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Structural Dynamics Of The Actin Binding Cleft Of *Dictyostelium* Myosin II Analyzed By Stopped Flow Time-Resolved FRET

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Using site-directed fluorescence labeling, transient time resolved FRET ($[TR]^2$FRET) and time resolved fluorescence anisotropy, we have measured actin- and nucleotide-induced structural changes within the actin binding cleft of *Dictyostelium* myosin II. In a recent report using time-resolved pulsed EPR spectroscopy, distances were measured between paramagnetic probes attached to the upper and lower 50 kDa subdomains of *Dictyostelium* myosin II (Klein et al., *PNAS*, 105:12867-72). These results support the hypothesis that the actin-binding cleft closes partially upon actin binding, but also suggested that both open and closed conformations are simultaneously present, with nucleotides and actin controlling the open-closed equilibrium. Due to technical constraints, those EPR distance measurements were limited to frozen samples. In the present study, time-resolved fluorescence is used to probe the cleft in solution under more physiologically relevant conditions, including the transient phase of the ATPase reaction. Fluorescent probes were attached to engineered Cys residues in the upper and lower 50 kDa subdomains and used to measure the distance across the cleft. Single probes attached to either subdomain were used in combination with fluorescent nucleotides to monitor the coupling between the actin-binding cleft and the active site. Here we use a combination of transient time resolved FRET and transient time resolved fluorescence anisotropy to probe the equilibrium between open and closed cleft conformations. In the key experiments, a complete nanosecond time-resolved fluorescence decay was measured, defining the detailed distance distribution between probes, every 0.1 ms following rapid mixing (stopped flow), thus yielding high-resolution structural information on the sub-millisecond time scale. The results provide new insights into the coupling between the actin-binding cleft, the active site, and actin binding.

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Structural Dynamics of the Myosin Relay Helix Resolved by DEER and Time-Resolved FRET

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We have used DEER (double electron-electron resonance) and TR-FRET (time-resolved fluorescence resonance energy transfer) to study conformational changes within the myosin II relay helix. Major structural changes during the myosin II ATPase cycle take place in the force-generating domain. Crystal structures show that the converter domain, the relay helix, and SH1 helix have different conformations in the proposed pre- and post- powerstroke structural states of myosin. This dramatic structural change is detected in solution studies by intrinsic fluorescence [1, 2] and EPR of a spin label attached to SH1 [3]. In the present study, we focus on the relay helix as a crucial structural element involved in coupling between the force-generating domain and the nucleotide-binding pocket. Cysteine mutations were introduced into a Cys-lite construct of *Dictyostelium discoideum* (*Dicty*) myosin in the lower 50k domain (either D515C or A639C) and the C-terminal end of the relay helix (K498C). These constructs were selectively modified with either MSL/MSL or IAEDANS/DABCYL pairs, and the distance between probes was measured in different myosin conformations trapped with nucleotides or nucleotide analogs Two conformations of the relay helix (with distinct probe-to-probe distances, presumably corresponding to the ''straight'' and ''bent'' states of the relay helix) were resolved. Observed distances were in good agreement with existing crystal structures, but at least two distinct structural states were present in certain biochemical states (e.g, with bound ADP.BeF_3, ADP.V_i, ADP.AlF_4). The mole fraction of the ''bent'' conformation was higher with post-hydrolysis analogs (ADP.V_i, ADP.AlF_4) bound at the active site. Our results reveal structural rearrangements within a single subdomain of myosin and provide insights into the coupling between ATP binding and changes in the force-generating region.

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A Glimpse at Loop 1 Movement in Smooth Muscle Using Intrinsic Tryptophan Fluorescence

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Smooth muscle myosin has two N-terminal isoforms that result from alternative splicing of loop 1. Loop 1 contains a seven amino acid insert (QGPSFSY) in one isoform (SM-B) that is absent in the other (SM-A). It has been shown that the presence of the insert causes a two-fold increase in the rate of in-vitro actin sliding velocity and actin-activated ATPase (Rovner *et al.*, *J. Muscle Res. Cell Motil.* **18**:103, 1998). Based on these results and its proximity to the active site it was hypothesized that loop 1 plays a role in modulating the release of ADP (Spudich, *Science* **372**:515, 1994). However, little is known about the conformation of loop 1 in different nucleotide states, as it is absent in crystal structures. To examine the position of loop 1 and its potential role in ADP release we have engineered a single tryptophan residue into loop 1 at position 215. Using the intrinsic tryptophan fluorescence from W215 and fluorescent analogs of ADP and ATP we have looked at the position of loop 1 as a function of temperature. The results suggest two conformations of loop 1 in the ADP state, both an open and closed form. The distance between loop 1 and the active site decreases for both nucleotides from 25-15°C. At 10 °C loop 1 moves away from the active site in the ADP state, while there is no additional movement in the presence of ATP. This is the first data to demonstrate movements of loop 1 associated with different nucleotide states of the myosin active site, giving insight into how it may contribute to nucleotide release.

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Importance In The Powerstroke Of Interaction Between The Relay Helix And Helix HQ Of Myosin

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Ideas on mechano-chemical transduction by myosin have matured greatly through crystallography and are ripe for testing *in vivo*. One approach for doing so is termed reversion analysis, in which pairs of compensating mutations are identified. Suppression of one missense mutation by another reveals an interaction at the amino acid level that may be direct or indirect, long-lived or fleeting, but nonetheless physiologically relevant. Reversion analysis thus can test current ideas and, importantly, uncover interactions underlying dynamic or strain-dependent myosin states likely not represented in crystals. We used random mutagenesis to induce suppressors of *Caenorhabditis elegans* myosin/UNC-54 mutation E524K (=E500 of chicken myosin V), located on helix HQ at the predicted actin-binding region.

Worms with E524K alone display disorganized A-bands and have a paralysis that worsens with increasing temperature. Thermodynamically, the heat-sensitivity suggests loss of a salt-bridge. The comparable residue in other myosins forms in the rigor-like and post-rigor crystallographic structures, but not in the pre-powerstroke one, a salt-bridge with a lysine on the relay helix (K460 of myosin V; =K483 of UNC-54). Thus, in the paralyzed worms, electrostatic repulsion between E524K and K483 potentially destabilizes interactions between helix HQ and the relay helix, thereby hindering the powerstroke.

Twenty independent lines of suppressed worms were recovered from a screen of 10^6 mutagenized haploid genomes, and the suppressors mapped to seven residues: near the P-loop, V187I; in the actin-binding domain, E524K to E/T, L547F, A548V, and M579I/L/V; on the SH1-helix, C712Y; and in the converter domain, D724N. Consistent with the crystallographic structures of myosin, the suppressors can be interpreted as diminishing unfavorable interaction between E524K and K483, thus permitting the relay helix to reorganize properly as myosin progresses through the crossbridge cycle.

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New Mechanism of Actin Activation of Myosin

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Actin activation of myosin ATPase activity is a general property of actomyosin systems, however, its role in the mechanochemical transduction is still not unveiled. Recently, we showed that in the absence of actin the rate limiting step of the ATPase cycle is not the phosphate release step but the preceding conformational change and actin activates directly this step to constitute the power stroke. Here we report that the rate of the power stroke step is initiated directly by the interaction of actin and the prolin rich (PR) loop which is located in the proximal part of the relay helix. Deletion of the PR loop does not change any of the kinetic properties of *Dictyostelium* myosin motor domain in the absence of actin. Actin binding of the PR loop deleted mutant decreased only three fold in rigor and in the presence of nucleotides. Also, ATP induced actin dissociation was only slightly affected by the deletion. Nevertheless, we found that PR loop deletion caused dramatic effect on the actin activation: basal ATPase activity is not increased by actin. Surprisingly, the motility is not reduced if this interaction between the PR loop and the

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 flS1 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 AlF_4 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 AlF_4. Previous work (Kraft et al. (2005) Proc. Natl. Acad. Sci. USA **102:**13861-66) has shown via single-fiber X-ray diffraction that AlF_4 traps two distinct pre-powerstroke myosin states in activated muscle. The first state (ADP.AlF_4-I) produces a weak actin binding, disordered myosin whereas the second state (ADP.AlF_4-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.AlF_4-II state whereas the ADP.AlF_4-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-Vi 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 T_0 isdue 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 T_0 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