. The strong form was observed along Ca^{2+} -actin filaments, in which the density of HMM molecules in the clusters was similar to full decoration. The weak form that one HMM molecule was bound per 5~10 actin protomers was observed along Mg^{2+} -actin filaments. Since physiological actin molecules are expected to carry Mg^{2+} at the metal binding site, we further examined the weak form. The weak cooperative binding occurred in the presence of sub-micromolar ATP, and did not in the absence of nucleotides or in the presence of ADP. HMM(G680V)-YFP, which keep the A-M-ADP-Pi state, also formed clusters along Mg^{2+} -actin filaments. These data imply that the weak cooperative binding occurs or initiates at A-M-ADP-Pi state, which is a critical intermediate in force generation by actomyosin motors.

3P-130 ミオシン・アクチン系におけるドッキング過程の分子動力学シミュレーション

Molecular dynamics simulation for the docking process of myosin against an actin filament

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Myosin in the ADP or nucleotide-free state is attracted to an actin filament. The potential energy associated with this attractive force decreases during the docking process and can be converted into other form of energy such as mechanical work. Based on this possibility, Masuda has proposed a 'Driven-by- Detachment (DbD) mechanism' to explain the chemical-to-mechanical energy conversion in the actomyosin system. In this theory, the energy of ATP hydrolysis is mainly used to detach myosin from actin, and not directly for the power stroke.

As the first step to reproduce the elementary processes assumed in the DbD mechanism, the docking of a myosin molecule against an actin filament is calculated with a molecular dynamics (MD) method (Gromacs code with Gromos96 force field and SPC water model).

The initial configuration was build from a three-dimensional structure of an actomyosin complex (PDB ID: 1M8Q). One molecule of myosin and seven molecules of actin were extracted from the structure. Four actin molecules located at the both ends of the filament were fixed to the space. The remaining three actins and one myosin were moved according to the MD simulation. The calculation was conducted over 30 ns with a step of 2 fs.

Initially the myosin is located close to the actin molecule on the minus side of the filament but about 1.5 nm away from the second actin on the plus side. After 4 ns, by changing the positions and the configurations, the myosin made a close contact with the second actin as predicted in previous studies.

3P-131 キネシン頭部の選択的な前方着地におけるネックリンカーの役割 Role of the neck linker on the preferential forward stepping of kinesin

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Kinesin is a motor protein that walks along microtubules by alternately moving two motor 'heads'. At the last meeting, we reported that the mutant kinesin whose neck linker was artificially extended using poly-Gly insertion showed reduced velocity and failed to maintain one-head-bound state even while waiting for ATP, demonstrating that the optimal size of the neck linker is important for head-head coordination. To further investigate the role of the neck linker on the processive motility, we observed conformational changes of this mutant kinesin moving along microtubules under low ATP concentration using single molecule FRET. The neck linker extended mutant spent most of the time in two-head-bound state, which is clearly different from the wild-type kinesin that spent most of the time in one-head-bound state. This result indicates that prior to ATP-binding the detached head immediately rebind to the microtubule. To examine which binding site the detached head rebinds, we replaced one of the heads to a mutant head that cannot hydrolyze ATP and detach from the microtubule. Single molecule FRET measurement showed that the tethered wild-type head rebinds to the rear tubulin-binding site as well as the forward-binding site with nearly equal probability. These results suggest that maintaining one-head-bound state while waiting for ATP is important for preferential binding of the tethered head to the forward-binding site and for preventing futile ATP hydrolysis.

3P-132 一分子 FRET 法による尾部によるキネシン 1 の運動制御機構 Regulation of kinesin-1 motility by tail domain as studied by single molecule FRET

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The molecular motor kinesin-1 is involved in intracellular transport by moving along the microtubule by a hand-over-hand manner. The C-terminal tail domain of kinesin-1, which binds to the cargo, is also known to be involved in the regulation of cargo transport by kinesin. Recent studies demonstrated that the tail

directly binds to the motor domain (head), prohibiting the ADP release from the head. However, it is still unclear how the tail interrupts kinesin's motility while it moves along the microtubule. In this study, we used single molecule FRET technique to visualize the interaction between tail and head in kinesin molecules bound on the microtubule. We constructed heterodimer kinesin in which donor and acceptor dyes are introduced to the head and the tail in one of the polypeptides, and measured the FRET efficiency using total internal reflection microscopy. In the presence of AMP-PNP, where kinesin dimer predominantly takes two-head-bound state, the FRET efficiency was low, but in the presence of small amount of ADP, where kinesin takes one-head-bound state, we observed both high and low FRET states. The population of the high FRET state, which presumably represents tail binding to the head, decreased when the ionic strength was increased, implying that the interaction is likely to be electrostatic. These results suggest that the tail specifically binds to the tethered head of kinesin dimer, preventing the head to step forward.

3P-133 蛍光 ATP を用いたキネシンの運動解析 Movement analysis of kinesin on microtubules using fluorescent ATP

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Kinesin is a dimeric motor protein that hydrolyzes ATP to generate force and move without dissociating from a microtubule (MT). Recent studies have shown that kinesin takes one 8-nm step per ATP hydrolyzed with two motor domains (heads) acting alternately, while when to convert the chemical energy of ATP hydrolysis to mechanical force as a step is not yet well known. Simultaneous observation of both the movement of the kinesin labeled with fluorescent dye and the fluorescently labeled nucleotide binding and dissociation will lead to elucidation of the timing of the mechanochemical coupling. In this study, we focused on the influence of two types of fluorescent ATP analogues, Alexa Fluor 647 ATP and BODIPY TR ATP, on the behavior of kinesin by measuring changes in velocity of kinesin with total internal reflection fluorescence microscope. First, in an in vitro motility assay, the speeds of MTs moving across surfaces coated with multiple kinesin molecules was 520.0 nm/s with 100 μM nonlabeled ATP, and 299.2 nm/s with 100 μM Alexa Fluor 647 ATP, respectively. Surprisingly enough, however, with 100 μM BODIPY TR ATP, MTs did not slide at all. Second, in a single-molecule assay, the speeds of single kinesin molecules moving along MTs was 158.4 nm/s with 5 μM nonlabeled ATP, 59.2 nm/s with 5 μM Alexa Fluor 647 ATP, respectively. With BODIPY TR ATP, no movements of kinesin were observed. These results show that Alexa Fluor 647 ATP does not radically interfere with the behavior of kinesin and should be useful for further study.

3P-134 腸内連鎖球菌 V 型 ATPase の膜内性サブユニット (NtpI) の発現と精製

Expression and purification of the membrane subunit NtpI of *Enterococcus hirae* V-ATPase

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The vacuolar ATPases (V-ATPases) function as ATP-dependent proton pumps in the membranes of acidic organelles, in plasma membranes of eukaryotic cells. The V-ATPases are structurally and mechanistically related to the F-ATPase (ATP synthase). These ATPases consist of a globular catalytic domain (V_1 or F_1), which hydrolyze/synthesize ATP, and an integral membrane domain (V_o or F_o), which pumps ions across the membrane. The rotation mechanism of catalytic domain (V_1 or F_1) has been clear. However, the ion transport mechanism of membrane domain (V_o or F_o) is not still clear, because the high-resolution structure of the membrane-embedded proton channel (subunit a) has not been determined. V-ATPase in *Enterococcus hirae* transports Na^+ rather than H^+ . It is generally accepted that the Na^+ is pumped at the interface between the rotating NtpK ring (atomic structure available) and a static membrane component NtpI. To understand details of the ion transport mechanism, it is essential to obtain the atomic structure of NtpI.

We have recently established over-expression and purification protocols of the NtpI with GFP and His-tag in *Saccharomyces cerevisiae*. 3 milligrams of NtpI was purified from 1 liter culture after several column chromatographies. Ternary NtpI-E-F complex was also purified by gel filtration chromatography applied on mixture of the purified NtpI and NtpE-F complex. Further, we will report the kinetics of reconstituted NtpI-proteoliposomes, and the progress of crystallization trials.

3P-135 LIS1 による dynein の運動制御のメカニズム LIS1 regulates dynein motility in a dimerization-dependent manner