

N-terminus of actin is abolished. These results indicate a new communication pathway in myosin. Based on the seesaw model of the relay helix movement, actin pulls the PR loop which directly moves the relay region and accelerates the rate of the power stroke. The kinetic scheme suggests that actin activation is not required directly for the motor functions and motility. The increased ATPase biases the kinetic paths towards the actin bound forms even in weak actin bound states, thus this effect reduces the possibility of futile cycles of the actomyosin ATPase.

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Light Chain Domain Orientation Determined by Time-resolved FRET

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We have used site-directed time-resolved FRET to determine light-chain domain transitions in expressed *Dictyostelium* full-length myosin S1. Myosin S1 is proposed to undergo large rotations of the lever-arm in response to the nucleotide state. Previous fluorescence experiments (Shih et al., 2000) have shown that S1 adopts two populations of pre-stroke states and the equilibrium between these states is driven by nucleotide. To observe these population changes in detail, and to observe the effect of actin, we have used time-resolved fluorescence resonance energy transfer (TR-FRET) to measure the distribution of distances between the labeled catalytic domain (A250C) and a labeled RLC (M129C). In the present work, we show that the lever arm adopts multiple orientations in solution. In the absence of nucleotide, the light-chain domain probe is at a mean distance of 8.4 nm from the catalytic domain probe. This distance extends to >9.0 nm when fS1 is bound to actin. The addition of ATP gives three roughly equal populations of distances at 3.6, 4.3, and >9.0 nm. ADP.V_i stabilizes the shorter of these populations, indicating that ADP.P_i induces a conformational change of the light-chain domain. This work was supported by grants from NIH (AR32961, AR07612). We thank Igor Negrashov for excellent technical assistance.

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Measuring Myosin Light Chain Domain Orientation in the Pre-Power Stroke AIF₄ States with a Bifunctional Spin Label in Skinned Muscle Fibers

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We are using electron paramagnetic resonance and site-directed spin labeling to measure the orientation of the light chain domain of skeletal muscle myosin in pre-power stroke states trapped by the phosphate analog AIF₄. Previous work (Kraft et al. (2005) Proc. Natl. Acad. Sci. USA **102**:13861-66) has shown via single-fiber X-ray diffraction that AIF₄ traps two distinct pre-powerstroke myosin states in activated muscle. The first state (ADP.AIF₄-I) produces a weak actin binding, disordered myosin whereas the second state (ADP.AIF₄-II) produces a strong binding, stereospecific actomyosin complex. This weak-to-strong transition is a clear indication of the initiation of the power stroke before phosphate release, but it does not reveal the state of the light chain domain (LCD), which undergoes a rotation during the power stroke. We measured the orientation of the light chain domain by exchanging the native regulatory light chain (RLC) of skinned rabbit psoas muscle fiber bundles with a Di-Cys mutant RLC labeled with a bifunctional spin label. Our group has shown previously (Thompson and Naber et al. 2008 Biophys J, in press) that a bifunctional methanethiosulfonate spin label binds rigidly to myosin and reports protein orientation accurately. EPR spectra of oriented fibers show that the LCD produces a distinct orientation from rigor and relaxation in the ADP.AIF₄-II state whereas the ADP.AIF₄-I state is indistinguishable from relaxed muscle, supporting the hypothesis that the II state represents a state early in the power stroke with a distinct LCD orientation. This work was supported by NIH (AR32961, AR007612). We thank Bernhard Brenner and Theresia Kraft for guidance.

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Probing The Divalent Cation-binding Region Of The Myosin Regulatory Light Chain During Muscle Contraction Using EPR

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We are using electron paramagnetic spectroscopy (EPR) to determine whether the myosin light chain domain (LCD) rotates as a rigid unit during muscle contraction. To accomplish this we are making orientational measurements at selected positions in the LCD using site-directed spin labeling of single-cysteine mutants of RLC. In addition, we are using double-cysteine RLC mutants to measure distance changes using dipolar EPR. We created two RLC mutants that contain a single cysteine (I35C or A55C) in the helices of the helix-loop-helix of the rat ventricular RLC (vRLC) divalent cation-binding site. Starting with each single-cysteine mutant, a second cysteine was added in

the opposite helix (I35C/L49C and A55C/A32C) for distance measurements. The four RLC mutants were labeled with the spin probe MTSL and separately reconstituted into rabbit psoas muscle fiber bundles. The EPR spectra of the labeled fiber bundles in rigor were sensitive to muscle fiber orientation, for both I35C- and A55C-RLC mutants, indicating that the spin labels were highly oriented at both sites, making them ideal for measuring orientational changes. For both single-cysteine RLC mutants, myosin in rigor showed two populations of oriented spin labels that became substantially disordered in relaxation, while contraction induced order in a small fraction of the spin labels. The EPR spectra of the fibers with double-cysteine RLC mutants showed that the spin labels were less sensitive to muscle orientation, so they are suitable for distance measurements with myofibrillar preparations at low temperatures. This work is supported by NIH grant AR052360 to OR and by the Minnesota Supercomputing Institute.

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Orthovanadate Slows Kinetics Of The Acto-Myosin Interaction In Skinned Muscle Fibers By Competition Between Myosin-ADP-P_i and Myosin-ADP-V_i Cross-Bridges For Actin Sites

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Orthovanadate (V_i) is a phosphate analog that has been used to study the relationship between biochemical reactions and structural and mechanical transitions in the cross-bridge cycle, with great success. In solution, V_i binds to myosin with high affinity, forming a stable myosin-ADP-V_i complex. In permeabilized muscle fibers, V_i binds to an actin-myosin-ADP state during cross-bridge cycling, and suppresses isometric tension, isometric stiffness and unloaded shortening velocity. We investigated the effects of V_i on the mechanics and kinetics of the actin-myosin interaction in skinned fibres from rabbit psoas (sarcomere length 2.4 µm, temperature 12 °C). After correction for myofilament compliance, the observed changes in stiffness indicate that the reduction in T₀ is due to a proportional reduction in the number of myosin cross-bridges attached to actin (Caremani et al., *Biophys J* 480a/2289-Pos, 2007). The effect of [P_i] (range 0-15 mM added [P_i]) on T₀ and the rate constant of force development following a period of unloaded shortening (k_D) in the presence of V_i (0.1 mM) suggest that V_i acts as a competitive inhibitor of P_i for the myosin-ADP state (Caremani et al., *Biophys J* 128a/621-Pos, 2008). Simulations show that the effects of V_i on force, stiffness and rate of force redevelopment, as well as on the actomyosin ATPase (Wilson et al., *Biophys J* 68:216-226,1995) and velocity of unloaded shortening (Chase et al., *J Physiol* 460:231-246, 1993), can be explained with a straightforward modification of the kinetic scheme of Dantzig et al., *J Physiol* 451:247-278, 1992 to include myosin-ADP-V_i heads competing with myosin-ADP-P_i heads for actin sites. Supported by NIH (R01 AR049033.03) and MiUR (Italy).

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Single Molecule Kinetic Measurements Of Non-muscle Myosin IIB Using Optical Tweezers

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Non-muscle myosin IIB (NMIIB) is a cytoplasmic myosin which is ubiquitously expressed in eukaryotic cells, particularly in the central nervous system and cardiac tissues. Our previous solution kinetic study of the single headed, subfragment-1 (S1) construct of NMIIB showed that this myosin spends a significant amount of its actomyosin ATPase cycle in the strongly bound state. In the presence of actin, the rate of ADP release is slow (~0.35 s⁻¹), comparable to the steady-state ATPase rate (0.13 ± 0.01 s⁻¹). Furthermore, ADP-affinity for NMIIB is the highest reported so far for the myosin super-family (<0.15 µM). These unique kinetic parameters are advantageous for NMIIB, whose function *in vivo* has been shown to be associated to cortical tension generation and maintenance. To study the kinetics of single molecules of NMIIB-S1 constructs interacting with a single actin filament, we used a dual-beam optical tweezer apparatus to perform single molecule kinetic/mechanical studies using the 'three-bead' assay. We measured the lifetimes of unitary actomyosin interactions and determined the actin-detachment kinetics with varying ATP concentrations. Optical trapping results showed that at physiological ATP concentration (1 mM), the rate of detachment of acto-NMIIB-S1 interactions was ~0.3 s⁻¹, similar to the ADP release rate and steady-state ATPase rate reported from solution kinetic studies. Decreasing the ATP concentration (10 µM) did not alter this rate of detachment. Additional single molecule experiments were performed by adding

excess ADP into the NMIIB environment (1 mM ADP, 1 mM ATP), which prolonged the strongly bound states further, exhibiting behavior of a molecule with high ADP affinity. We will discuss these kinetic measurements, as well as the mechanical measurements, extracted from the optical tweezers experiments in the context of NMIIB's role as a tension generating motor *in vivo*.

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Protein-surface Interactions and Functional Geometry of Surface-adsorbed Myosin Motor Fragments

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Biophysical studies with myosin motor fragments (heavy meromyosin; HMM and subfragment 1; S1) adsorbed to artificial surfaces, are important for elucidation of actomyosin function. In spite of the widespread use of such *in vitro* motility assays and single molecule studies, little is known about the adsorption geometry and effects of protein-surface interactions on the motor properties. Here, we investigate these factors with focus on HMM using quartz crystal microbalance with dissipation (QCM-D) and total internal reflection fluorescence (TIRF) spectroscopy based ATPase assays. In the latter, we monitored the turnover of Alexa-fluor647-ATP (Alexa-ATP) by surface adsorbed HMM. Studies were performed with HMM/S1 adsorbed to model hydrophilic (SiO₂) or hydrophobic (trimethylchlorosilane [TMCS] - derivatized) surfaces. The results suggest that adsorption of HMM is weakened on SiO₂ (but not on TMCS) at high (245 mM) compared to low (65 mM) ionic strengths. The changes in ionic strength were also associated with structural changes in the protein layer according to QCM-D studies. Moreover, the TIRF based ATPase assay suggested a larger fraction of HMM molecules with low catalytic activity on SiO₂. These and other TIRF and QCM-D results, suggest that HMM preferentially adsorbs to negatively charged hydrophilic surfaces via the actin-binding region. In contrast, the majority of the HMM molecules seem to adsorb via their C-terminal tail on moderately hydrophobic surfaces. In the latter case the catalytic sites appear to be close to, but not immobilized on the surface. The results with HMM were compared to, and found consistent with, QCM-D and TIRF-data obtained with S1 motor fragments.

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Simultaneous Measurement Of Actin Sliding Velocities And Actin-myosin Dissociation Kinetics

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Myosin is an enzyme that couples ATP hydrolysis with mechanical force and motion generation through its cyclic interactions with actin filaments. In order to better understand how actin-myosin mechanics and biochemistry are linked, we have developed a novel *in vitro* motility assay that allows us to measure actin sliding velocities, V , and actin myosin dissociation kinetics, T_{on} , simultaneously. To measure T_{on} during a motility assay, we monitor changes in actin filament dynamics near a myosin binding site using nanometer tracking of actin filaments. Actin-myosin binding damps actin dynamics, and T_{on} is estimated as the average duration of these binding events. With this approach, we observe that the duration of actin myosin binding events decreases linearly with increasing ATP concentrations, resulting in an estimated ATP induced dissociation rate constant of $0.4 \mu\text{M}^{-1} \text{s}^{-1}$, consistent with previous kinetic measurements. This assay provides a powerful tool for simultaneous measurement of actin-myosin mechanics and the kinetics and allows us to determine the mechanochemistry of a single myosin molecule functioning within the context of an ensemble system.

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Actin-Myosin Binding Kinetics in Relation to Actin Sliding Velocities

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Muscle shortening velocities are widely thought to be solely limited by ATP-induced actin-myosin dissociation; however, recent studies indicate that muscle shortening velocities are influenced significantly by the kinetics and energetics of myosin's weak-to-strong binding transition. To test this hypothesis, we have developed a novel assay that allows us to measure actin-sliding velocity, V , and actin-myosin binding, k_{on} , and dissociation, k_{off} , kinetics in the same flow chamber. Using this assay we study the effects of different modulators of muscle mechanics such as blebbistatin, vanadate, sucrose, ATP, ADP, and P_i on k_{on} , k_{off} , and V . In general, when k_{off} is varied (e.g., by varying [ATP]), we observe

a strong correlation between actin-myosin dissociation kinetics and V as predicted by detachment-limited models of V . However, certain modulators such as blebbistatin and vanadate affect V by altering weak-to-strong binding kinetics, k_{on} . This assay will allow us to better understand the mechanism by which changes in k_{on} affect V .

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Electoron Microscopy on The Comprehensive Structural Change during Actomyosin Sliding

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We have been studying the transient intermediate configuration of myosin crossbridges during *in vitro* sliding, utilizing a novel procedure to quantitatively compare quick-freeze replica microscopic images with the atomic models in various conformations. Snapshots of actin-sliding myosin predominantly consisted of kinked configuration to hold actin filaments inside its curvature. The images of those myosin heads cannot be reconciled with any crystal structures documented in the data-base. We also found that such unusual configuration well resembles the images of myosin molecules whose two reactive thiols are chemically-crosslinked; the putative intermediate analogue. Since myosin heads were too small for single-particle-analysis coupled with conventional electron cryo-microscopy, we attempted to determine its structure from above high-contrast replica images after classification and averaging in each class. Euler-angles of the motor-domain and the leverarm were estimated as reported, and the molecule's envelope was reconstructed from a few projections utilizing a new procedure. By including such novel configuration with different attachment angles to actin, we proposed a revised scheme on the structural change during crossbridge-cycle that comprehensively explains all the observed images under sliding conditions. Since the leverarm of the novel intermediate was kinked to the opposite direction of that in ADP/Pi-bound head, flipping must occur sometime after the head dissociates from actin filament. We thus sought for crossbridges in any other configurations in the original replica images, and found a minor fraction that resembles the ADP/Vi-form. The existence of such structure might add further information on the time-sequence of the conformational change, suggesting that once dissociated myosin in ADP/Vi-form first reattaches to actin, followed by flipping of the leverarm to form the predominant, oppositely-kinked crossbridges. We will also show some results with a new landscape-marker probe that might be useful for the electron microscopic structural determination.

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A Viscous Drag Model of Frictional Loading Assays for Myosin Motors

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The myosin superfamily is a diverse family of actin binding proteins that hydrolyze ATP and generate force. The *in vitro* motility assay, which combines the strengths of solution biochemistry with the ability to measure myosin force and motility, has allowed the molecular events that couple ATP hydrolysis to mechanical work to be probed. Several groups have used modifications of the *in vitro* motility assay to study the effects of load on actin filament velocity. One such method uses alpha-actinin to exert a frictional load on sliding actin filaments that are being propelled over a bed of a given myosin isoform. This method, which has been used to measure myosin isometric force, relies on the ability to determine the amount of alpha-actinin necessary to stop actin translocation over the bed of myosin. However, careful examination of frictional loading assay data shows that even in the presence of very high concentrations of alpha-actinin, actin filament sliding was never observed to completely stop, suggesting a non-linear relationship between the amount of alpha-actinin added and the frictional load acting on the myosin. In order to better characterize this behavior, we developed a model in which the alpha-actinin behaves as a viscoelastic rather than purely elastic element, giving a velocity dependence to the frictional load imposed by the alpha-actinin. This correction allows for the determination of the myosin isometric force in the absence of a clear zero velocity point. Furthermore, this model describes the absolute resisting force imposed by the alpha-actinin as function of velocity, allowing for the measurement of isotonic kinetics using the motility assay. Supported by NIH-HL077280, AHA-0435434T (J.R.M.), and AHA-0815704D (to M.J.G.).