Structural Dynamics of the Actin–Myosin Interface by Site-directed Spectroscopy

Vicci L. Korman1, Sarah E. B. Anderson1, Ewa Prochniewicz1, Margaret A. Titus2 and David D. Thomas1*

1Department of Biochemistry Molecular Biology and Biophysics, University of Minnesota, Minneapolis, MN 55455, USA
2Department of Genetics Cell Biology and Development University of Minnesota, Minneapolis, MN 55455 USA

We have used site-directed spin and fluorescence labeling to test molecular models of the actin–myosin interface. Force is generated when the actin–myosin complex undergoes a transition from a disordered weak-binding state to an ordered strong-binding state. Actomyosin interface models, in which residues are classified as contributing to either weak or strong binding, have been derived by fitting individual crystallographic structures of actin and myosin into actomyosin cryo-EM maps. Our goal is to test these models using site-directed spectroscopic probes on actin and myosin. Starting with Cys-lite constructs of both yeast actin (ActC) and the Dictyostelium myosin II motor domain (S1dC), site-directed labeling (SDL) mutants were generated by mutating residues to Cys in the proposed weak and strong-binding interfaces. This report focuses on the effects of forming the strong-binding complex on four SDL mutants, two located in the proposed weak-binding interface (ActC5 and S1dC619) and two located in the proposed strong-binding interface (ActC345 and S1dC401). Neither the mutations nor labeling prevented strong actomyosin binding or actin-activation of myosin ATPase. Formation of the strong-binding complex resulted in decreased spin and fluorescence probe mobility at all sites, but both myosin-bound probes showed remarkably high mobility even after complex formation. Complex formation decreased solvent accessibility for both actin-bound probes, but increased it for the myosin-bound probes. These results are not consistent with a simple model in which there are discrete weak and strong surfaces, with only the strong interface forming under strong-binding conditions, nor are they consistent with a model in which surface residues become rigid and inaccessible upon complex formation. We conclude that all four of these residues are involved in the strong actin–myosin interface, but this interface is remarkably dynamic, especially on the surface of myosin.

Abbreviation used: SDL, site-directed labeling.
E-mail address of the corresponding author: ddt@ddt.biochem.umn.edu

Introduction

Force is generated in muscle contraction when the actin–myosin complex isomerizes from a disordered weakly bound (W) state to an ordered strongly bound (S) state.1–3 It is proposed that the W state is characterized primarily by non-stereospecific electrostatic interactions, and the S state is characterized primarily by stereospecific hydrophobic interactions. Although there are high-resolution X-ray structures for myosin and actin by themselves, there are none for the actomyosin complex in either the W or S state. It is particularly important to obtain direct information about the structural dynamics of the actin–myosin complex in solution, because there is evidence that both actin and myosin have their surface and dynamics changed by the actomyosin interaction,4 that these effects are different in the weak and strong states1,4 and that these changes are functionally important.5 Chemical crosslinking, fluorescence resonance energy transfer, peptides, and antibodies have been used to identify regions of interaction between...
Site-directed Probes of the Actin–Myosin Interface

1108

myosin and actin, such as the N and C termini of actin and the 20–50 kDa junction of the myosin heavy chain. More recently, several models of the actomyosin interface were created by fitting the crystal structures of actin and myosin into density maps derived from actomyosin cryo-EM data.6–11 While there is general agreement among the models, there are points of disagreement, such as the extent of the interface and the involvement of specific residues. These models are particularly uncertain where they suggest substantial changes from crystal structures. It is not clear whether the W residues are also involved in the S interface or that the two interfaces are unique. Our goals are to test these models using site-directed labeling (SDL) and to refine them if necessary.

SDL requires incorporating a specific labeling site into a protein, usually accomplished by starting with a “Cys-lite” protein (having no reactive Cys side-chains) and mutating a selected amino acid residue to Cys. To create the actin and myosin SDL mutants, model systems are needed for the mutagenesis and expression of the mutant protein. Work by the Reisler & Rubenstein groups has established yeast (Saccharomyces cerevisiae) as a model system for mutagenesis and purification of actin mutants.12–16 Dictyostelium discoideum (Dicty.) myosin II has similar affinity for actin and the same general kinetic mechanism as muscle myosin, thus making it a good model system for myosin mutagenesis and expression.17,18 Both of these model systems have Cys-lite constructs available making them ideal for our studies.19,20

The site-directed placement of Cys allows for the attachment of an assortment of probes to the protein. Most prominently, SDL has been used in conjunction with EPR21 or fluorescence22 to determine protein structure, dynamics, and interactions. Specifically, if a label is placed at a proposed site of protein–protein interaction, the probe’s rotational mobility and solvent accessibility are expected to decrease upon addition of the other protein.23

In the present study, we prepared four single-Cys mutants, two located in the proposed weak-binding interface (one on actin and one on myosin) and two located in the proposed strong-binding interface (one on actin and one on myosin). Experiments were performed with both fluorescent and spin probes attached to each site. We examined how solvent accessibility and probe rotational mobility were perturbed by formation of the strong-binding (rigor) complex, formed in the absence of nucleotide. The results were interpreted in terms of existing models of the actomyosin complex.

Results

Choice of labeling sites

We chose mutations that are at or near the proposed interface region but are not likely to affect binding affinity. Therefore, when placing potential labeling sites in the proposed W interface, we selected non-charged residues, actin Val5 (close to charged residues Asp2 and Glu4) and myosin Ser619 (close to charged residues Arg620, Lys622, Lys623, and Glu624). We selected residues in the S interface that are not essential for rigor binding but are close to residues that are. Actin residue ile345 was chosen as an S labeling site because earlier work has shown that mutating this residue to Ala did not weaken rigor binding, while mutating residue 341 did.14 Myosin residue Gly401 is in the FHC loop. It has been shown that some mutations in this region of myosin affect actomyosin complex formation;24 but this residue is not one of the functionally important hot spots (Figure 1). Since we have one mutant on each protein in each interface, these labeling sites are designated below as MW (S1dC-S619C), AW (ActC-V5C), MS (S1dC-G401C), and AS (ActC-I345C) (Figure 1).

Functional properties of labeled SDL mutants

We measured binding and ATPase activity to determine whether the mutant proteins retained function after labeling (Table 1). All labeled proteins had at least 75% of the Cys sites labeled, so the functional assays reflect the properties of labeled proteins. As in the spectroscopic experiments, rabbit skeletal actin or S1 was used to test the function of the labeled proteins in the complex. In general, although labeling had some effects, all labeled proteins had significant activity, in most cases within a factor of 2 of that of wild-type. Neither of the actin sites showed a significant functional effect of mutation or labeling, while the most significant effects of labeling were at the MS site (Table 1).

Since the spectroscopic measurements in the present study were performed in the absence of...
nucleotide, the most important functional measurement is the ability of each labeled SDL mutant to form a strong-binding actomyosin complex in the absence of nucleotide (rigor). This was verified by sedimentation binding measurements (Figure 2, Table 1). Rabbit skeletal myosin S1 exhibits a tight stoichiometric binding to rabbit skeletal F-actin ($K_d = 0.013 \pm 0.006$ μM, Figure 2(a), □), and this binding was just as tight when wild-type yeast actin was used ($K_d = 0.017 \pm 0.002$ μM, Figure 2(b), ■). Each of the labeled SDL myosin mutants bound to rabbit skeletal F-actin very tightly, with a $K_d$ value indistinguishable from that observed with rabbit S1, with the exception of M3-IAEDANS (Figure 2(a)), for which $K_d$ was about ten times greater ($0.15(\pm 0.03)$ μM, Figure 2(a), green triangle). However, in spectroscopy experiments on labeled S1dC, the actin concentration was always at least 2 μM, well above this $K_d$ value, and there was a twofold ratio of actin to S1dC, so virtually all of the labeled S1dC was bound to actin. Myosin S1 bound equally tightly to each of the labeled yeast actin SDL mutants (Figure 2(b)). We conclude that the EPR and fluorescence data below comes from labeled protein in a strong actin–myosin complex, and all labeled proteins are capable of going through the actomyosin ATPase cycle.

**Rotational dynamics**

We determined the probe’s nanosecond rotational mobility at each of the four sites for the labeled protein by itself and in the actin–myosin complex, using both EPR and fluorescence anisotropy. Nanosecond probe rotation makes EPR lines narrower and more intense and decreases the splitting between the outer extrema. A quantitative estimate of the amplitude of these nanosecond motions, which reflects steric restriction at the labeled site, is obtained from the maximum splitting and reported as $\theta_c$, the half-cone angle (equation (2)). In the absence of actin–myosin complex formation (black spectra in Figure 3), most of the spectra are quite narrow, having the three-line spectral shape that implies a “weakly immobilized” spin label characterized by a large cone angle (greater than 70°, as indicated in Figure 4), as expected for a relatively unconstrained probe on the surface of a protein. The only exception is A5 (ActC345), which shows the broad spectrum characteristic of a “strongly immobilized” probe (Figure 3, lower right), having a cone angle less than 30° (Figure 4). The splitting increased upon formation of the rigor complex in all four cases. In the case of A5, the spectrum in the absence of S1 contained two components, suggesting two conformational states, and S1 eliminated the narrower of the two components. When the two-component spectrum was analyzed as described,

\begin{table}
\begin{tabular}{|c|c|c|c|}
\hline
Sample & High-salt ATPase (s\(^{-1}\)) & Actin-activated ATPase (s\(^{-1}\)) & $K_d$ (μM) \\
\hline
RS S1\(^a\) & & & 0.013±0.006 \\
Unlabeled M\(_d\) & 4±2 & 1.2±0.4 & \\
M\(_d\)-MTSSL & 7±3 & 1.0±0.4 & 0.010±0.004 \\
M\(_d\)-IAEDANS & 3±1 & 0.5±0.2 & 0.009±0.002 \\
Unlabeled M\(_o\) & 5±2 & 1.2±0.4 & \\
M\(_o\)-MTSSL & 7±4 & 0.236±0.001 & 0.05±0.03 \\
M\(_o\)-IAEDANS & 3±1 & 0.4±0.3 & 0.15±0.03 \\
Unlabeled WT actin & & & \\
A5-IAEDANS & 0.056±0.008 & 0.008±0.002 \\
A5-MTSSL & 0.09±0.01 & 0.008±0.002 \\
A5-IAEDANS & 0.044±0.009 & 0.010±0.002 \\
\hline
\end{tabular}
\end{table}

The unlabeled control samples underwent the same incubations and washes as the labeled samples.

\(^a\) High-salt Ca/K-ATPase.

\(^b\) Actin-activated rates have had the corresponding basal (S1 alone) activity subtracted from the total.

\(^c\) $K_d$ was determined as illustrated in Figure 2.

\(^d\) Rabbit skeletal myosin S1. Actin-activated ATPase or $K_d$ was measured for mutant protein (based on S1dC or ActC) plus unlabeled rabbit skeletal actin or S1. For actin-activated ATPase assays with S1dC, 0.075 mg/ml of S1 was incubated with 25 μM rabbit skeletal actin and 1 mM ATP. In the case of the actin-activated ATPase assays with yeast actin, 2 μM F-actin was incubated with 25 μM rabbit skeletal S1 and 2.5 mM ATP. Buffer conditions are given in Materials and Methods. All experiments were done at 25°C.
for S sites (11(±2)°) than for W sites (6(±3)°), and was about the same for probes on myosin (9(±4)°) as for probes on actin (8(±3)°).

**Solvent accessibility**

Fluorescence intensity provides information that is complementary to that of dynamics. A change in intensity indicates that the probe environment has changed upon complex formation. If a decrease in intensity is shown to correspond to a decrease in fluorescence lifetime, this implies a decrease in quantum yield, which suggests increased solvent

---

**Figure 2.** Binding of labeled SDL mutants. The fraction of actin having bound myosin is plotted against the concentration of free myosin determined from sedimentation; $K_d$ was determined by fitting the data with equation (1). (a) Labeled myosin SDL mutants and rabbit skeletal F-actin. Rabbit skeletal myosin S1 control (■, fit shown as continuous line), M_w-MTSSL (●), M_s-MTSSL (●), M_w-IAEDANS (▲), and M_s-IAEDANS (▲, fit shown as green line). (b) Rabbit skeletal myosin S1 and labeled F-actin SDL mutants. Wild-type yeast actin control (■, fit shown as continuous line), A_w-IAEDANS (●), A_s-IAEDANS (●,A_w-MTSSL (◆),A_s-IAEDANS (◆) and A_w-IAEDANS (◆).

---

**Figure 3.** EPR spectra. MTSSL was bound to myosin SDL mutant (left) or actin SDL mutant (right), at the W site (top) or S site (bottom). Black, labeled protein alone. Red or green, rigor complex, in which the spin-labeled mutant protein was bound to an excess of rabbit actin (left) or rabbit skeletal S1 (right). Each overlaid pair of spectra was normalized by dividing by the double integral, so a direct comparison of amplitude is valid within a pair. Each pair is plotted so the maximum peak-to-peak amplitude is the same for all four pairs.

---

**Figure 4.** Probe mobility for SDL mutants. $\theta_c$ is the cone angle of probe nanosecond dynamics, determined from equation (2). Top, probe in proposed W interface. Bottom, probe in proposed S interface. Left, probe on myosin. Right, probe on actin. Black: labeled protein alone. Red and green, actomyosin complexes. Error bars, SEM.
exposure. Fluorescence intensity increased upon complex formation at both actin sites and decreased at both myosin sites (Figure 5). In all four cases, similar changes were observed in the average fluorescence lifetime (data not shown), confirming that the changes were in fluorescence quantum yield.

While a decrease in fluorescence quantum yield can be caused by increased solvent accessibility, there are other possible explanations, such as increased environmental polarity. A more direct measure of solvent accessibility can be obtained by determining the effects of solute quenching. In the present study, steady-state and time-resolved fluorescence was measured as a function of the concentration of the quencher (acrylamide), and a Stern–Volmer analysis was performed. The steady-state and time-resolved data were consistent with each other, but the steady-state data were less reliable due to artifacts from scattering and static quenching, so only the time-resolved data are shown (Figure 6). The slope of the Stern–Volmer plot, \( K_Q \), is proportional to the probe’s solvent accessibility.

Figure 5. Fluorescence emission spectra. IAEDANS was bound to myosin SDL mutant (left) or actin SDL mutant (right), at the W site (top) or S site (bottom). Black, labeled protein alone. Red or green, rigor complex, in which the spin-labeled mutant protein was bound to an excess of rabbit actin (left) or rabbit skeletal S1 (right).

Figure 6. Acrylamide quenching of IAEDANS fluorescence. Top, W interface. Bottom, S interface. Left, probe on myosin. Right, probe on actin. Free label standard Cys-IAEDANS ( ), labeled protein alone ( ), actomyosin complex (red or green circle). Lines are the best fit to equation (4). Slope, \( K_Q \), is proportional to accessibility.
accessibility. Fractional accessibility was calculated as the ratio $K_Q/K_{Q0}$ where $K_{Q0}$ is the value obtained for free Cys-IAEDANS (Figure 6, $K_{Q0} = 5.2(\pm 0.05) \text{M}^{-1}$). In most cases, $K_Q$ for protein-bound probes (●) was always much less than $K_{Q0}$, indicating partial protection from solvent (Figure 6).

In the case of the W interface (Figure 6, top), rigor complex formation decreased accessibility as expected at the actin site ($A_W$) by more than a factor of 2, but the opposite effect was observed at the myosin site ($M_W$). This was consistent with the opposite effects observed in the fluorescence emission spectra (Figure 5, top). At the S interface (Figure 6, bottom), the myosin site ($M_S$) gave nonlinear quenching at higher acrylamide concentrations (data not shown), so data were analyzed only in the lower concentration range, where quenching was linear with [Q]. The data for $M_S$ in the absence of actin showed high accessibility, consistent with the proposed location of myosin residue 619 on an exposed loop, and complex formation caused only a slight decrease in accessibility. The contrast with the large decrease in fluorescence quantum yield at this site (Figure 5) indicates a conformational change in addition to an accessibility change. $A_S$ showed much less accessibility than $M_S$, consistent with the much lower rotational mobility at this site, and complex formation caused only a slight decrease in accessibility (Figure 6, bottom).

**Discussion**

The goal of this study was to characterize four sites in the proposed actomyosin interface, in the proposed weak and strong-binding domains of actin and myosin, and to determine how these sites are affected by actin–myosin binding in the absence of nucleotide (rigor). The results of this study are summarized in Figure 7. In all four cases, for both kinds of probes, we found that probes on both actin and myosin were less mobile in the complex (Figure 7), consistent with their participation in the interface. However, accessibility did not follow such a simple pattern, complex formation decreased accessibility of actin sites, as expected, but increased accessibility of myosin sites. Another difference between actin and myosin is that the myosin-bound probes are more mobile and accessible than the actin-bound probes, especially in the complexes, consistent with the myosin surface being more dynamic. The comparison between W and S sites is also different for actin and myosin; in actin, mobility and accessibility tend to be less for W than for S, while in myosin the opposite is observed. More insight can be gained by discussing each site individually.

**$M_W$: myosin residue 619**

Residue 619 of myosin is at the start of a surface-exposed loop that extends to residue 629, also known as the 50k/20k loop or loop 2, which is proposed to be part of the W binding domain of myosin. In many crystal structures, all or part of this loop is too disordered to be resolved, but the involvement of this loop in myosin W binding to actin is strongly suggested by the functional effects of mutation. We selected this residue for SDL to obtain direct information about the structural dynamics of the 50k/20k loop and its possible involvement in W and S binding.

The EPR spectra of $M_W$ (Figure 3) indicate that this site is very mobile, with a cone angle $\theta_0$ of 83° (Figure 4), consistent with the lack of resolution of this loop in crystal structures. The more hydrophobic IAEDANS probe was less mobile at this site, but its $\theta_0$ value was still quite high (49°), indicating considerable rotational freedom (Figure 4). Actin binding constrains the mobilities of both probes, reducing the cone angles by about 10°. These results are consistent with analysis of electron microscopy data, which suggested that loop 2 undergoes a disorder-to-order transition upon rigor binding to actin. However, we conclude that the ordering is only partial; and these probes still undergo large-amplitude nanosecond rotations.

Even though we observed a decrease in probe mobility upon complex formation, both the decrease in fluorescence intensity (Figure 5) and the increase in acrylamide quenching (Figure 6) indicate that residue 619 becomes more solvent-exposed upon actin binding. This result suggests that actin binding does not cover this site directly; the decrease in probe mobility is due to a conformational change that actually increases this site’s exposure. Electron microscopy and mutational studies suggest that loop 2 plays a role in actomyosin nucleotide release. The observed conformational change could be related to the role...
that loop 2 plays in the transition between the different actomyosin states.

**M₅:** myosin residue 401

Like residue 619 (M₆), residue 401 (M₅) is located on a surface loop of myosin, which extends from residue 397 to residue 405. It is known as the “cardiomyopathy loop”, since residue 397 (equivalent to 403 in human β-cardiac myosin) is a hot spot for mutations that result in FHC.²⁵ Previous studies have shown that mutating residues in this loop affect actin-activated myosin ATPase activity,²⁴,³⁶ and the deletion of residues 398 to 405 weakens S-state binding.²⁴ This residue was chosen for SDL to provide structural dynamics of the myopathy loop upon S binding to actin.

As observed for M₆, the probe mobility at M₅ is quite high, indicating that the myopathy loop has high flexibility and that acting binding only reduces this mobility slightly (Figures 4 and 7). Another similarity with M₆ is that solvent accessibility at M₅ is slightly (and surprisingly) increased by actin binding, as indicated by decreased fluorescence quantum yield (Figure 5) and increased quenching (Figure 6), although the latter change was not statistically significant. Thus, there is evidence that both myosin sites 619 (M₆) and 401 (M₅) are sterically affected by the strong binding with actin, but that the interaction causes a conformational change that actually exposes these sites more to solvent and leaves the side-chains quite dynamic. Labeling M₅ had a larger functional effect than labeling any of the other sites, including M₆, confirming the functional importance of the loop that contains M₅. In particular, the large increase in K₅ upon labeling supports the proposed role of this loop in stabilizing the strong actin–myosin interaction.

**A₅:** actin residue 5

Residue 5 was selected for SDL because it is near the acidic N terminus, which is predicted by the actomyosin models to be involved in W-state binding. Mutational studies have confirmed that the acidic residues D₁, E₂, D₃, and D₄ are important for weak myosin binding.¹²,¹³,³⁷ While this region is clearly implicated in the weak-binding actomyosin complex, it is unclear whether it would also be involved in strong binding. Mobility and accessibility both decrease upon strong myosin binding (Figure 7), consistent with this site’s involvement, but mobility remains quite high, indicating that this site remains flexible even in the rigor complex. These observations agree with 3-D reconstructions, which suggest that the N terminus remains disordered even after rigor binding.¹⁰

**A₃:** actin residue 345

We chose to label residue 345 because it is in a hydrophobic surface region of actin that has been proposed to play a crucial role in the strong-binding interaction with myosin. This particular site is of particular interest because the nearby residue 341 has been shown to be crucial for rigor (S) binding,¹⁴ while mutation of residue 345 to Cys and attachment of labels does not affect this binding (Table 1, Figure 2). In contrast to the other three labeling sites, both probes at this site showed low rotational mobility (Figure 7), indicating that this is a relatively well-ordered site in which the probes probably bind in a pocket. Both rotational mobility and accessibility were decreased by actin binding, providing strong support for this site’s direct involvement in the S interface.

**Conclusions**

We have used site-directed spin and fluorescence labeling to probe the structural dynamics of the actin–myosin interface under strong-binding (S) conditions. Results from the two actin sites were straightforward, probe mobility and accessibility decreased upon complex formation, consistent with a simple model in which the actin surface becomes covered by the myosin surface without extensive conformational changes. However, the myosin results were more complex, mobility decreased but solvent accessibility increased, strongly suggesting conformational changes at the myosin surface. While formation of the strong-binding complex resulted in decreased probe mobility at all four sites, both myosin sites and one of the actin sites showed remarkably high probe mobility even after complex formation. These results are not consistent with a simple model in which there are discrete weak and strong interfaces, with only the strong interface forming under strong-binding conditions, nor are they consistent with a model in which surface residues become rigid and inaccessible upon complex formation. In fact, the actin–myosin interface remains quite dynamic, especially on the surface of myosin, even when myosin and actin bind strongly to each other.

**Materials and Methods**

**Yeast actin mutagenesis, expression, purification, and labeling**

The site-directed mutagenesis of yeast actin was carried out in *E. coli* using the QuikChange kit (Stratagene) with the following primers (mutated codons underlined): ActC5F 5’-CCATGGATTTCTGAGTGTGCTGTTTGG-3’; ActC5B 5’-CCAAAGACACACACTCAGAATTCCATGG-3’; ActC345F 5’-GGATTGGTGGT TCT-3’; ActC345B 5’-GGTAGTC-AAGAAGCCAAAGCAAACGACCCACCAAATCC-3’. The background construct was pRS2CA, a pRS-based plasmid in which Cys374 has been mutated to Ala (a gift from Dr Rubenstein, University of Iowa). Once the additional desired SDL mutation was confirmed by DNA sequencing, the plasmid was transformed into a haploid strain of *S. cerevisiae* in which the genomic copy of the actin gene
has been disrupted, with the only source of wild-type actin expression coming from plasmid pCENWN. The pRS3A plasmid contains the TRP1 selectable marker gene, while the pCENWN plasmid contains the URA3 selectable marker gene. This allows for the isolation of cells that only express the SDL mutant actin by growing cells in selective media. DNA sequencing was used to confirm that the SDL mutant is the only actin that is expressed by the cells. The cells were then grown to stationary phase in YPD at 30 °C for large-scale preparations.

Wild-type yeast actin from bakery yeast (Saccharomyces carlsbergensis, which has an actin sequence identical with that of S. cerevisiae) and mutant actin from recombinant yeast, were purified using a DNase affinity column, with the following modifications: G-buffer used in the column purification contained 0.4 mM CaCl₂, 0.2 mM ATP, and 10 mM Tris (pH 8.0), actin was eluted from the DNase column with 40% formamide in G-buffer, and the eluted actin was then applied to a DEAE column for further purification and concentration.

Purified actin was dialyzed overnight against the standard G-buffer (0.2 mM CaCl₂, 0.2 mM ATP, 10 mM Tris, pH 7.5) at 4 °C, concentrated using Biomax filters (Millipore, 10 kDa cutoff), and then polymerized by adding 3 mM MgCl₂ and incubating at room temperature for 1 h. Actin concentration (approximately 50 μM) was determined by BioRad protein assay, and 10× molar excess of label (either MTSSL or IAEDANS) was incubated with F-actin at room temperature for 2 h. The reaction was stopped by adding 100-fold molar excess of either dithiothreitol (DTT) (in the case of IAEDANS) or G418, 10 mM ATP, and 10 mM Tris (pH 8.0) at 4 °C using Biomax filters (Millipore, 10 kDa cutoff). StcD (approximately 30 μM) was then incubated with a sevenfold excess of label (MTSSL or IAEDANS) for 4 h on ice. The IAEDANS labeling reaction was stopped by adding 100-fold molar excess of DTT. The free label was removed by dialyzing overnight at 4 °C or by extensive washing using the Biomax filters. Myosin S1 was purified from rabbit fast skeletal muscle.41 Rabbit fast skeletal muscle F-actin was isolated from acetone powder as described, except that the polymerization buffer contained 3 mM MgCl₂.

### ATPase assays

All functional and spectroscopic measurements were performed at 25 °C. ATPase activity was determined by measuring the production of inorganic phosphate.42,43 For the myosin high-salt Ca⁺²- /K⁺-ATPase assay, StcD myosin was incubated at a concentration of 0.0125 mg/ml in 10 mM CaCl₂, 600 mM KCl, 5 mM ATP, 50 mM Mops (pH 7.5). The quantification of inorganic phosphate in the actin-activated ATPase assay was based on either the method of Lanzetta et al.43 in the case of experiments with yeast actin. To measure actin-activated ATPase of StcD myosin, 0.075 mg/ml StcD was incubated with 25 μM rabbit F-actin and 1 mM ATP in 10 mM Mops (pH 7.5), 3.5 mM MgCl₂, and 10 mM KCl. For ATPase measurements with yeast actin, 2 μM of yeast F-actin (ST or mutant) was incubated with 20 μM rabbit muscle S1, 10 mM imidazole (pH 7.5), 2 mM KCl, 1 mM MgCl₂, and 2.5 mM ATP.

### Co-sedimentation binding assay

The co-sedimentation binding assay was based on the previous method, except that the Ca⁺²/K⁺-high salt ATPase buffer was used with Dicty myosins, since this gives the highest activity for StcD. The dissociation constant K₄ for the actin–myosin complex was determined by fitting the data with:

\[
[\text{AM}] = \frac{[\text{AMmax}][\text{Mfree}](K_d + [\text{Mfree}])}{1 + [\text{Mfree}]} \quad (1)
\]

where [Mfree] is the concentration of free myosin determined from the ATPase activity of the supernatant, and the concentration of the bound complex [AM] was then calculated as [Mtotal]−[Mfree]. This fit, performed by non-linear least-squares minimization using Origin 7, returned values for K₄ and AMmax at infinite [Mfree]. The data (Figure 2) were then plotted as y versus y[AM], where y is the fraction of total actin that has myosin bound, y = [AM]/[AMmax].
To characterize yeast actin mutants, $K_d$ values were determined by incubating a variable concentration of rabbit skeletal myosin S1 with 1.5 μM labeled SDL mutant F-actin, in 10 mM Tris (pH 7.5), 1 mM MgCl$_2$, 2 mM KCl and 0.1 mg/ml of BSA. To characterize S1dC mutants, $K_d$ values were determined by incubating various concentrations of the labeled S1dC mutant with 2 μM wild-type F-actin, 10 mM Tris (pH 7.5), 1 mM MgCl$_2$, 2 mM KCl and 0.1 mg/ml of BSA. To characterize S1dC mutants, $K_d$ values were determined by incubating various concentrations of the labeled S1dC mutant with 2 μM wild-type F-actin, 10 mM Tris (pH 7.5), 1 mM MgCl$_2$, 2 mM KCl and 0.1 mg/ml of BSA.

EPR

Spectra were acquired using a Bruker EleXsys 500 spectrometer with the SHQ cavity. Samples (approximately 20 μl in a 0.6 mm i.d. quartz capillary) were maintained at 25 °C using the Bruker temperature controller with a quartz Dewar insert. The field modulation frequency was 100 kHz, with a peak-to-peak amplitude of 3 G. The microwave power was 12.6 mW, and the modulation frequency was 100 kHz, with a peak-to-peak amplitude of 3 G. The microwave power was 12.6 mW, producing moderate saturation (so that signal intensity was at least 50% of maximum) without significant effect on the spectral lineshape. The sample buffer was 2 mM KCl, 1 mM MgCl$_2$, 10 mM Tris (pH 7.5). The concentration of the spin-labeled protein (yeast actin or S1dC) was at least 20 μM, well above the dissociation constant measured by cosedimentation (Figure 2), and the concentration of the unlabeled protein (rabbit skeletal S1 or actin) was at least twice as great, ensuring that the spin-labeled protein was all in the strongly bound actin-S1 complex.

If we assume that the spin label undergoes only sub-nanosecond rotational motions, the amplitude of these motions is given by the half-cone angle:

$$\theta_c = \cos^{-1}[0.5(1 + 8S)^{1/2} - 0.5] \quad (2)$$

where the order parameter $S = (T_{1i} - T_0)/(T_0 - T_0)$. $2T_{1i}$ is the separation between the outer extrema, $T_0$ (35.66 G) is the rigid-limit value of this splitting, determined from a frozen sample, and $T_0$ (15.85 G) is the isotropic-limit value, measured at the zero-crossing points from a solution of freely tumbling spin-label. $\theta_c = 90^\circ$ indicates unrestricted rotation (isotropic limit, $S = 0$), while $\theta_c = 0^\circ$ indicates complete restriction (rigid limit, $S = 1$).

Fluorescence

Steady-state fluorescence was performed with either the Spex Fluorolog or the ISS-K2 fluorescence spectrophotometer at 25 °C with an excitation wavelength of 346 nm. Time-resolved fluorescence was performed with an instrument developed by Dakota Technologies, Inc. (Fargo, ND). The actin-bound IAEDANS was excited with the third harmonic (355 nm) of a 1 ns pulse from Nd:YAG laser (9 kHz repetition rate). Time-dependent fluorescence emission $I(t)$ was extracted by a 440 nm glass cut-off filter, detected with the emission polarizer at the magic angle, and digitized with an oscilloscope (Tektronics TDS 3032B), using a dwell time of 0.5 ns/channel. Data acquisition was completed within 20 s. The time-dependent fluorescence emission $I(t)$ was analyzed by fitting the data to a convolution of the instrument-response function with the function:

$$I(t) = A_1 \exp(-1/r_1) + A_2 \exp(-1/r_2) \quad (3)$$

The average lifetime $\langle \tau \rangle$ was determined from $\langle \tau \rangle = (A_1/r_1 + A_2/r_2)/(A_1 + A_2)$. The buffer used for fluorescence experiments was 10 mM imidazole (pH 7.5), 2 mM KCl and 1 mM MgCl$_2$ with 2 μM labeled protein. To create the rigor complex, 4 μM complementary unlabeled protein was added to 2 μM labeled protein. Solvent accessibility was determined from fluorescence quenching as a function of acrylamide concentration, with both steady-state and time-resolved data recorded from the same sample.

Solvant accessibility of the fluorescent probe IAEDANS was determined from the emission intensity or the average lifetime, as a function of quencher (acylamide) concentration. The solvent accessibility of the probe to acrylamide was determined by plotting $(\tau_0)/\langle \tau \rangle$ or $F_0/F$ against acrylamide concentration and fitting the data to the Stern–Volmer equation:

$$\langle \tau \rangle = \tau_0 (1 + K_Q Q) \quad (4)$$

where $\tau_0$ is the average lifetime the absence of quencher, $F_0$ is the emission intensity in the absence of quencher, $Q$ is the quencher concentration and $K_Q$ is the Stern–Volmer quenching constant. Fractional accessibility was calculated as the ratio $K_Q/Q_{0}$, where $Q_{0}$ is the value obtained for Cys-IAEDANS, a standard sample corresponding to complete accessibility. To prepare this sample, 0.1 mM IAEDANS was reacted with 0.2 mM cysteine in 2 mM KCl, 1 mM MgCl$_2$, 10 mM imidazole (pH 7.5) at 25 °C for 2 h.

Rotational mobility of the fluorescence probe was determined from the steady-state anisotropy, $r = (I_v - G_h)/(I_v + 2G_h)$, where $I_v$ and $I_h$ are the vertically and horizontally polarized emission signals, respectively, following vertically polarized excitation, and $G$ is a polarization correction factor. The order parameter $S$ was then calculated from the anisotropy according to $S = (r(r/0.4)^{1/2})$. As in the case of the spin label, this calculation assumes that the rotational correlation time is less than 1 ns. The cone angle $\theta_c$ was calculated from the order parameter $S$ according to equation (2).

Acknowledgements

This work was supported by grants to D.D.T. from NIH (AR32961) and the Muscular Dystrophy Association, and to V.L.K. from NIH (AR47755) and the American Heart Association (to V.L.K). We thank Dr Peter Rubenstein and Dr James Spudich for the gift of the Cys-lite yeast actin construct and Cys-lite Dicty, myosin motor domain construct, respectively. We thank Yuri Nesmelov for assistance with EPR spectroscopy, and Igor V. Negreshov for his work on the fluorescence instrumentation and analysis software. We thank Zach Ryan, Shawn Galdeen, and Luke Olsen for their advice and assistance in Dicty, myosin mutagenesis, expression, and purification.

References


Edited by J. Karn

(Received 3 August 2005; received in revised form 7 October 2005; accepted 11 October 2005)
Available online 2 November 2005