

mutant G220C chimera protein fused with GFP at the neck-linker has been prepared and labeled IAE-DANS. The FRET efficiencies between the fluorescent probe DANS at C220 and GFP in the presence and absence of nucleotides were analyzed.

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Analysis of Conformational Change of Novel Rice Kinesin K16 Using Small Angle X-ray Solution Scattering and EPR

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We have previously revealed that rice kinesin K16 has several unique enzymatic characteristics comparing with conventional kinesin. The most interesting property is that the ADP-free K16 motor domain (MD) is very stable, contrast to conventional kinesin that is very labile in ADP-free state. Recently, we have successfully dissolved the crystal structure of ADP bound K16 motor domain. The overall structure of the K16MD is similar to that of conventional kinesin MD, as expected from the high similarity of amino acid sequence. However, neck-linker region showed an ordered conformation in a position quite different from conventional kinesin. In this study, we designed the K16MD chimera protein fused with GFP at the neck-linker in order to monitor the conformational change of the neck-linker during ATP hydrolysis by small angle X-ray solution scattering and EPR. We determined the Radius gyration (Rg) values of K16-GFP in the presence or absence of nucleotides by X-ray solution scattering. The Rg of nucleotide-free K16-GFP was about 42Å. In the presence of ADP and ATP, the Rg values were 38Å and 39Å, respectively. These results may suggest that the neck-linker of nucleotide free K16 is in the docked conformation, on the other hand, the neck-linker of nucleotide bound state is in the novel conformation observed in crystal structure. We also analyzed conformational change of K16 in the solution by EPR. We constructed K16 mutants which have single cysteine at 331, 335 or 340 and labeled with 4-maleimido-2,2,6,6-tetramethyl-1-piperidinyloxy. But we could not observe notable change of mobility during ATP hydrolysis in the absence of microtubule for the three mutants. Currently, we are analyzing the distance between kinesin core region at 47 and neck linker at 328 using the dipolar EPR method.

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The Effect Of Loads on the Collective Behavior of Neurospora Kinesin

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The motor protein kinesin converts the energy from ATP hydrolysis and Brownian motion into directed movement. There is increasing evidence suggesting that several kinesin motors cooperate to transport cargoes. Recent experiments also suggest that the collective behavior of kinesin differs significantly from single-molecule behavior. We study the collective behavior of *Neurospora* kinesin (Nkin) in vitro. By laser trapping latex beads attached to microtubules through biotin-streptavidin linkages, we are able to apply forces to microtubules being transported by several kinesins attached to the coverslip. The density of motors on the coverslip is related to the average number of motors involved in the transport. We experimentally characterize the transport for a range of loads and motor densities.

Unconventional Myosins

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Photo-Control of Myosin Va using Photoresponsive Calmodulin

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¹Soka Univ., Tokyo, Japan, ²Univ. of Massachusetts, Worcester, MA, USA. Myosin Va is a processive motor that has a role as an organelle transporter in various cells. Myosin Va consists of motor domain, neck domain, coiled-coil region, and globular tail domain (GTD). The neck domain carries six IQ motifs, which act as the binding site for calmodulin (CaM) or CaM-like light chains. The GTD inhibits the Ca²⁺/CaM dependent actin-activated ATPase activity of myosin Va. CaM is a physiologically important Ca²⁺-binding protein that participates in numerous cellular regulatory processes. CaM has a dumbbell-like shape in which two globular domains are connected by a short α -helix. Each of the globular domains has two Ca²⁺-binding site called as EF-hand. CaM undergoes a conformational change upon binding to calcium, which enables it to bind to specific proteins for a specific response. N- (4-phenylazophenyl) maleimide (PAM) is a photochromic compound that undergoes *cis-trans* isomerization by ultraviolet

(UV) - visible (VIS) light irradiation reversibly. Previously we have demonstrated that the binding of the CaM to the target peptide is controlled by the isomerization of PAM. PAM was incorporated into CaM mutants that have a single reactive cysteine residue. The binding of PAM-CaM (N60C), PAM-CaM (D64C) and PAM-CaM (M124C) to M13-YFP were apparently photo-controlled by UV-VIS light irradiation reversibly at the appropriate Ca²⁺ concentration. In the present study, we have tried to photo-control the function of myosin Va using the PAM-CaM by UV-VIS light irradiation reversibly. The part of endogenous CaM of myosin Va heavy meromyosin (M5aHMM) was substituted by exogenous PAM-CaM. The M5aHMM substituted by PAM-CaM (M5aHMM/PAM-CaM) showed normal range of actin-activated ATPase activity. Currently, we are examining to photo-control the actin-activated ATPase activity of M5aHMM/PAM-CaM in the presence of exogenous GTD.

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Modification Of Loop 1 Affects The Nucleotide-Binding Properties Of Myo1c, The Adaptation Motor In The Inner Ear

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Myo1c is a ubiquitously expressed mammalian class I myosin that serves as a component of the hair cell's adaptation-motor complex in the inner ear. We have recently shown that a truncated form of Myo1c consisting of the motor domain and a single IQ domain, Myo1c^{11Q}, has kinetic properties similar to full-length Myo1c (Adamek et al, 2008). We also showed that the ATPase cycle of Myo1c shows a unique response to Ca²⁺, inhibiting the ATP hydrolysis step 7-fold and accelerating ADP release by 10-fold. Here we probed the role of loop 1, a flexible loop near the nucleotide-binding region, in defining the properties of Myo1c by creating six chimeras. We found that replacement of the charged residues in loop 1 with alanines or the whole loop with a series of alanines did not alter the ATPase, transient kinetics properties and Ca²⁺-sensitivity of Myo1c^{11Q}. Substitution of loop 1 with that of the corresponding region from tonic smooth muscle myosin II (Myo1c^{11Q}-tonic) or replacement with a single glycine (Myo1c^{11Q}-G) accelerated ADP release 2-3-fold from A.M in Ca²⁺, whereas substitution with loop 1 from phasic muscle myosin II (Myo1c^{11Q}-phasic) accelerated ADP release 35-fold. Myo1c^{11Q}-tonic translocated actin in vitro twice as fast as wild type and Myo1c^{11Q}-G 3-fold faster. The changes induced in Myo1c showed no resemblance to the behaviour of the loop donor myosins or to the changes observed with similar Myo1b chimeras (Clark et al, 2005).

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Mechanics of myosin V near stall

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Motor proteins of the myosin family are driving many types of cellular motility. Functions are diverse, ranging from muscle contraction to endocytosis, cell locomotion, intracellular transport or signal transduction in hearing. Recent structural, kinetic and single molecule mechanical studies however revealed that the basic mechanisms of chemo-mechanical energy transduction are shared amongst myosin motors. This includes a working stroke in two phases coupled to the release of Pi and ADP and strain dependence of ADP release. Many details of the basic mechanism still remain unclear, including the effect of stall forces on the mechanics of a single motor head. Here we have used a single-headed myosin V construct (6 IQ) to investigate whether the conformational change associated with the working stroke can be reversed at high loads. We used optical tweezers to apply forces near stall for the processively moving dimeric motor (>2pN). We observed backstrokes of ~15nm, consistent with a reversal of the main conformational change of a single myosin V motor head. The dwell times of backstrokes were dependent on load. Implications of these findings for processive movement of the native, dimeric motor are discussed. Supported by MRC and NIH.

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Force Dependence of a Myo1b Truncation Mutant

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Myosin-IIs are the single-headed members of the myosin superfamily that associate directly with cell membranes and play roles in regulating membrane dynamics. We previously characterized the force dependence of the widely expressed myosin-I isoform, myo1b, using an optical trap and a novel isometric force clamp. This myo1b isoform, which contains five IQ-motifs, is highly strain sensitive, with forces of < 2 pN decreasing the rate of actin detachment > 75 fold. We estimated the distance parameter (distance to the

transition state of the force dependent step) to be 12 nm by a fit of the force dependence of actin attachment times in the presence of 50 micromolar ATP. In this study, we examine the force dependence of a myo1b truncation mutant that contains only the IQ motif closest to the motor domain. Although the unloaded biochemical kinetics of this mutant are nearly identical to the 5 IQ construct, we found the actin detachment rate to be substantially less force sensitive. These experiments suggest that the length of the regulatory domain modulates force sensitivity.

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Single-molecule Measurements Of Myo1c-PIP2 Detachment Forces Using Optical Tweezers

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Optical tweezers has become one of the most efficient techniques to accurately measure small forces (pN) and displacements (nm) upon interactions of individual biological molecules, particularly molecular motors. In the current study we extend the application of optical tweezers to measure protein-lipid detachment forces at the single-molecule level. Myo1c is a single headed, force-generating motor that links cell membranes to the underlying actin cytoskeleton. Actin binding occurs via the motor domain, while the tail domain interacts with phosphatidylinositol 4,5-bisphosphate (PIP2). To determine the forces required to detach the myo1c tail domain from PIP2, we used spherical supported bilayers composed of 1 μ m diameter silica beads coated with 2% PIP2 and 98% dioleoyl-phosphatidylcholine (PC). The efficiency of coating was verified by examining the distribution of fluorescently labeled lipids, and the specificity of binding was confirmed by sedimentation assays. The myo1c tail domain binds to the spherical supported bilayers containing a PC/PIP2 mixture, but not PC alone. Additionally, myo1c dissociates from the PC/PIP2 beads in the presence of inositol 1,4,5-trisphosphate, which effectively competes with PIP2 for binding. For the laser trap measurements, 2 μ m diameter silica pedestals were immobilized under a layer of nitrocellulose on a coverslip and were coated with either anti-his-antibody or neutravidin to anchor tetra-His-tagged or biotinylated constructs of myosin-I in a specifically oriented manner. The bilayer coated beads trapped by a laser beam were brought into contact with spherical pedestals decorated with the myo1c tail domain. Upon repeated contact and retraction cycles, binding events and subsequent disruption forces were measured. The most probable rupture force of the PIP2/myosin-I tail interactions are ~ 7.1 pN at a loading rate of 360 pN/s.

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High Speed Imaging For Myosin VI

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Myosin-family molecular motors have been a subject of extensive research recently. While there is a general consensus on how do these motor molecules perform their function, myosin VI presents a serious challenge to a conventional view. It has been reported that myosin VI moves processively in a hand-over-hand manner toward the minus end of actin filament, opposite to other classes of myosin. The head takes large steps (50 - 70 nm) relative to its short lever arms (7.2 nm), and frequently moves backward with half the size of a forward step. Using a video rate FIONA technique, we observed the above features under the several ATP concentrations (10 μ M ATP, $\sim 67 \pm 19$ nm, N=206; 100 μ M ATP, $\sim 73 \pm 13$ nm, N=362; 1000 μ M ATP, $\sim 73 \pm 19$ nm, N=105). The back-steps frequently occur ($\sim 8\%$), and its size is half of the forward step (42 ± 14 nm, N=57). The mystifying observation of a large step size and its large distribution has been explained in terms of the proximal tail domain to unwind which allows the myosin VI molecule to stretch out. However, this hypothesis does not support the exerted mechanical force, which was measured in myosin VI to be 2 pN without slowing.

We believe that the major action takes place during the rapid stepping transition (<30 msec) between a quick release of a rear head from the actin molecule and a strong rebinding event. To address all the above questions, we are currently working on the direct observation of the one head dynamics using a dark-field imaging microscopy with much-improved microsecond time resolution.

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Temperature Dependent Energy Transfer Measurements Reveal Flexibility in the Upper 50 kDa Domain of Myosin V

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Our previous work has demonstrated that labeling myosin V in the upper 50 kDa domain with the biarsenical dye FIAsh can serve as an acceptor for fluorescence resonance energy transfer studies with mant labeled nucleotides and

IAEDANS actin. These FRET studies suggest that myosin V can adopt a conformation in which the nucleotide binding pocket and the actin binding cleft are in a closed conformation. Our studies suggest the upper 50 kDa domain may be highly flexible in certain nucleotide-states which allows tight binding to nucleotide and actin. Molecular geometric simulations demonstrate the upper 50 kDa domain is most flexible in the myosin V.ADP state, consistent with this state having a high affinity for ADP and actin. Currently, we examined the temperature dependence of the FRET signal between mantADP and MV FIAsh. We found that at low temperature (4-15°C) a high FRET state dominates (closed pocket) while at high temperature (30-37°C) a low FRET state dominates (open pocket). This transition is reversible suggesting a temperature-dependent conformational change. We also found that FIAsh labeled G440A MV, a non-hydrolyzable mutant, has a similar temperature-dependent transition in the presence of mantATP. In contrast, the transition does not occur in the presence of mantADP.BeFx or with the non-hydrolyzable E44A MV mutant in the presence of mantATP. Our results suggest coordination of the gamma-phosphate of ATP rigidifies the upper 50 kDa domain which results in a weak actin affinity state (open actin binding cleft and closed nucleotide binding pocket). However, upon phosphate release the upper 50kDa domain becomes more flexible which allows myosin to adopt a conformation in which it has a high affinity for both nucleotide and actin (closed nucleotide binding pocket and actin binding cleft).

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Kinetics Of Myo1c Association To And Dissociation From Phosphoinositide-containing Vesicles

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Myo1c is a single-headed unconventional myosin that associates with negatively-charged lipids through electrostatic interactions. A putative pleckstrin homology (PH) domain has been identified in the myo1c tail that binds PI(4,5)P₂ with high affinity. However, the kinetics of association and dissociation, as well as the influence of membrane phospholipid composition and Mg²⁺ on the kinetics, remain unknown. Stopped-flow measurements were made using the increase in light scattering that occurred upon myo1c-tail binding to 100 nm diameter large unilamellar vesicles (LUVs).

We found that the association of myo1c-tail with phosphatidylcholine (PC) LUVs containing 2% PI(4,5)P₂ followed a 2-exponential time-course. The rate of the predominant, fast phase depended linearly upon the total lipid concentration. The apparent second order rate constant in the absence of Mg²⁺ was approximately diffusion-limited, indicating that no conformational change occurs upon binding. The molar ratio of anionic lipid was increased by adding phosphatidylserine (PS) or additional PI(4,5)P₂ to LUVs or by situating PI(4,5)P₂ in a more physiologically relevant lipid background (phosphatidylethanolamine, PC, PS, phosphatidylinositol, and sphingomyelin). None of these conditions increased the apparent association rate constant much more than two-fold. Dissociation of myo1c-tail was measured by chasing with excess inositol 1,4,5-trisphosphate (InsP₃). The presence of additional anionic phospholipid reduced the observed dissociation rate constant by orders of magnitude (3.2 s^{-1} vs. 0.03 s^{-1}). This suggests that once myo1c-tail interacts via its putative PH domain with PI(4,5)P₂, additional electrostatic interactions between positively-charged regions of the tail and negatively-charged lipids help to stabilize binding. The presence of Mg²⁺, known to interact with polyvalent anions, did not alter these trends, though it did increase the dissociation rate for all lipid compositions. Finally, we measured the dissociation rate of myo1c-tail from InsP₃ and found that it approximates the dissociation rate from PI(4,5)P₂.

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Single Molecule Investigation of the Acto-Myosin-10 Complex Using Optical Tweezers

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Recent cell biological studies of myosin-10 have revealed that myosin-10 is essential to cellular processes such as filopodia extensions and phagocytosis. Steady-state and transient kinetic biochemical studies of the ATPase cycle of a single-headed, subfragment-1 like (S1), construct of myosin-10 show that it has an intermediate duty-cycle ratio. It remains tightly bound to actin for about 16% of its total ATPase cycle time but spends around 90% associated with actin in both weak and strongly bound states. Furthermore, the acto-myosin-10-S1 complex has two ADP bound states and a surprisingly low affinity for actin, comparable to that of the rigor complex between actin and skeletal muscle myosin II. To study the mechano-chemical coupling of myosin-10, we used the