Amplitude of the actomyosin power stroke depends strongly on the isoform of the myosin essential light chain

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We have used time-resolved fluorescence resonance energy transfer (TR-FRET) to determine the role of myosin essential light chains (ELCs) in structural transitions within the actomyosin complex. Skeletal muscle myosins have two ELC isoforms, A1 and A2, which differ by an additional 40–45 residues at the N terminus of A1, and subfragment 1 (S1) containing A1 (S1A1) has higher catalytic efficiency and higher affinity for actin than S1A2. ELC’s location at the junction between the catalytic and light-chain domains gives it the potential to play a central role in the force-generating power stroke. Therefore, we measured site-directed TR-FRET between a donor on actin and an acceptor near the C terminus of ELC, detecting directly the rotation of the light-chain domain (lever arm) relative to actin (power stroke), induced by the interaction of ATP-bound myosin with actin. TR-FRET resolved the weakly bound (W) and strongly bound (S) states of actomyosin during the W-to-S transition (power stroke). We found that the W states are essentially the same for the two isoenzymes, but the S states are quite different, indicating a much larger movement of S1A1. FRET from actin to a probe on the N-terminal extension of A1 showed close proximity to actin. We conclude that the N-terminal extension of A1 serves as binding sites for one essential light chain (ELC) and one regulatory light chain (RLC) and is proposed to function as a lever arm that amplifies small structural changes in the CD into the larger motions by which myosin exerts force on actin. In mammalian muscle, RLC modulates the interaction of the myosin head with actin through phosphorylation of residues near the N terminus (8, 9), whereas the functional role of ELC is not as well understood. However, important insight into ELC function has been gained by studying its distinct isoforms. Fast skeletal muscle has two ELC isoforms, A1 and A2, which differ primarily at their N-terminal regions, where A1 contains an additional domain containing 40–45 aa (10). In contrast, slow skeletal muscle and heart contain only the longer A1 isoform (11). In cardiac muscle, both ventricular and atrial ELC isoforms have N-terminal extensions of approximately the same length as their A1 counterparts in skeletal muscle and are important regulators of cardiac contractility (12, 13). In smooth muscle and nonmuscle myosins, only the short (A2) isoforms are expressed (14, 15). Functional differences between the A1 and A2 isoforms are observed only in the presence of actin (16), resulting in much higher catalytic efficiency for the A1 isoform (17) and higher in vitro actin motility for A2 (18).

Cryo-EM of skeletal muscle acto-S1 (19) suggests that the N terminus of A1 is near the C terminus of actin in the absence of nucleotide (Fig. 1). Removal of ELC from skeletal muscle myosin strongly inhibits the in vitro motility of actin and actomyosin force while retaining ~50% of ATPase activity (12, 20–22). Myosin S1 containing A1 (S1A1) showed higher apparent affinity for actin (lower $K_{\text{ATPase}}$) than S1A2, suggesting that intermolecular contacts between A1 and actin result in stronger and slower interaction of myosin cross-bridges with actin (18, 23, 24). Differences have been observed even between the two long isoforms of cardiac A1, indicating different cycling kinetics of cross-bridges containing different ELCs (25). In smooth muscle myosin, A2 modulates the actomyosin interaction through phosphorylation of RLC (26). It is clear that the mechanism of regulation of actomyosin by ELC warrants further study.

We hypothesize that functional differences associated with the two ELC isoforms in skeletal muscle involve isoform-specific structural explanation for previous functional observations, with implications for muscle pathophysiology and therapeutic design.

Significance

Myosin is an enzyme that uses energy from ATP to exert force on another protein, actin, resulting in muscle contraction. Force is generated in the power stroke, when the actin–myosin complex transitions from a weak-binding structure (ATP bound to myosin) to a strong-binding structure (ADP). We detected the amplitude of the power stroke directly by measuring fluorescence resonance energy transfer from actin to myosin, with high time resolution following excitation with a pulsed laser. We found that the amplitude of the power stroke is strongly dependent on the form of the essential light chain that is bound to myosin. This provides a structural explanation for previous functional observations, with implications for muscle pathophysiology and therapeutic design.

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effects on the structural states of both actin and myosin in the actomyosin ATPase cycle. We have previously shown that S1A1 is more effective than S1A2 in restricting the amplitude of actin’s intrafilament rotational dynamics, particularly in the presence of saturating ATP (27), suggesting that complexes of S1A1 and S1A2 with actin are structurally different during the ATPase cycle. To test this hypothesis, we have expressed and purified A1 and a truncated A1 with 44 residues deleted from the N terminus (referred to below as A2) and prepared homogeneous samples of S1A1 and S1A2. We labeled actin at C374 with a donor probe Alexa Fluor 568 (AF568), and we labeled each ELC at its native C-terminal cysteine (C180 in A1, C136 in A2) with an acceptor probe Alexa Fluor 647 (AF647) (Fig. 1). For a better understanding of the role of A1’s N-terminal extension in actin-myosin interaction, we engineered a cysteine at position 16 (A16C) of the A1 (Fig. 1) and labeled it with DABCYL maleimide, to serve as the acceptor for fluorescence resonance energy transfer (FRET) from IAEDANS (donor) attached to actin C374. Structural transitions in the labeled acto-S1 complexes during transitions from weakly to strongly bound states were detected using time-resolved FRET (TR-FRET). A key advantage of TR-FRET is its ability to resolve and measure distances within a bound complex directly, without interference from unbound proteins (28, 29). This is particularly important for measurements in the presence of ATP, which decreases the affinity of myosin for actin, so the bound complex is only a fraction of the total protein population. The results indicate that the W-to-S structural transition in the actomyosin complex during the ATPase cycle is strongly modulated by ELC isoforms, with profound implications for contractile function.

Results

Enzymatic Properties of Labeled Isoenzymes. We have examined the effects of labeling and exchange of A1 and A2 into myosin S1 by measuring functional properties of the two isoenzymes, S1A1 and S1A2. The interaction of these isoenzymes with actin is reflected in their enzymatic activities (SI Methods, Fig. S1A, and Table 1). $V_{\text{max}}$ and $K_{\text{ATPase}}$ of S1A1 are independent of the position of the probe (C180 or C16). $V_{\text{max}}$ of S1A1 is $\sim$40% that of S1A2, whereas the apparent actin affinity ($1/K_{\text{ATPase}}$) is more than seven times greater (Table 1), so the catalytic efficiency ($V_{\text{max}}/K_{\text{ATPase}}$) of acto-S1A1 is nearly four times that of acto-S1A2. These results are similar to those reported previously for unlabeled proteins (23, 30), indicating that labeling and ELC exchange did not perturb the enzymatic properties of the two isoenzymes. This is consistent with a previous report that the rate of product release is the same for both isoenzymes, but for S1A1 other processes become rate limiting (17). $K_{\text{d}}$ measured by cosedimentation (Fig. S1B) confirmed the much greater actin affinity of S1A1 compared with S1A2 (Table 1), consistent with previous reports for unlabeled proteins (31).

Strongly Bound Complex. In the absence of ATP, actin affinity (measured by cosedimentation) and quenching of pyrene-labeled actin were essentially the same for both isoenzymes, showing that both isoenzymes, S1A1 and S1A2, bind actin stoichiometrically with submicromolar affinity. Time-resolved fluorescence shows that binding of S1 (labeled in the C-terminal lobe of ELC with the AF647 acceptor) to actin (labeled at C374 with the AF568 donor) leads to a faster decay (shorter lifetime) of the donor in both isoenzymes (Fig. 2A, black to green), indicating FRET. Analysis of FRET decays ($F_{\text{D,A}}$) (SI Methods) of strongly bound complexes (Fig. 2B, green) showed that the mean distance $R_s$ between donor and acceptor probes in the strongly bound complex is independent of the fraction of bound acceptor-labeled S1, at substoichiometric as well as excess concentrations of added S1 (Fig. 3A), showing that FRET is not affected by the proximity of multiple acceptors and that the unbound S1 does not contribute to FRET. The distance $R_s$ was quite dependent on the ELC isoform (Figs. 2B, green, and 3A), but the width of the distribution (FWHM = $\Gamma$) was not. The shorter $R_s$ value in S1A1 (than in S1A2) is consistent with our previous results suggesting that interaction of the N terminus of A1 with actin (Fig. 1) results in increased rigidity of actin (27).

Weakly Bound Complex. Resolution of the structural states of the W complex required acquisition of TR-FRET decays during the brief steady state of the actomyosin ATPase cycle. AF647-labeled S1 (5–10 μM final concentration) was added to AF568-labeled actin (1 μM final concentration), followed by mixing with a saturating concentration of ATP (1–3 mM). ATP decreased the rate of decay, indicating a decrease in FRET (Fig. 2A, red) and thus an increase in interprobe distance (Fig. 2B). We recorded a series of TR-FRET waveforms at intervals of several seconds and analyzed them globally (SI Methods) to determine the distance distribution corresponding to the W state (Fig. 2B, red), the mole fraction of actin having bound myosin (Fig. 2C, $X_B$), and the mole fractions in the W and S complexes (Fig. 2C, $X_W + X_S = X_P$). For both S1A1 and S1A2, the steady state of ATP hydrolysis persisted ~1 min after addition of ATP, as reflected in the constant values of bound mole fractions ($X_B$) of S1A1 and S1A2 (Fig. 2C). Acquisition of high signal/noise waveforms at intervals of several seconds, as required to accurately capture the steady-state signal (Fig. 2, red) was made possible by our direct waveform recording technology.

Table 1. Interaction of labeled S1 isoforms with labeled actin in the presence of saturating ATP

<table>
<thead>
<tr>
<th>S1</th>
<th>$V_{\text{max}}$ s$^{-1}$</th>
<th>$K_{\text{ATPase}}$ μM</th>
<th>$K_{\text{d}}$ μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1A1</td>
<td>9.2 ± 0.3</td>
<td>4.9 ± 0.7</td>
<td>12.3 ± 1.0</td>
</tr>
<tr>
<td>S1A1(A16C)</td>
<td>8.8 ± 0.6</td>
<td>4.9 ± 1.7</td>
<td>6.7 ± 1.1</td>
</tr>
<tr>
<td>S1A2</td>
<td>24.8 ± 4.7</td>
<td>36.7 ± 13.4</td>
<td>47.2 ± 5.0</td>
</tr>
</tbody>
</table>

$SE$s were calculated by the fitting program.
(32). The steady-state value of $X_B$ was $0.64 \pm 0.02$ for S1A1 and $0.38 \pm 0.01$ for S1A2. With increasing time of data acquisition, leading to exhaustion of ATP, in all cases $X_B$ gradually increased up to the level of the strongly bound complex (Fig. 2C). Control experiments (Fig. S2) showed that the dissociation of the weakly bound acto-S1 complex by adding 0.1 M KCl completely eliminated FRET, indicating that all S1 is capable of binding ATP, thus weakening the interaction with actin.

For acto-S1A1 with AF647 at C180, the $W$ and $S$ states are clearly distinct in structure (Fig. 2B, Top); the mean distance between probes on actin and C180 of A1 decreases from $9.6 \pm 0.2$ nm in $W$ to $7.9 \pm 0.1$ nm in $S$. The widths $\Gamma_W$ and $\Gamma_S$ are not significantly different. For acto-S1A2 with AF647 at C136, the $W$ and $S$ states show only a small difference in the actin–C136 distance (Fig. 2B, Bottom): $R_W = 9.7 \pm 0.2$ nm and $R_S = 9.3 \pm 0.1$ nm. However, the width of the distance distribution in the $W$ state ($\Gamma_W = 6.0 \pm 1.2$ nm) is nearly double that in the $S$ state ($\Gamma_S = 3.6 \pm 0.5$ nm). Thus, the $W$→$S$ transition in acto-S1A2 is associated primarily with a disorder→order transition. The distance of separation ($R_W$) is independent of the fraction of bound myosin for both isoforms (Fig. 3B), indicating that, as in the FRET analysis of strongly bound complexes (Fig. 3A), the mean distances obtained in FRET analysis of weakly bound complexes are not affected by the presence of multiple acceptors and that the unbound S1 does not contribute significantly to FRET. We conclude that the $W$→$S$ structural transition in actomyosin, as detected by FRET between actin and ELC, is strongly modulated by the ELC isoform, and the extent of the transition is greater for S1A1 than for S1A2.

**FRET from Actin to Probe Near the N Terminus of A1.** To test the hypothesis that the N terminus of the A1-ELC interacts with actin (Fig. 1), we performed TR-FRET from actin 374, labeled with the IAEDANS donor, to S1A1, labeled at A16C of ELC with the DABCYL acceptor (Fig. 4). Analysis (Fig. 4, Inset) shows that in the $S$ state, the distance between IAEDANS-labeled actin and the N terminus of A1 is $R_S = 2.9 \pm 0.2$ nm, whereas in the $W$ state this distance increases to $R_W = 3.6 \pm 0.2$ nm. These distances are clearly short enough to support the proximity (suggesting tethering) of the N terminus of A1-ELC to actin, and the distance change is in the same direction as that observed for the probe in the C lobe (Fig. 2B, Top).

**Discussion**

The distance between probes on actin and myosin depends strongly on the ELC isoform and the presence of ATP (Figs. 2–4), suggesting the model in Fig. 5. In the weak-binding state $W$, the mean distance $R_W$ between probes on actin and the C lobe of ELC (C180 in A1 and C136 in A2) was essentially the same for acto-S1A1 ($9.6 \pm 0.2$ nm) and acto-S1A2 ($9.7 \pm 0.2$ nm), but the width $\Gamma_W$ was much greater in acto-S1A2 (Fig. 2B), indicating increased disorder (Fig. 5, Top Right). In the strong-binding state $S$, substantial ELC-dependent differences were detected. In acto-S1A1, the mean distance $R_S$ between actin and C180 was $7.9 \pm 0.1$ nm, indicating a decrease by $1.7 \pm 0.3$ nm during the $W$→$S$ transition. A significant decrease in distance was also observed with the acceptor near the N terminus of A1, from $3.6 \pm 0.2$ nm ($W$) to $2.9 \pm 0.2$ nm ($S$) (Fig. 4). In acto-S1A2, the mean distance $R_S$ between actin and C136 was significantly longer than in acto-S1A1, $9.3 \pm 0.1$ nm, so the change during the $W$→$S$ transition for the A2 complex was much smaller ($0.4 \pm 0.3$ nm). Assuming that the actin-ELC distance depends on the orientation of the LCD (lever arm) relative to actin, these results indicate that the LCD in S1A1 undergoes substantial rotation during the $W$→$S$ transition (Fig. 5, Left), whereas the extent of this rotation in S1A2 is much less and is primarily an order–disorder transition (Fig. 5, Right). Previous steady-state FRET measurements using acto-S1A1 also detected a
change in the LCD position relative to actin during the W-to-S transition (33), but the lack of time resolution prevented accurate analysis of the bound W complex, and no studies were done on S1A2.

The detected distance $R_S = 9.3 \pm 0.1$ nm between probes on C374 of actin and C136 of A2 in the S acto-S1A2 complex is in good agreement with the predicted 9.0-nm distance between α-carbons of these residues in the currently available structural model of skeletal acto-S1, based on the crystal structure of S1A2 (6, 34). Starting from the post-power-stroke position, we modeled the W-to-S transition in acto-S1A2, assuming upward rotations of LCD for three experimentally reported angles 70° (smooth muscle myosin) (7), 18° (skeletal muscle myosin) (35), and 36° (scallop myosin) (36) (SI Methods, Molecular Modeling of the Power Stroke, Fig. S3, and Tables S1 and S2). Of these three models the 18° power stroke observed for skeletal muscle myosin agrees best with our FRET data (Fig. S3 and Tables S1 and S2). Our FRET data clearly do not agree with a much larger LCD movement (70°) proposed on the basis of crystallographic studies on smooth muscle myosin (7). This suggests that the extent of the power stroke is myosin specific, and changes predicted for one myosin isoform cannot be easily applied to another myosin isoform.

Role of the N-Terminal Extension of A1 in the Structural States of the Actomyosin Complex. The only published structural model for acto-S1A1 (Fig. 1) was generated by computational modeling of the N-terminal extension of A1 into the acto-S1A2 complex, assuming that the position of the C terminus of ELC and of the whole LCD is the same for both, S1A1 and S1A2. Our data indicate that this assumption is not correct. In the S complex of S1A1, the distances from actin 374 to probes on A1-ELC are substantially shorter than computed by the model in Fig. 1: C180 was observed at 7.9 ± 0.1 nm and C16 at 2.9 ± 0.2 nm (Figs. 2B, 3, and 4), whereas the model in Fig. 1 predicted 9.0 nm and 3.6 nm, respectively. The most probable explanation for the shorter observed distances in the strongly bound acto-S1A1 complex is that the binding of the N-terminal extension of A1 to actin shifts the LCD position toward the actin filament (Fig. 5). This result is consistent with the previously published results of electron microscopy (19), functional mutations (37), cross-linking (30), and molecular modeling (38). In contrast to the ELC-dependent distances in the strongly bound complex, in the weakly bound complex the distance within acto-S1A1 (9.6 ± 0.2 nm) was essentially the same as in acto-S1A2 (9.7 ± 0.2 nm). Thus, the modeling of the W position of LCD in acto-S1A2 probably applies to acto-S1A1. Despite similar mean distances, the structural states of weakly bound acto-S1A1 and acto-S1A2 complexes are clearly different, as the width of the distance distribution is greater for S1A2 (6.0 ± 1.2 nm) than for S1A1 (3.6 ± 0.5 nm) (Fig. 2B). This can be explained by stabilization of the position of LCD by the actin-bound N terminus in the W state of S1A1 (Fig. 4).

Our findings lead to a structural model of the W-to-S transition in which the acto-myosin power stroke, proposed as a swing of the LCD domain in response to binding and hydrolysis of ATP in the CD (6), is regulated by the interaction of the N-terminal extension of A1 with actin (Fig. 5). In this model, the W-to-S transition in acto-S1A1 is associated with a shift of the N-terminal extension on actin, which facilitates the downward swing of the LCD and stabilizes the post-power-stroke state of LCD. In the absence of the N-terminal extension (S1A2), the power stroke has much smaller amplitude and is characterized primarily as an order-to-disorder transition. This model is consistent with our previous studies focused on actin dynamics, where the W-to-S transition was much greater for acto-S1A1 than for acto-S1A2 (27).

Previous spectroscopic studies on skeletal muscle fibers detected the power stroke of the LCD, but effects of ELC isoforms were not evident. These studies (EPR, fluorescence...
although all myosins go through the actomyosin ATPase cycle, the differences between ELC and the CD show substantial differences between muscle myosin. The structure of the converter region and the interactions are not required for a power stroke. However, nonmuscle myosins (45) with short, A2-like ELC indicated that the interaction of the ELC isoform on LCD movement. Subsequent EM of the myosin power stroke was based on the crystal structure of actomyosin in pre (red)- and post (green)-power-stroke states, based on TR-FRET measured from actin C374 to the C lobe and the N terminus of ELC (gray arrows). S1A1 (left) undergoes a substantial W-to-S transition relative to actin, whereas this movement is much smaller for S1A2. The N-terminal extension of A1 is shown as a curved blue line.

polarization, and fluorescence anisotropy (35, 39, 40) revealed incorporation of labeled light chains (RLC as well as ELC) into fibers, and the effects of specific ELC isoforms were probably obscured by (i) incomplete replacement with labeled ELC, (ii) unknown isoform composition in myosin heterodimers, and (iii) the inability to distinguish between free and actin-bound heads. In the present study, we have overcome these limitations by measuring TR-FRET on actin-bound heads containing exclusively one ELC isoform.

The importance of the N-terminal extension of A1 for the extent of the power stroke is most evident in cardiac myosin, where A1 is the only isoform of ELC and truncation of its 43 N-terminal residues negatively affects cardiac contractility (41). However, the lack of the N-terminal extension is not necessarily detrimental for muscle function (42). Absence of the N-terminal extension generates lower force (41), probably causing a less prominent power stroke in S1A2, and is not related to abnormal cardiac function. On the other hand, in the skeletal muscle of aged rats, replacement of A1 in a fraction of heads by A2 improved contractility by increasing the rate of shortening (43). This is consistent with the hypothesis that the N-terminal extension enhances iso-metric force while slowing the speed of unloaded shortening. These effects show that ELC isoforms regulate muscle contractility, but also indicate that this regulation is muscle specific.

Relationship to Myosin Crystal Structures. The conventional model of the myosin power stroke was based on the crystal structure of skeletal muscle S1 containing a truncated form of A1, which is equivalent to S1A2 (6), so this model was unable to predict the effect of the ELC isoform on LCD movement. Subsequent EM and crystallographic studies on smooth muscle (7), scallop (44), or nonmuscle myosins (45) with short, A2-like ELC indicated that the N-terminal extension is not required for a power stroke. However, these myosins are structurally different from skeletal muscle myosin. The structure of the converter region and the interactions between ELC and the CD show substantial differences between skeletal, smooth (7), and scallop myosins (44). Furthermore, although all myosins go through the actomyosin ATPase cycle, the equilibria and kinetics within the cycle are myosin specific. The isoform-specific transmission of nucleotide-induced structural changes from the CD to the LCD, which is the essence of the power stroke, is probably necessary to accommodate diverse functional roles of actomyosins in muscle and nonmuscle cells.

Conclusion

The N-terminal extension in the A1 light chain of myosin ELC enables S1A1 to form a complex with actin that is structurally different from that of S1A2 in both pre- and post-power-stroke states. This result provides a compelling explanation for the dependence of muscle function on the ELC isoform, with potentially profound implications for the function and regulation of striated muscle. Future FRET studies are needed to test and refine these models (Fig. 5).

Methods

Protein Preparations and Labeling. Actin was prepared disc from rabbit skeletal muscle by extracting acetone powder in cold water, as described before (3). A total of 140 μM Alexa Fluor 568 C2 maleimide and/or 700 μM 1-5 IAEDANS, used as donor, freshly dissolved in dimethylformamide, was added to 0.2 mM F-actin and the sample was incubated for 18 h at 4 °C. Labeling was terminated by adding 10 mM DTT, and actin was ultracentrifuged for 30 min at 350,000 × g. The F-actin pellet was suspended in G-Mg buffer (5 mM Tris, 0.5 mM ATP, 0.2 mM MgCl₂, pH 7.5) followed by clarification at 300,000 × g for 10 min. Actin was then polymerized for 30 min at 25 °C in the presence of 3 mM MgCl₂ and ultracentrifuged, and then the pellet was suspended in F-Mg buffer (3 mM MgCl₂, 10 mM Tris, pH 7.2) containing 0.2 mM ATP. The labeled F-actin was immediately stabilized against depolymerization and denaturation by adding 1 Meq of phalloidin.

S1 was prepared by s-cytotryptic digestion of rabbit skeletal muscle myosin. A pQE60 vector containing recombinant A1 ELC was a generous gift from J. Borejdo (University of North Texas, Fort Worth, TX). The A2 ELC was prepared from A1, using PCR, by deleting 44 aa from the N terminus of A1. The 5′ primer with the BsaI restriction site was designed to replace E45 of the A1 as the start site of A2 and the 3′ primer was designed to incorporate the XbaI site to the stop codon at the C terminus. The PCR product was subcloned into TOPO, digested with BsaI and XbaI restriction enzymes, and then ligated into the BsaI site of the pE-SUMOstar vector containing His and SUMO tags. Engineering of A16C was done by quick change in cys-lite A1. The A1 and A2 sequences were confirmed by DNA sequencing. Native A1 was transformed into Escherichia coli M15 and A16C and A2 was transformed into E. coli BL21A1-competent cells. Transformed cells were grown for large-scale preparation in LB media supplemented with 100 μg/mL ampicillin. A1 expression was induced by adding 1 mM IPTG. A16C and A2 expression was induced by 1 mM IPTG and 0.2% 3-aminobenzamide. His-tagged ELCs were purified from bacterial cell pellets, using Talon (Clontech) affinity resin; concentrated; dialyzed to 10 mM Tris, pH 7.5; and stored frozen at --80 °C in 150 mM sucrose.

Before labeling, ELCs were reduced with 5 mM DTT for 60 min on ice. Excess DTT was removed using Zeba spin columns (Pierce) in 50 mM Tris, pH 7.5. C180 of A1 and C136 of A2 (250 μM) were labeled with fivefold molar excess of Alexa Fluor 647 C3 Maleimide (Invitrogen) in 6 M GuHCl, 5 mM EDTA, and 1 mM Tris(2-carboxyethyl)phosphine for 18 h at 23 °C. Unbound dye was removed by absorbing ELCs to phenyl Sepharose in PS buffer [50 mM Tris and 45% (wt/vol) saturated ammonium sulfate, pH 7.5] and then washing the resin with PS buffer. Labeled ELC was eluted in 50 mM Tris, pH 7.5; and stored frozen at --80 °C in 150 mM sucrose.

Fluorescence waveforms were acquired using a high-performance time-resolved fluorescence spectrometer constructed in this laboratory, which uses direct waveform recording rather than the conventional method of time-correlated single-photon counting (32). As shown previously, when identical samples are studied, this direct waveform recording instrument offers 10 times higher throughput than time-correlated single-photon counting while providing at least comparable performance in signal/noise, accuracy, and resolution of distinct components. AF568 actin and IAEDANS actin were excited using a passively Q-switched microchip YAG laser.
actomyosin ATPase was achieved. For S1A2, several successive decays were measured every ∼5 s for 10 min to ensure that the steady state of the actomyosin ATPase was achieved. For S1A2, several successive decays were averaged, as needed to obtain sufficient signal/noise for the bound complex.

Supporting Information

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SI Methods

Exchange of ELCs in S1. A total of 50 μM S1 was incubated with 300 μM labeled A1/A2 in 100 mM imidazole (pH 7.0), 2 mM DTT, 2 mM EDTA, and 4.7 M NH₄Cl for 25 min on ice, followed by extensive dialysis in 10 mM imidazole (pH 7.0). Dialed sample was applied to the Talon affinity resin to remove unexchanged S1. S1 containing labeled and bound A1 and A2 together with free ELCs was eluted in Talon elution buffer (200 mM imidazole and 300 mM KCl, pH 7.0). The S1A2 sample was exchanged into digest buffer [25 mM Tris (pH 7.5), 150 mM NaCl, 5 mM [ME] to cut the SUMO tag. The tag was digested by adding SUMO protease (Ulp1) at a 1:5 weight ratio followed by 1 h incubation at 4 °C. S1 was finally brought into 10 mM Tris, pH 7.5. Free ELC, Ulp1, and tags were removed by pelleting S1 with actin (20 min for 300,000 × g) added at 1:1 molar ratio. The acto-S1 pellet was suspended in the release buffer [3 mM MgCl₂, 10 mM Tris (pH 7.5), 0.1 M KCl, and 3 mM ATP]. Ultra centrifugation of this sample at 300,000 × g for 20 min separated actin from S1 and released S1 containing labeled A1/A2 was obtained in the supernatant. Supernatants containing final S1A1 and S1A2 were concentrated and exchanged into F-Mg buffer for experiments.

The concentrations of unlabeled proteins were determined by measuring absorbance at 280 nm, assuming the following molecular weights and absorption of 0.1% protein A2 (S1, 110,000 Da, A280 0.75; A1, 21,894 Da, A280 0.13; A2, 29,117 Da, A280 0.15; A2, 16,725 Da, A280 0.13). The concentration of labeled S1A1/S1A2 and actin was measured by the Bradford assay (BioRad), using unlabeled S1 and ELCs of known concentration as a standard.

Steady-State ATPase and Binding SI Methods (Fig. S1 and Table 1). Actin-activated ATPase activity was measured at 25 °C in F-Mg buffer containing 3 mM ATP at a constant concentration of S1 and actin was measured by the Bradford assay (using quinine sulfate as the standard). The affinity of labeled S1A1 was determined as described previously (2). The concentration of unlabeled proteins were determined by measuring absorbance at 280 nm, assuming the following molecular weights and absorption of 0.1% protein A2 (S1, 110,000 Da, A280 0.75; A1, 21,894 Da, A280 0.13; A2, 29,117 Da, A280 0.15; A2, 16,725 Da, A280 0.13). The concentration of labeled S1A1/S1A2 and actin was measured by the Bradford assay (BioRad), using unlabeled S1 and ELCs of known concentration as a standard.

TR-FRET Data Analysis. Fluorescence waveforms were analyzed using nonlinear least-squares fitting as described previously (3–5). The observed donor-only waveform FDob(t) was fitted by a simulation FDim(t), consisting of a multieponential decay FD(t) (Figs. 2A and 4) convolved with the instrument response function IRF(t).

$$F_D(t) = \sum_{i=1}^n A_i \exp\left(-\frac{t}{\tau_i}\right)$$

$$F_{Dim}(t) = IRF(t-t')F_D(t')dt'$$

where TD is the donor-only fluorescence lifetimes. The ensemble-average lifetime is given by

$$\langle \tau_{D} \rangle = \frac{A_I T_D}{A_I}$$

The observed donor plus acceptor waveform FD+Aobs(t) was fitted by a multieponential function, using the same approach. The ensemble-average FRET efficiency, which is equivalent to the result of a steady-state fluorescence measurement (6), is given by

$$\langle E_{D+A} \rangle = 1 - \frac{\langle \tau_{D+A} \rangle}{\langle \tau_D \rangle}$$

To resolve structural states, a distribution of donor–acceptor distances ρ(t) was assumed,

$$F_{DA}(t) = A_I \exp\left(-\frac{t}{\tau_D} \left(1 + \left(\frac{R_{0i}}{R}\right)^6\right)\right) dr,$$

where R₀ is the lifetime-weighted Förster distance,

$$R_0^6 = 9,786 J \kappa^2 k_{rad}^2 \kappa_{rad}^4,$$

where K is the overlap integral between the donor emission and acceptor absorption spectra, n is the refractive index (1.4), κ² is the orientation factor (2/3, assuming random orientation), and k_rad is the radiative decay rate for the donor. Eq. S7 follows directly from the Förster theory’s assumption (4, 7) that the energy transfer rate constant K₂ (= R₀² R⁻¹/k_rad) depends on the donor–acceptor distance R but not on the donor-only lifetime τD. R₀ between AF568 actin and AF647 S1 isoforms was calculated as 8.2 nm with a quantum yield of 0.69 for Alexa 568 actin (using quinine sulfate as the standard) and R₀ between IAEDANS actin and Dabcyl S1A1 was calculated as 3.3 nm with a quantum yield of 0.32 for IAEDANS actin (using quinine sulfate as the standard).

The distance distribution ρ(R) (Eq. S6) was assumed to be a sum of n Gaussian components, each corresponding to a structural state of the actomyosin complex, with its central distances Rᵢ and with a full width at half maximum, FWHMᵢ or Γᵢ and mole fraction xᵢ,

$$\rho(R) = \sum_{i=1}^n x_i \sigma_i^{-1}(2\pi)^{-1/2} \exp\left(-\frac{[R-R_i]^2}{2\sigma_i^2}\right)$$

$$\sigma_i = \frac{FWHM_i}{2\sqrt{(\ln 2)^{1/2}}} \sum_{j=1}^n x_j = 1.$$

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The observed waveform $F_{D+Absh}(t)$ was fitted by $F_{D+Asim}(t)$.

$$F_{D+Asim}(t) = \int_{-\infty}^{+\infty} IRF(t-t') \cdot F_{D+Asim}(t') dt'$$  

where $X_B$ is the fraction of donor-labeled actin bound to and transferring energy to acceptor-labeled myosin. Thus, binding ($X_B$) is determined independently of the mole fractions of resolved structural states ($x_i$ in Eq. S8).

Donor-Only Fluorescence Decays Are Best Fitted with Two Exponential Components. The donor-only fluorescence decay $F_D(t)$ for AF568 actin (Fig. 2A) was fitted by a multieponential function, with the result that two lifetime components are necessary and sufficient to fit the data (Eq. S3, $n = 2$), based on the residual plots (Fig. S4A) and the $\chi^2$ values (sum of residuals at each data point, Fig. S4B). The results show clearly that the fit is improved by increasing $n$ from 1 to 2, but not by increasing $n$ from 2 to 3. Similar analysis was also done for IAEDANS-labeled actin and three lifetime components are necessary. To increase precision in the FRET analysis, the two donor lifetime values were globally linked.

Strongly Bound Complex Shows Best Fit with a Single Attached State. To resolve structural states of the actomyosin complex in the absence of ATP, we used $F_D(t)$ as input, to fit $F_{D+Absh}(t)$ and thus determine the mole fraction of unbound donor ($1 − X_B$) and interprobe distance distribution corresponding to the bound complex, $F_{DA}(t)$ (Eqs. S6–S9). The goodness of fit was evaluated to minimize $\chi^2$, and the best fit to $F_{DA}(t)$ was obtained with a one-Gaussian component. A second Gaussian component did not improve the fit. Thus, in the rigor (no ATP) state, actomyosin is described by a two-state model: a mole fraction ($1 − X_B$) of unbound donor, a mole fraction $X_S$ of the $S$ state with a mean distance $R_S$ and a FWHM of $\Gamma_S$. The distance ($R_S$) and width ($\Gamma_S$) were independent of the added myosin concentration (0.5–10 μM) to 1 μM actin (Fig. 3A). In subsaturating conditions, the value of the mole fraction $X_S$ obtained from the fitting was indistinguishable from the added myosin concentration; i.e., $X_B = X_S$. In the presence of excess myosin, $X_B$ was indistinguishable from 1.

Three States Are Necessary and Sufficient to Fit the Steady-State TR-FRET Data. To resolve structural states of the actomyosin complex in the presence of ATP, we used $F_D(t)$ as input, to fit $F_{D+Absh}(t)$ and determine the interprobe distance distribution (Eqs. S6–S9). We tested models with one, two, and three Gaussian components (Eq. S8, $n = 1, 2,$ and 3). The goodness of fit was evaluated to minimize $\chi^2$ (Fig. S3). The fit was consistently improved by increasing the number of components $n$ from 1 to 2, but not from 2 to 3 (Fig. S3). Thus, two-Gaussian distance distributions were required to fit the TR-FRET data. We assume that during the steady state of ATP hydrolysis the actomyosin complex is a mix of pre (W)- and post (S)-power-stroke structural states represented by two-Gaussian distances $R_W$ and $R_S$ with mole fractions $X_W$ and $X_S$, respectively. Thus, in the presence of ATP, actomyosin is described by a three-state model: a mole fraction ($1 − X_B$) of unbound donor, a mole fraction $X_S$ of the $S$ state with a mean distance $R_S$ and a FWHM of $\Gamma_S$, and a mole fraction $X_W$ ($= X_B − X_S$) of the $W$ state, with a mean distance $R_W$ and a FWHM of $\Gamma_W$ (Fig. 2B, red). The results are summarized in Figs. 2–4. The same two-Gaussian analysis was successfully applied previously to analyze FRET within smooth muscle myosin regulatory light chain (4) and the myosin relay helix (8), generating high-resolution structural information that was confirmed by independent molecular dynamics simulations (4) or by dipolar electron-electron resonance (DEER) EPR spectroscopy (8).

Molecular Modeling of the Power Stroke. The most commonly considered model for the positions of the LCD in the actomyosin complex at the end of the power stroke (S state, no nucleotide) was obtained by fitting crystal structures of rabbit actin and chicken skeletal myosin into an EM envelope (9), as depicted in Fig. S3 (myosin cyan). We modeled the W structure (beginning of power stroke) by superimposing the CD of the $S$ state with that of nucleotide analog-bound chicken smooth muscle myosin (Fig. S3A, myosin green) (Protein Data Bank ID: 1BR4) (10). In this model for the power stroke, the axial angle between the LCD domains is 70° (10). Using the fulcrum point Gly-770 (11) that defines the connection of the CD and LCD of chicken skeletal myosin (9), we used the MMTSB program to rotate the LCD axially by 18° (Fig. S3B, magenta) and 36° (Fig. S3C, orange), as measured previously by EPR in muscle fibers (12, 13). From each model, we measured distances between the labeled sites (Cys136 of ELC and Cys374 of actin) for the three nearest actin protomers (labeled 1, 2, and 3 in Fig. S3 and Table S1). We calculated the average FRET efficiency $E_{av}$ predicted from each distance (14), and the apparent interprobe distance $R_{pp}$ was calculated as $R_{pp} = R_0(1 − E_{av}/E_{av})^{1/6}$ (Table S1). Our result shows that the FRET-determined distances in the weakly bound actomyosin complex are closest to the distances predicted from 18° rotation of the LCD.

References:

Fig. S1. (A) Actin-activated ATPase ($V_{\text{max}}$ and $K_{\text{ATPase}}$ in Table 1) of labeled S1 isoforms. (B) Binding of labeled S1 isoforms to labeled actin in the presence of ATP ($K_d$ in Table 1).

Fig. S2. Complete dissociation of S1A2 from actin in the presence of 100 mM KCl ($W + \text{ATP} + \text{KCl}$).
Fig. S3. Molecular models showing different orientations of LCD in acto-S1A2. (A) Nucleotide-bound (W) smooth muscle myosin (green) at the beginning of the power stroke shows a 70° rotation of the LCD compared with nucleotide-free (S) skeletal muscle myosin (cyan). (B and C) Nucleotide-bound skeletal muscle myosin (B, magenta) was rotated 18° and scallop myosin (C, orange) was rotated 36° at Gly770 in the fulcrum. Closest actin monomers are numbered 1–3 from the top. Distances from Cys136 to actin monomers 1, 2, and 3 and average energy transfer ($E_{ave}$) are summarized in Table S1.

Fig. S4. Fluorescence lifetime fit of AF568 actin. (A) Normalized residual plot of fits with increasing lifetime components. (B) $\chi^2$ values of lifetime components.

Fig. S5. Time-resolved FRET resolves two structural states of the actomyosin complex in the presence of ATP. Examples of the fluorescence waveforms AF568 actin only ($F_D$) or AF568 actin in the presence of AF647 myosin ($F_D + A + ATP$) are in Fig. 2A. (A) Normalized residual plots show that 2G is better than 1G, whereas 3G does not improve the fit. (B) The $\chi^2$ values confirm that the 2G model is necessary and sufficient to fit the data.

Table S1. FRET results predicted by models in Fig. S3

<table>
<thead>
<tr>
<th>Rotation of LCD, °</th>
<th>Distances for actin protomers 1, 2, 3: R1, R2, R3</th>
<th>$E_{ave}$</th>
<th>$R_{app}$, nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>0°, S</td>
<td>9.2, 12.3, 8.8</td>
<td>0.36</td>
<td>9.0</td>
</tr>
<tr>
<td>70°, W</td>
<td>7.1, 8.4, 9.9</td>
<td>0.30</td>
<td>8.3</td>
</tr>
<tr>
<td>18°, W</td>
<td>8.4, 11.0, 9.3</td>
<td>0.33</td>
<td>9.4</td>
</tr>
<tr>
<td>36°, W</td>
<td>8.8, 9.6, 9.2</td>
<td>0.47</td>
<td>9.2</td>
</tr>
</tbody>
</table>
Table S2.  \(W\)-to-S transitions for experimental and modeled complexes (Fig. S3) of actin and S1A2

<table>
<thead>
<tr>
<th></th>
<th>(R_S)</th>
<th>(R_w)</th>
<th>(R_w/R_S)</th>
<th>(R_w - R_S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed</td>
<td>9.3 (\pm) 0.01</td>
<td>9.7 (\pm) 0.2</td>
<td>1.04 (\pm) 0.04</td>
<td>0.4 (\pm) 0.4</td>
</tr>
<tr>
<td>Model B, 18°</td>
<td>9.0</td>
<td>9.4</td>
<td>1.04</td>
<td>0.4</td>
</tr>
<tr>
<td>Model C, 36°</td>
<td>9.0</td>
<td>9.2</td>
<td>1.02</td>
<td>0.2</td>
</tr>
<tr>
<td>Model A, 70°</td>
<td>9.0</td>
<td>8.3</td>
<td>0.92</td>
<td>−0.7</td>
</tr>
</tbody>
</table>

\(R_S\) and \(R_w\) for modeled structures are \(R_{app}\) for S and \(W\) complexes from Table S1 and were compared with experimentally observed distances.