Conformationally Trapping the Actin-binding Cleft of Myosin with a Bifunctional Spin Label*

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Background: The actin-binding cleft of myosin is proposed to close upon force generation.
Results: Crosslinking the cleft with a bifunctional spin-label weakens binding, eliminates actin activation, and disorders the actomyosin interface.
Conclusion: Restricting the cleft traps an actomyosin state with structural dynamics intermediate between strongly and weakly bound.
Significance: We have determined the structural dynamics of an elusive intermediate at the threshold of force generation.

We have trapped the catalytic domain of Dictyostelium (Dicty) myosin II in a weak actin-binding conformation by chemically crosslinking two engineered cysteines across the actin-binding cleft, using a bifunctional spin label (BSL). By connecting the lower and upper 50 kDa domains of myosin, the crosslink restricts the conformation of the actin-binding cleft. Crosslinking has no effect on the basal ATPase activity of isolated myosin, but it impairs rigor actin binding and actin-activation of myosin ATPase. EPR spectra of BSL provide insight into actomyosin structural dynamics. BSL is highly immobilized within the actin-binding cleft and is thus exquisitely sensitive to the global orientation and rotational motions of the myosin head. Conventional EPR shows that myosin heads bound to oriented actin filaments are highly disordered with respect to the actin filament axis, in contrast to the nearly crystalline order of myosin heads in rigor. This disorder is similar to that of weakly bound heads induced by ATP, but saturation transfer EPR shows that the disorder of crosslinked myosin is at least 100 times slower. Thus this cleft-crosslinked myosin is remarkably similar, in both actin affinity and rotational dynamics, to SH1-SH2 crosslinked BSL-myosin S1. We conclude that, whether myosin is trapped at the actin-myosin interface or in the force-generating region between the active site and lever arm, the structural state of myosin is intermediate between the weak-binding state preceding phosphate release and the strong-binding state that succeeds it. We propose that it represents the threshold of force generation.

Myosin is the molecular motor that converts chemical energy from ATP hydrolysis into mechanical force to produce muscle contraction. The biochemical steps of ATP hydrolysis are accompanied by a sequence of myosin structural transitions. Force generation by myosin is associated with the transition from a state of weak actin binding, characterized by dynamic disorder, to an ordered, strong actin-binding state (1). The weak-to-strong transition is associated with large-scale rotation of the light-chain domain (LCD) relative to the catalytic domain (CD) (1–5). The rotation of the LCD, acting as a lever arm, displaces the associated actin filament, generating movement.

The large-scale structural change of the LCD with respect to the CD is accompanied by smaller-scale structural changes within functionally important subdomains of the myosin CD, including the nucleotide-binding pocket, the actin-binding cleft, and the force-generating region (converter, relay helix, SH1 helix). These subdomains must be structurally coupled so that force generation results from the productive coordination of actin binding and ATP hydrolysis. A generalized picture of the force-generating powerstroke includes rotation of the LCD coupled to a closing of the actin-binding cleft, opening of the nucleotide pocket, and ordering of the bound myosin head. However, spectroscopy has revealed that a single biochemical state, as defined by the ligands of myosin, can produce multiple structural states, as revealed by high-resolution spectroscopy in the SH1 and relay helices of the force-generating domain (6–8) and the actin-binding cleft (9).

The actin-binding cleft of myosin is a solvent-filled cavity that separates the upper 50 kDa (U50) domain from the lower 50 kDa (L50) domain, each of which contributes to the actin-binding interface. Crystallographic data has led to the proposal that the actin-binding cleft closes as actomyosin transitions from weak binding to strong binding (10). Crystal structures of myosin II, the principal isoform of muscle, are consistently observed with an open cleft, but a high-resolution structure of myosin V is proposed to represent the closed cleft state (11). Cryo-EM data has indicated that the actin-binding cleft is structurally sensitive to nucleotide and actin binding (12–15).

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3 The abbreviations used are: LCD, light-chain domain; BSL, bifunctional spin label; 3,4-bis-(methanethiosulfonfyl-methyl)-2,2,5,5-tetramethyl-2,5-di-hydro-1H-pyrro1-1-yloxy; MSL, maleimide spin label; N-[1-oxyl-2,2,6,6-te-tramethyl-4-piperidinyl]maleimide; EPR, electron paramagnetic resonance; STEPR, saturation transfer electron paramagnetic resonance; S1dC, Dictyostelium discoideum myosin II catalytic domain; Dicty, Dictyostelium.
Spectroscopic measurements on myosin in solution have demonstrated that for isolated sites within the cleft, changes in cleft conformation do occur in response to nucleotide and actin binding. Solvent exposure of the myosin cleft was probed at an engineered tryptophan at position F425W in smooth muscle myosin (16) (equivalent to Ser-416 in Dicty myosin II). Weak-binding (M-ATP, M-ADP-P) states were more solvent-accessible than strong-binding (M-ADP) states in the absence of actin, and actin decreased solvent accessibility, suggesting cleft closure. Actin-induced cleft closure was also suggested by EPR-observed changes in the interspin distance between sites 416 and 537 of Dicty myosin II (17), and by pyrene excimer fluorescence at these same positions (18). We used spin labels attached to several pairs of sites across the cleft to show that the actin-binding cleft exists in a conformational equilibrium between open and closed structural states in all biochemical states, with actin and nucleotides shifting the equilibrium toward the closed and open states, respectively (9). In support of these results, it has been suggested that intrinsic flexibility in the U50 domain of the actin-binding cleft is necessary for myosin to rapidly adopt the strongly bound conformation preceding force generation (13, 19, 20).

In the present study, we ask how restricting the conformational flexibility of the actin-binding cleft affects actomyosin functional interaction and structural dynamics at the actomyosin interface. We have chemically crosslinked two engineered cysteines across the actin-binding cleft using a bifunctional methanethiosulfonate spin label (BSL) attached to residues 416 and 583 of the Dicty catalytic domain. We determined the effects of this crosslinking on myosin and actomyosin ATPase activity, and actin binding affinity, and we used both conventional and saturation transfer EPR to determine the orientation and rotational dynamics of crosslinked myosin when attached to oriented actin filaments. The results provide direct insight into the early steps of actomyosin force generation.

**EXPERIMENTAL PROCEDURES**

**Preparation of Proteins and Muscle Fibers**—Cysteine mutations for spin labeling were introduced into a Dicty myosin II gene truncated at residue 762 (S1dC), containing only a single (non-reactive) cysteine at position 655 (9). These proteins were expressed and purified from Dicty orf+ cells (9). Glycerinated rabbit psoas muscle fiber strips were prepared and stored in a 2m M MgCl2, 1m M EGTA, pH 7.0) and glycerol (21). Actin used in F-buffer (10 mM Tris, 2 mM MgCl2, pH 7.5) was stabilized with acetone powder and purified as described previously (22). F-actin in F-buffer (10 mM Tris, 2 mM MgCl2, pH 7.0) and glycerol (21). Actin used in F-buffer (10 mM Tris, 2 mM MgCl2, pH 7.5) was stabilized with acetone powder and purified as described previously (22). F-actin in F-buffer (10 mM Tris, 2 mM MgCl2, pH 7.0) and glycerol (21). Actin used in F-buffer (10 mM Tris, 2 mM MgCl2, pH 7.0) and glycerol (21).

**Spin-labeling of S1dC**—S1dC was spin-labeled at sites 416 and 583 with the monofunctional label MSL (N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl) maleimide) (Toronto Research Chemicals, North York, ON) overnight on ice using 20 μM myosin and 100 μM MSL in labeling buffer (30 mM Tris, 50 mM KCl, 3 mM MgCl2, pH 7.5). The unbound spin label was removed by desalting the labeled protein using two 7 kDa cutoff Zeba size-exclusion spin columns (Thermo Fisher Scientific Inc., Rockford, IL). The same S1dC mutant was labeled with BSL (3,4-bis-(methanethiosulfonyl-methyl)-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-1-yl-oxy) (Toronto Research Chemicals, North York, ON). A BSL stock solution was prepared in dimethylformamide (DMF). Labeling was carried out at 20 μM myosin and 100 μM BSL in labeling buffer. The reaction was complete within 5 min, based on EPR observation of spin label immobilization. After 1 h, unreacted BSL was removed as described above for monofunctional labeling.

**Determining Extent of Crosslinking by SDS-PAGE**—SDS-PAGE was used to separate BSL-crosslinked from uncrosslinked S1dC, based on gel migration rate. S1dC (1 μM) in Laemmli sample buffer (BioRad), with or without 5% β-mercaptoethanol (Sigma-Aldrich), was applied to a 15% Tris-glycine polyacrylamide gel and run at 150 V for 4 h. Crosslinked and non-crosslinked S1dC was quantified by densitometry using Image J (23).

**Myosin ATPase Activity**—Basal myosin ATPase activity was measured as the release of inorganic phosphate (24) at 25 °C. High-salt calcium ATPase activity was measured in a solution containing 0.0125 mg/ml myosin, 10 mM CaCl2, 600 mM KCl, 5 mM ATP, and 50 mM MOPS (pH 7.5). Actin-activated ATPase activity was measured by detection of ADP generated by the NADH-coupled ATPase assay (25) using 0.5 μM S1dC, 2 mM ATP, and increasing concentrations of phallolidin-stabilized F-actin in 10 mM Tris, 2 mM MgCl2, (pH 7.5).

**Actomyosin Binding Affinities**—The equilibrium binding of actin and myosin was measured by both cosedimentation and fluorescence quenching of pyrene-actin. In cosedimentation assays, varying concentrations of actin were mixed with 1 μM S1dC in F-buffer followed by centrifugation at 340,000 × g using a TLA100 rotor (Beckman Coulter) at 25 °C, to pellet the actomyosin complex. Supernatant and pellet samples were each run on 12% Tris-glycine SDS-PAGE gels that were stained with Coomassie G (Sigma-Aldrich); and band intensity was analyzed by densitometry using Image J (23). The equilibrium constant for dissociation of S1dC from actin (Kd) was determined by fitting with a quadratic binding function with maximal fraction bound constrained to 1. To further assess the weak or strong mode by which S1dC binds to actin, the quenching of pyrene-actin was measured (26). Actin was labeled at Cys-374 with pyrene iodoacetamide (Invitrogen, Carlsbad, CA) (27), and fluorescence was measured for 1 μM pyrene-actin in F buffer, using a Varian Cary Eclipse fluorometer (Varian Inc., Palo Alto, CA) with excitation and emission at 350 nm and 407 nm, respectively. Quenching was assumed proportional to the fraction of actin protoners with strongly bound myosin (25).

**EPR Spectroscopy**—EPR spectra were obtained at X-band (9.5 GHz) with a Bruker (Billerica, MA) E500 spectrometer, using a TE102 (Bruker 4102ST) cavity at a temperature of 4 °C. The sweep width was 120 G (1024 points), sweep time was 40 s, and the center field value Hc was set proportionally to the microwave frequency (Hc = ν/2.803 MHz/G, corresponding to a g value of 2.0027, the value of gₓ for a typical nitroxide) so that all spectra were equivalently aligned.

For EPR experiments on spin-labeled myosin in solution, samples were dialyzed into EPR buffer (10 mM Tris, 2 mM MgCl2, pH 7.5), adjusted to a final concentration of 100 μM, and placed into a flame-sealed glass capillary (50 μl Wiretrol,
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Drummond Scientific, Broomall, PA). For experiments on actin-bound myosin, skinned muscle fiber strips were dissected into bundles of ~0.5 mm diameter. The fibers were then soaked in the 100 μM spin-labeled S1dC solution. After at least 2 h of soaking the fibers were washed several times with EPR buffer to remove any unbound S1dC.

Oriented EPR samples were prepared as previously described (28, 29). S1dC-decorated muscle fiber bundles were blotted to remove excess buffer, cut into 0.5 cm lengths, and aligned perpendicular to the long axis of a quartz tissue flat cell; the fiber axis was manually oriented parallel or perpendicular to the external magnetic field. Spectra of randomly oriented fibers were obtained after mincing the fibers with a razor blade. Some spectra were also obtained with a fiber bundle in a capillary tube oriented either parallel or perpendicular to the magnetic field, using a peristaltic pump to perfuse the fiber such that the buffer was replaced every 30 s (28). This was useful for removing traces of free spin label that was slowly released. Perfusion of a fiber bundle for one hour did not decrease the EPR signal by more than 5%, indicating that virtually all of the labeled myosin was bound to actin.

Conventional EPR spectra ($V_1$) spectra were recorded at a microwave field amplitude of $H_1 = 0.14$ G, with modulation frequency $v_m = 100$ kHz (first harmonic), peak-to-peak modulation amplitude $H_m = 1$ G, and modulation phase $\phi_m = 0$ degrees (maximum signal). The $V_1$ spectrum of a randomly oriented sample was analyzed to determine the rotational correlation time using Equation 1,

$$\tau_R = a[1 - (T'_1/T||)]^b$$  \hspace{1cm} (Eq. 1)

where $a = 0.54$ ns, $b = -1.36$, $T'_1$ is the splitting between the outer extrema, and $T_{||}$ is the rigid-limit value of $T'_1$ (30). Spectra of spin-labeled S1dC attached to actin in oriented muscle fibers were analyzed to determine the orientational distribution of the nitroxide spin label relative to the muscle fiber axis, using computational simulation and least-squares minimization, as described previously (28, 31, 32). STEPR ($V_2$) spectra were recorded as described previously (33), with $H_1 = 0.25$ G, $v_m = 50$ kHz, $H_m = 5$ G, phase-sensitive detection at 100 kHz (second harmonic), $\phi_m = 90$ degrees, and the filter time constant equal to twice the conversion time. The rotational correlation time was determined from $V_2$ spectra by comparison with reference spectra (33).

The extent of spin labeling was quantified by digital analysis of EPR spectra. The double integral of the $V_1$ spectrum of 100 μM S1dC was obtained at low power (1 milliwatt) and compared with that of a sample of known spin label concentration, to obtain the number of spin labels per myosin. For labeling di-Cys myosin, we found precisely two (1.95 ± 0.04) monofunctional MSL labels and one (1.03 ± 0.05) bifunctional BSL label per myosin.

**Molecular Modeling**—Spin labels were modeled on crystal structure 1FMV using Discovery Studio 2.5.5 (Accelrys Software Inc., San Diego) and visualized using the PyMOL Molecular Graphics System, Version 1.5.0.3 (Schrodinger, LLC).

RESULTS

A BSL, with two cysteine-reactive functional groups, was reacted with a double-Cys myosin mutant to form a crosslink containing two mixed (label-protein) disulfide bonds. The mutations S416C and D583C, across the actin-binding cleft from each other (Fig. 1A), were introduced into a Cys-lite Dicty myosin II (S1dC) background suitable for site-directed spectroscopy, as described previously (9). This approach is feasible because S1dC mutants can be expressed in milligram quantities (9), which is not true for muscle myosin. The EPR spectrum of BSL-S1dC was stable at 4 °C for at least 1 day, but at 25 °C, spin label immobilization was partially reversed (less than 5%) on the time scale of hours, presumably because of disulfide exchange to produce an intraprotein disulfide bond and free spin label (34). The conventional EPR ($V_1$) spectrum (Fig. 1B) indicates that the label is strongly immobilized on the nanosecond time scale, i.e. that it is rigidly fixed to the protein, as expected for the bifunctional attachment of BSL (1, 28, 29, 35).

Dipolar EPR measurements between MSL-labeled S416C and D583C (Fig. 2, A and B) were used to determine the distance between these two labeling sites in solution. Fitting the EPR spectrum to a Gaussian distribution shows that the myosin cleft samples two major conformations, in which residues 416 and 583 are separated by ~0.8 and 1.2 nm (Fig. 2C) (9). The distance between the two sulfur atoms in BSL is ~0.9 nm, establishing the feasibility of crosslinking cysteine residues 416 and 583 with BSL.

**Extent of BSL-Crosslinking**—In addition to monitoring BSL immobilization by conventional EPR, the extent of myosin crosslinking by BSL was determined by separating the cross-
linked and non-crosslinked myosins by SDS-PAGE under non-reducing conditions (Fig. 3). The crosslinked myosin migrates more slowly through a polyacrylamide gel so that the band is shifted to higher apparent molecular weight. When BSL is reacted with myosin at a molar ratio of 0.5 BSL per myosin, two bands are observed (Fig. 3). The lower band (\(88 \text{kDa}\)) represents non-crosslinked myosin, while the upper band (\(100 \text{kDa}\)) represents crosslinked myosin. When a 5-fold molar excess of BSL is used, crosslinking is essentially complete, as shown by a single band at 100 kDa. No gel shift is observed for unlabeled or MSL-labeled myosin (Fig. 3). Since the BSL-crosslink consists of two mixed protein-label disulfide bonds, it can be reversed by treatment with a reducing agent such as β-mercaptoethanol (βME) or dithiothreitol (DTT). Inclusion of βME in the gel sample results in reduction of the BSL-crosslink so that the sample migrates at the same rate as the unlabeled or MSL-labeled myosin (Fig. 3).

**Myosin ATPase Activity**—In the absence of actin, the basal myosin ATPase activity (0.010 ± 0.002 s\(^{-1}\)) was not affected by crosslinking (0.012 ± 0.002 s\(^{-1}\)). However, crosslinking of the myosin cleft with BSL induces a virtually complete loss of actin-activated ATPase activity, in comparison with unlabeled or MSL-labeled myosin (Fig. 4, A and B). Consistent with SDS-PAGE gel-shift data (Fig. 3), treatment of BSL-crosslinked myosin with a reducing agent reverses the functional effect of crosslinking, with no effect on unlabeled or MSL-labeled myosin (Fig. 4B).

**Actomyosin Interaction**—Cosedimentation of myosin with actin in the absence of nucleotide (i.e. in rigor) shows that unlabeled and MSL-labeled 416.583 S1dC bind actin strongly, with...
the actin concentration is in large excess above the whenever we study EPR of BSL-S1dC in the presence of actin, spectra of the actin-bound complex. In EPR studies below, weaker than in rigor, it is still strong enough to obtain EPR values of 0.03 ± 0.01 μM (UL), 0.4 ± 0.1 μM (MSL), and 20.6 ± 0.2 μM (BSL). B, fluorescence quenching of 1 μM pyrene-actin by 20 μM S1dC. Data represent mean ± S.E., n = 3.

FIGURE 5. BSL weakens interaction of myosin with actin in the absence of ATP. A, fraction of myosin S1dC bound to actin from cosedimentation. Data were fit by the quadratic binding function $y = \frac{[(M)_t + [A]_t + K_d]}{[(M)_t] + [A]_t + K_d^2 - 4[M]_t[A]_t} \frac{1}{2[A]_t}$ where $M$ = [myosin] and $A$ = [actin], yielding $K_d$ values of 0.03 ± 0.01 μM (UL), 0.4 ± 0.1 μM (MSL), and 20.6 ± 0.2 μM (BSL). B, fluorescence quenching of 1 μM pyrene-actin by 20 μM S1dC. Data represent mean ± S.E., n = 3.

Myosin Rotational Dynamics and Orientation—Conventional EPR ($V_1$) is sensitive to rotational motion in the range from about 0.01 to 600 ns (1, 38). The $V_1$ spectrum of BSL-labeled S1dC in solution has the characteristic lineshape of a strongly immobilized spin label (Fig. 6A, black), confirming its rigid bifunctional binding to two Cys. Actin binding, causes a small increase in label restriction (Fig. 6A, green), increasing the splitting between the outer peaks by 0.90 ± 0.05 G. If, as indicated by further studies below (Fig. 6B), we assume that actin-bound S1dC is at the rigid limit for conventional EPR (rotational correlation time $\tau_R > 1 \mu s$), $\tau_R$ for BSL-S1dC free in solution is 200 ± 20 ns (Equation 1), corresponding to the expected value at 4 °C for a rigid protein the size of S1dC (29, 39). This result confirms that BSL is rigidly bound to S1dC, but it does not provide much information about the rotational dynamics of BSL-S1dC on actin.

This requires saturation transfer EPR (STEPR, $V_2'$), which is sensitive to rotational correlation times $\tau_R$ as long as 600 μs (1, 38, 40). Thus STEPR is ideal for measuring global rotational motions of large proteins within macromolecular assemblies, such as actomyosin (1, 38). Unlike conventional EPR (Fig. 6A), STEPR is extremely sensitive to the affect of actin binding on the rotational dynamics of BSL-S1dC (Fig. 6B). Rotational motion of BSL-S1dC on actin is at or near the rigid limit of 600 μs for STEPR (Fig. 6B, green), at least 6000 times slower than S1dC free in solution (Fig. 6B, black), indicating that any remaining rotational motion is in the millisecond range or slower. The rotational motion of actin-bound BSL-S1dC is at
at least 60 times slower than previously observed for MSL-S1dC weakly attached to actin in the presence of saturating ATP (41–44) and is virtually identical to that observed for strongly bound MSL-S1dC in rigor (45).

EPR is not only sensitive to spin-label mobility but also to the orientational distribution of a spin-label with respect to the applied magnetic field. In a well-oriented muscle fiber, aligned either parallel or perpendicular to the magnetic field, the EPR spectrum directly reports the angle between the spin-label principal axis and the fiber axis (31). The parallel and perpendicular spectra can be used together to determine the degree of orientation of the spin-label relative to the fiber axis. The orientation of BSL- or MSL-labeled myosin bound to actin, with respect to the actin filament axis, was determined from EPR spectra of skinned muscle fibers in the presence with labeled myosin (Fig. 6C). As shown previously, myosin spin-labeled with MSL at SH1 (T688C) is highly ordered with respect to the filament axis as indicated by the large differences observed between EPR spectra acquired in the parallel and perpendicular orientations (Fig. 6C, MSL) (31). In contrast, fibers decorated with BSL-S1dC yield EPR spectra showing little difference between the parallel and perpendicular orientations, indicating nearly random orientation (Fig. 6B).

The relatively static disorder of myosin on actin, due to the cleft crosslink is in good agreement with that observed for myosin trapped by crosslinking SH1 and SH2 in the force-generating domain of myosin obtained from muscle (28, 29) or Dicty (46). Virtually all of the BSL–myosin in skinned fiber bundles in Fig. 6 must be bound to actin, because the STEPR spectrum is at or near the rigid limit of STEPR, indicating a rotational correlation time $\tau_R \geq 1 \text{ ms}$, which is at least 5000 times slower than free myosin in solution (1). In addition, it is not plausible that the orientational disorder is due to a significant population of unbound myosin, since the $K_D$ of the cleft-crosslinked S1dC is 20.6 $\mu M$ (Fig. 5), which is at least 18 times less than the concentration of unoccupied actin in a vertebrate muscle fiber (47).

**DISCUSSION**

We have found that the actin–myosin interaction is impacted profoundly, both functionally and structurally, by conformationally restricting the myosin actin-binding cleft through chemical crosslinking with a BSL. The crosslink forms rapidly and completely between a pair of labeling sites, 416 and 583, in the U50 and L50 domains of the actin-binding cleft. The ATPase activity of isolated myosin is not affected by this crosslink, but actin cannot activate it (Fig. 4), and myosin’s affinity for actin in the absence of ATP is decreased by $\geq 20$-fold (Fig. 5B). Even when it binds to actin, this crosslinked myosin loses its ability to quench pyrene-actin fluorescence (Fig. 5A), consistent with the formation of a weak actomyosin bond. Structurally, crosslinking the cleft induces an actin-bound myosin catalytic domain with a high degree of orientational disorder but very slow rotational motion on the microsecond time scale (Fig. 6). The disorder is similar to that reported previously for weakly bound heads induced by ATP, but the rotational motion is orders of magnitude slower, similar to that of strongly bound heads in rigor. These results are virtually identical to those obtained with myosin crosslinked between SH1 and SH2 in the force-generating domain (28, 29), indicating that a similar intermediate was trapped. Fig. 7 illustrates these results in the context of the actomyosin ATPase cycle.

**Effects of Cleft Crosslinking on Actomyosin Functional Interaction**—During the actomyosin ATPase cycle, myosin transitions from a dynamically disordered, weak actin-binding state (W, Fig. 7, left) to an ordered, strong actin-binding state (S, Fig. 7, right). This transition from W to S also involves a shift in the populations of the actin-binding cleft structural states (“open” versus “closed”), with $S$ stabilizing the closed conformation. Extensive experimental analysis has sought to define the biochemical and structural properties of $W$ and $S$ states of the actomyosin complex. The $W$ state appears to be stabilized by hydrophobic interactions between loop 2 of the L50 domain of myosin and actin (13, 15, 48, 49). $K_D$ for actomyosin weak binding using the Dicty myosin II motor domain has been previously reported as $\sim 60 \mu M$ (50). In contrast, strong binding is characterized by $K_D < 1 \mu M$, stabilized by predominantly electrostatic interactions with the U50 domain (13, 49, 51–53). Generally, mutations within both the weak- and strong-binding interfaces decrease myosin affinity for actin and reduce actin-activated ATPase activity. Crosslinking the cleft produces an intermediate $K_D$ of 20 $\mu M$ (Fig. 5A), and complete loss of actin-activated myosin ATPase activity. Since 416.583 BSL-labeled S1dC interacts with actin but its ATPase activity is not activated by actin, we hypothesize that the crosslink stabilizes a conformation of the actin-binding interface that can interact with actin at weak-binding contacts while still containing bound Pi. Together these results indicate that crosslinked myosin is trapped in a weak actin-binding state and suggest that BSL crosslinking the actin-binding cleft severely diminishes the coupling between the actomyosin interface and the myosin active site. Structurally, the trapped state is probably characterized by a partially open actin-binding cleft that preferentially stabilizes a weak-binding interface and a closed nucleotide pocket, preventing Pi release (Fig. 7). We have previously shown that the actin-binding cleft exists in a conformational equilibrium between “open” and “closed” structural states in all biochemical states tested, with strong actin binding preferentially stabilizing the closed cleft conformation (9). We have now demonstrated directly the necessity of conformational flexibil-
ity within the actin-binding cleft, allowing the myosin cleft to properly transition between “open” and “closed” structural states, which is essential for actin-activation and transition to the strong actomyosin binding state. Consistent with our data, recent cryo EM data suggests that intrinsic flexibility of the U50 domain is necessary for myosin to properly transition to the strong binding conformation (13). In addition, simulations of the U50 domain from various crystal structures reveals that U50 flexibility is dependent on biochemical state and is proposed to underlie myosin ability to rapidly adopt its strong binding state (20).

Effects of Cleft Crosslinking on Actomyosin Structural Dynamics—As illustrated in (Fig. 7), cleft crosslinking creates an actomyosin complex with hybrid structural and dynamical properties: The orientational disorder of the actin-bound head is great (Fig. 6C, BSL), comparable to that induced in the absence of crosslinking by ATP (31, 54–57) and in sharp contrast to the high degree of orientational order observed in the absence of ATP for uncrosslinked myosin bound to actin (Fig. 6C, MSL) (29, 31, 54, 58). In contrast, the rotational motion of the crosslinked catalytic domain is very slow, undetectable on the submillisecond time scale (Fig. 6B, BSL), comparable to that observed in the absence of ATP for uncrosslinked myosin bound to actin (29, 39, 45, 59), and several orders of magnitude slower than the microsecond dynamics induced by ATP (41–44, 59, 60).

Comparison with Effects of other Internally Crosslinked Myosins—Although this is the first study to crosslink the actin-binding cleft of myosin, there have been numerous studies in which a crosslink was introduced between reactive cysteines SH1 and SH2 in the force-generating region of the catalytic domain of myosin, from rabbit skeletal muscle (28, 29, 61, 62) or Dicty myosin II (46). In all cases, the SH1–SH2 crosslink greatly reduced actin activation and actomyosin affinity, indicating that myosin was trapped in a weak-binding conformation. In one of these studies, rabbit skeletal myosin II was crosslinked with BSL at SH1 and SH2, yielding a $K_d$ for actomyosin interaction of 30 μM (29); similar to the 20 μM value observed here for cleft crosslinking by BSL in Dicty myosin II. Crosslinking SH1 and SH2 with BSL produced an actin-bound myosin catalytic domain with a high degree of orientational disorder and very slow rotational motion ($\tau_r \geq 1$ ms) (28, 29), just as we found in the present study with a crosslink in the cleft (Fig. 6). It is remarkable that conformational trapping of myosin at two distant locations, one in the actin-binding cleft and another in the force-generating region, have strikingly similar effects on actomyosin structure, dynamics, affinity, and function. This result strongly suggests that crosslinking the actin-binding cleft, like crosslinking SH1 and SH2, can trap myosin in an intermediate state between weak binding and rigor; i.e. early in force generation (Fig. 7). In the absence of crosslinking, this state is probably stabilized by ATP hydrolysis products (ADP + P$_i$) within the nucleotide binding pocket and shares the orientational disorder characteristic of ATP-induced weak binding, but the slow rotational motion of strong binding (Fig. 7) (29).

Allosteric Coupling—The similar effects of crosslinking the actin-binding cleft and SH1–SH2 region of the force-generating domain demonstrate that intrinsic conformational flexibility within the myosin catalytic domain and structural coupling between functionally important subdomains are required for force generation. Continued analysis of the intricate structural and biochemical coupling between myosin subdomains, in space and time, is needed to understand the mechanism of force transduction in actomyosin. Specifically, how tightly coupled is the communication pathway from the actin interface to the nucleotide pocket or to the force-generating domain? There is evidence for coupling between the actin-binding cleft and nucleotide pocket (63–65), as well as between the nucleotide pocket and the force-generating domain (66, 67). Recent work suggests that there is direct coupling between the actin-binding interface, particularly the myosin activation loop, and the relay helix in the myosin force-generating domain (68). We must next ask how conformationally trapping the actin-binding cleft affects the nucleotide-binding pocket structural dynamics, and the orientation of the myosin lever arm.

CONCLUSION

We produced a chemically modified Dicty myosin II catalytic domain, in which the actin-binding cleft is conformationally trapped by crosslinking the U50 and L50 domains with a bifunctional methanethiosulfonate spin label: Crosslinking produces a myosin catalytic domain that cannot be activated by actin and has weakened actin affinity. When bound to actin in an oriented muscle fiber, this protein is highly disordered with respect to the actin filament axis, and is characterized by very slow rotational motion on the microsecond time scale. Cleft-crosslinked myosin has greater binding affinity and slower motion on actin than that observed for ATP-induced weak-binding states. Thus it resembles the weak-binding state induced by crosslinking SH1 to SH2 in the force-generating domain, which is proposed to be the structural state that is at the threshold of force generation.

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