High-resolution helix orientation in actin-bound myosin determined with a bifunctional spin label

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Using electron paramagnetic resonance (EPR) of a bifunctional spin label (BSL) bound stereospecifically to Dictyostelium myosin II, we determined with high resolution the orientation of individual structural elements in the catalytic domain while myosin is in complex with actin. BSL was attached to a pair of engineered cysteine side chains four residues apart on known structural elements in the catalytic domain while myosin is in complex with actin. BSL was attached to a pair of engineered cysteine side chains four residues apart on known α-helical segments, within a construct of the myosin catalytic domain that lacks other reactive cysteines. EPR spectra of BSL-myosin bound to actin in oriented muscle fibers showed sharp three-line spectra, indicating a well-defined orientation relative to the actin filament axis. Spectral analysis indicated that orientation of the spin label can be determined within <2.1° accuracy, and comparison with existing structural data in the absence of nucleotide indicates that helix orientation can also be determined with <4.2° accuracy. We used this approach to examine the crucial ADP release step in myosin’s catalytic cycle and detected reversible rotations of two helices in actin-bound myosin in response to ADP binding and dissociation. One of these rotations has not been observed in myosin-only crystal structures.

Muscle | Actomyosin | Electron paramagnetic resonance | BSL

The myosin family of molecular motors is responsible for numerous vital functions in eukaryotes, including the contraction of striated muscle. Bundled within an intricate and highly regulated myofibril lattice, muscle myosin II converts the chemical energy released by ATP binding and hydrolysis into mechanical work, executing a series of structural transitions that generate force on actin and shorten each muscle cell (1, 2). Coupling of actin binding, nucleotide hydrolysis, and lever arm movement within myosin’s catalytic domain (CD) is essential for proper function of the contractile apparatus (3, 4).

Myosin function requires actin, and thus an understanding of its mechanism requires analysis of both proteins in complex. However, no crystals of actin–myosin complexes have been reported, so the resolution of actin-bound myosin structures is currently limited to that of electron microscopy. Furthermore, X-ray crystallography and electron microscopy produce only static structures in frozen or crystalline environments, which cannot accurately render the dynamics, disorder, and structural transitions that are essential to understanding function and pathology (4, 5).

In contrast, site-directed spectroscopy can be used to examine the actin–myosin complex under more physiological conditions. Both fluorescence and electron paramagnetic resonance (EPR) have been used in complement to examine the structural dynamics of myosin bound to actin (6, 7). EPR offers superior orientational resolution, due to the high sensitivity of the EPR spectrum to alignment of a spin label in the applied magnetic field. A well-placed spin label can provide direct information about orientation and dynamics in the vicinity of the labeling site, a strategy that has proven powerful in the study of myosin in oriented muscle fibers (7–9). However, conventional methods for site-directed spin labeling impose significant limits on the effective resolving power of EPR. Spin labels are typically incorporated into proteins through covalent attachment to Cys, resulting in a flexible linker that permits the label to undergo ns rotational motion independent of the peptide backbone. Such motion obscures the orientation dependence of the spectrum (10, 11).

Our solution is to eliminate local probe motions by using a spin label that becomes strongly and stereospecifically immobilized with respect to the target protein on attachment. In site-directed fluorescence, this has been achieved by using probes that react with di-Cys (12–14) or tetra-Cys (15) labeling sites, but these fluorescent probes are typically at least twice the size of spin labels, and fluorescence lacks the high orientational resolution of EPR (6, 7). The spin-labeled amino acid TOAC provides stereospecific attachment to the peptide backbone, but this probe is currently only practical for peptides on the order of 50 amino acids or less (16). For larger proteins, spin labels have been synthesized with bulky substituents to reduce mobility (17), or substitution with additional reactive moieties to confer bifunctionality (18–20). The smallest and simplest of these derivatives shares its basic structure with the widely used methanethiosulfonate spin label [1-oxyl-2,2,5,5-tetramethyl-3-pyrroline-3-methyl methanethiosulfonate spin label (MTSSL)], with a second MTS group that allows bifunctional targeting of two Cys residues (20) (Fig. L4). This bifunctional spin label (BSL) is rigidly immobilized when reacted with a pair of Cys residues, so that it undergoes negligible ns rotational motion relative to the protein and can thus be used reliably to measure μs protein rotational motions by saturation transfer EPR (21–23).

Crystallography has shown that BSL exhibits a rigid and stereospecific linkage when reacted with Cys residues apart on successive turns of an α-helix, with great potential for accurate spin-spin distance measurements (Fig. 1B) (24). However, the potential advantages of BSL for enhanced orientational resolution provide valuable insight into the mechanism of muscle contraction while showcasing a method with wide applicability to other oriented biological systems.

**Significance**

The interaction between actin and myosin is responsible for driving a vast array of essential biological processes, including the production of force in contracting muscle. However, the structural behavior of the two proteins in complex is not well understood, because high-resolution atomic models are not yet available by traditional methods. We use a bifunctional spin label and site-directed electron paramagnetic resonance spectroscopy to determine orientations of individual α-helices within the complex. We thus quantify for the first time, to our knowledge, structural changes within the motor domain of actin-bound myosin on nucleotide binding and dissociation. Our results provide valuable insight into the mechanism of muscle contraction while showcasing a method with wide applicability to other oriented biological systems.


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BSL Is Strongly Immobilized and Ordered on Myosin at Sites Across the Myosin CD. We generated two additional myosin constructs with bifunctional labeling sites on helix K (325C and 329C) and helix W (639C and 643C), located, respectively, in the upper and lower 50-kDa domains that form myosin’s actin-binding cleft (Fig. 2B). In all three cases, spectra of oriented fibers show very sharp lines that are quite sensitive to fiber orientation (Fig. 2D–F, Left), consistent with highly ordered probes. Spectra of minced fibers all exhibit identical lineshapes between sample cell orientations (Fig. 2D–F, Right) and show a very wide outer splitting, indicating strong immobilization on the ns timescale. Spectra of fibers oriented parallel to the field (Fig. 2D–F, Left, red) are clearly different across all sites, indicating that probes at these sites have distinct orientations relative to the fiber (actin) axis.

Results

Comparison of Spectra from Myosin Labeled with BSL and MTSSL. A solvent-exposed location on the C-terminal end of myosin’s relay helix was chosen for initial study, because crystal structures and spectroscopic studies of isolated myosin have shown that the orientation of this helical segment is sensitive to nucleotide binding (25–28). MTSSL was reacted monofunctionally at position 492 (Fig. 2A, Upper), and BSL was reacted bifunctionally at positions 492 and 496 (Fig. 2A, Lower, and B).

Skinned muscle fiber bundles, decorated with spin-labeled myosin S1dC in the absence of nucleotide (“rigor”), were oriented either parallel or perpendicular to the spectrometer’s applied magnetic field. Spectra from monofunctionally labeled (MTSSL) protein show virtually no dependence on fiber orientation (Fig. 2C, Left), indicating that the spin label ensemble is not well ordered in the myofilament lattice. In contrast, BSL spectra are highly sensitive to fiber orientation (Fig. 2D, Left), implying orientational order of the spin label relative to the myosin CD and of the myosin CD relative to actin.

Spectra of minced (randomly oriented) fibers show no dependence on sample orientation, as expected (red and blue spectra identical in Fig. 2C and D, Right), but the spectra of monofunctionally (C) and bifunctionally (D) attached probes are quite different. Because these samples lack orientation, such differences can only arise from ns rotational motion. The BSL spectrum has a “powder” lineshape, with a wide splitting of 71.2 ± 0.2 G between the outer extrema, indicating no significant ns rotational motion (Fig. 2D, Right). Thus, BSL is strongly immobilized and highly ordered at this site, enabling it to directly report structural states in an oriented system. In contrast, the spectrum of monofunctional MTSSL bound to the same site shows substantial narrowing (Fig. 2C, Right), indicating large-amplitude dynamic disorder on the ns timescale.

have not been explored. We hypothesize that if BSL is used in the context of an intrinsically oriented system (e.g., myosin in the myofilament lattice), the resolution of EPR will be sufficient to detect the orientation of individual protein structural elements with unprecedented accuracy (Fig. 1C and D).

Fig. 1. (A) Chemical structure of BSL. (B) BSL bound stereospecifically to an α-helix at positions i and i+4, as in ref. 24. (C) Angles θNB and φNB that define the orientation of the nitroxide spin label (defined by axes xN, yN, zN) relative to the applied magnetic field B, which directly determine the orientation dependence of the EPR spectrum. (D) Orienting the helically ordered muscle fiber (and thus the actin filament axis) with B permits direct measurement of the nitroxide orientation relative to actin.

Fig. 2. (A) MTSSL and BSL on the relay helix. (B) BSL labeling sites chosen on three stable helices throughout the myosin CD. (C–F) (Left) EPR spectra of the spin-labeled constructs on oriented fiber bundles, in the absence of nucleotide (rigor), with the fiber axis aligned parallel (red) and perpendicular (blue) to the magnetic field. (Right) EPR spectra of randomly-oriented (minced fiber) preparations of all spin-labeled constructs. (C) S1dC labeled at residue 492 with MTSSL (relay helix). (D) S1dC labeled at residues 492 and 496 with BSL (relay helix, bifunctional analog to C). (E) S1dC labeled at residues 325 and 329 with BSL (helix K). (F) S1dC labeled at residues 639 and 643 with BSL (helix W).
BSL Resolves Significant Structural Changes in the Force-Generating Domain of Myosin During ADP Binding and Release. We determined the orientational distribution in each sample through least-squares fitting of EPR data to spectral simulations (Fig. S3), as described previously (8, 9, 30). First, magnetic and hyperfine tensors were obtained by fitting spectra acquired from minced fibers (Fig. 2 D–F, Right), assuming immobile and randomly oriented spin labels. After fixing these values, analysis of oriented samples depends exclusively on $\theta_{NA}$ and $\phi_{NA}$ (Fig. 1). Each spectrum was assumed to arise from a distribution of static probe orientations relative to the applied magnetic field $B$ (here equivalent to the actin filament axis $A$). Spectra were fitted allowing for the presence of up to two independent populations, each characterized by central angles $\theta_{NA}$ and $\phi_{NA}$ and a Gaussian full width at half maximum ($\Delta \theta_{NB}$) describing static disorder of the ensemble. For each of the three BSL samples, one principal oriented Gaussian component was observed (Fig. S3), and that component is displayed in Fig. 3B, with parameters given in Table 1.

Helix Orientation with Respect to Actin. Angles in Fig. 3 and Table 1 correspond to $\theta_{NA}$ and $\phi_{NA}$, defined by the relative orientation of BSL and the actin filament (Fig. 4 A and B). To develop structural constraints that are independent of the spin label, we considered the spatial relationship between the label and its associated $\alpha$-helix. BSL is highly ordered at all sites tested, indicating that the label is stereospecific in its attachment, so our data support the use of a single model to define the label–helix relationship. We started with a crystal structure of BSL attached to T4 lysozyme at positions $i$ and $i + 4$ on an $\alpha$-helix [Protein Data Bank (PDB) ID code 3L2X] (24). We determined the axis of the labeled helix using a previously described technique (31) and defined a nitroxide reference frame using the geometry of BSL (Fig. 4C). Vector algebra yields two new angles, $\theta_{NH}$ and $\phi_{NH}$, which describe the orientation of the helix vector in the nitroxide frame (Fig. 4D). With both helix and actin vectors defined in the nitroxide reference frame, the angle between them ($\theta_{NH}$) describes the tilt of the myosin helix with respect to the actin filament (Fig. 4 E and F).

To verify our derivation, we compared the helix orientations calculated from our nucleotide-free EPR results to a recent model of the actomyosin complex, assembled from cryo-EM and crystallographic data on nucleotide-free myosin II in complex
with rabbit skeletal actin (32). In this model, θ_{AH} values were measured for the three labeled helices relative to the actin axis, and these values were compared with the centers of our experimental θ_{AH} distributions. Table 2 shows that each EPR experiment produces a θ_{AH} value that gives good agreement with the model calculation, with a difference of 12.7 ± 1.9° in the most extreme case.

Although all labeling sites on our myosin constructs were chosen within straight helices, the T4 lysozyme helix used in our derivation is kinked at the labeling site. It is likely that BSL’s conformation on straight helices is different from that observed in this crystal, and we hypothesized that this discrepancy could account for some of the observed disagreement in θ_{AH} Values. We varied the angles θ_{NH} and ϕ_{NH}, assuming the same values at all three sites, to minimize the difference between experimental and model-based results, yielding the optimized values given in Table 2. It is striking to observe that a small change in θ_{NH} and ϕ_{NH} is sufficient to bring all three of the experimental θ_{AH} angles to within 1° of their model-based predictions. This result demonstrates that we can obtain structural constraints for the actomyosin complex using our EPR-based method that are in precise agreement with previous literature while avoiding the inherent difficulties and caveats associated with frozen and crystalline samples. The agreement across all three labeling sites, when a single conformation of BSL is assumed, provides further evidence that BSL is indeed highly stereospecific in its attachment to α-helices.

### Effects of ADP on Helix Orientation

For the relay helix site, our results are consistent with crystal structures and previous spectroscopic studies on actin-free myosin, which show evidence of a nucleotide-dependent deformation in the C-terminal end of the relay helix (25, 33–35) (Fig. 5A). In each of these structures, a kink in the helix is introduced at residue M486. To model the changes in myosin reflected by EPR data, we assume that the observed reorientation of BSL corresponds to movement at the end of the relay helix, comprising residues 486–496. Analysis of MgADP spectra using our optimized θ_{NH} and ϕ_{NH} values (Table 2) yields a set of four potential relay helix orientations (θ_{AH}) in the presence of MgADP. Comparison with an alignment of myosin-only crystal structures highlights the most likely solution: a θ_{AH} value of 71.0 ± 2.4° (Fig. 5B, green) places the helix in good alignment with crystal structures of myosin with nucleotide and reflects a conformation highly similar to the MgADP-bound structures observed in crystallo. The other solutions bend the relay helix in a direction directly opposite from the crystal structures or deform the helix so severely as to disrupt its secondary structure. Thus, we conclude that the change observed by EPR correlates with a reorientation of the relay helix from 83.4 ± 1.8° to 71.0 ± 2.4° relative to the actin filament (Δθ_{AH} = −12.4 ± 3.9°; Fig. 5B and C). The helix also becomes more disordered with the addition of MgADP (ΔΔθ_{AH} = +6.2 ± 2.2°; Fig. 5C).

In contrast to the relay helix, the orientation of helix W is virtually invariant in myosin-only crystal structures. Thus, it is not clear whether our observed change corresponds to an internal bending of the helix, or to a reorientation of the entire helix. However, repeating the analysis discussed above and assuming that the helix behaves as a rigid rod, the best solution gives a change in θ_{AH} of −4.7° ± 1.1°, pulling helix W away from the bent relay helix.

### Discussion

**BSL Greatly Enhances the Resolution of EPR Within Oriented Systems.** Bifunctional derivatives of nitroxide spin labels simplify EPR spectra by reducing ns probe motion and disorder. Here we showed the power of this technology in intrinsically oriented systems, granting EPR the capacity to measure absolute orientations of individual protein structural elements.

Previously, it was possible to detect changes in protein structure by observing shifts in orientation and mobility of spin labels at well-chosen sites. Although informative, these results were typically unsuitable for accurate structural interpretation because of the weak coupling between probe and backbone (36). Measuring orientations precisely required the use of synthetically

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**Table 1. Orientation of the myosin-bound nitroxide spin label (N) relative to the actin (fiber) axis (A), derived from spectra of muscle fibers oriented parallel to the magnetic field (Fig. 3)**

<table>
<thead>
<tr>
<th>Labeling site</th>
<th>Actin-BSL axial angle, θ_{NA} (°)</th>
<th>Actin-BSL azimuthal angle, ϕ_{NA} (°)</th>
<th>Angular width FWHM, Δθ_{NA} (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Apo (rigor) 5 mM MgADP</td>
<td>Apo (rigor) 5 mM MgADP</td>
<td>Apo (rigor) 5 mM MgADP</td>
</tr>
<tr>
<td>492.496-BSL (relay helix)</td>
<td>70.5 ± 0.1  60.9 ± 0.2</td>
<td>27.0 ± 1.8  13.6 ± 2.4</td>
<td>11.9 ± 1.5  18.1 ± 0.7</td>
</tr>
<tr>
<td>325.329-BSL (helix K, U50)</td>
<td>88.2 ± 0.5  89.2 ± 0.6</td>
<td>33.3 ± 1.8  35.3 ± 1.0</td>
<td>10.0 ± 1.7  9.2 ± 0.2</td>
</tr>
<tr>
<td>639.643-BSL (helix W, L50)</td>
<td>28.0 ± 0.1  25.0 ± 0.3</td>
<td>32.6 ± 2.1  22.6 ± 1.8</td>
<td>10.4 ± 0.2  9.9 ± 0.7</td>
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Mean ± SEM (n = 3).
ADP Induces Internal Structural Changes Within the CD in Actin-Bound Myosin. The physiological significance of the myosin ADP release step has become increasingly apparent. Actin decreases myosin’s ADP affinity by a factor of roughly 100 (37, 38), implying crucial allosteric coupling between the nucleotide-binding pocket (ATPase) and the actin-binding interface (bridge-dynamics). Strain on the lever arm affects the rate of ADP release in several myosins (39–42), and single-molecule studies have correlated this rate with lever arm movement (39, 43). Thus, ADP release is directly related to a mechanical event that either represents the completion of the powerstroke, or functions as a load-dependent tuning mechanism (42). Electron microscopy of smooth muscle myosin bound to actin shows a significant effect of ADP on the orientation of the lever arm relative to the catalytic domain (44). Thus, a large body of evidence suggests an important structural change within the CD of myosin that affects both actin binding and lever arm position upon ADP release, but a direct measurement of this internal structural change has not previously been provided for actin-bound myosin.

We used BSL to probe specific sites on myosin while the protein is in complex with actin. We observe a reversible change in label orientation at the relay helix site on addition and removal of MgADP (Fig. 3 and Table 1). Deriving the orientation of the labeled helix from EPR data (Fig. 4), we calculate a change of \(-12.4 \pm 3.9^\circ\) in the orientation of the relay helix relative to actin (Fig. 5). These results align well with existing crystal structures of myosin alone, but have not previously been observed within the actomyosin complex. We also detect a change of \(-4.7 \pm 1.1^\circ\) in the orientation of helix W relative to actin. This change is not observed in myosin-only crystal structures and thus serves as a rare example of actin directly modulating myosin’s structural dynamics. Further characterization of this behavior with additional labeling sites may help to refine our understanding of the mechanism behind actin activation of myosin ATPase.

Table 2. Axial tilt angles of labeled helices with respect to actin in the absence of nucleotide

<table>
<thead>
<tr>
<th>Labeling site</th>
<th>Model-based (\theta_{AH}) (°)</th>
<th>Experimental (\theta_{AH}) (°)</th>
<th>Difference (°)</th>
<th>Optimized (\theta_{AH}) (°)</th>
<th>Experimental (\theta_{AH}) (°)</th>
<th>Difference (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relay helix (apo)</td>
<td>84.4</td>
<td>74.8 ± 1.8</td>
<td>-9.5 ± 1.8</td>
<td>83.4 ± 1.8</td>
<td>-1.0 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>Helix K (U50)</td>
<td>29.6</td>
<td>42.3 ± 1.9</td>
<td>12.7 ± 1.9</td>
<td>30.6 ± 1.9</td>
<td>0.9 ± 1.9</td>
<td></td>
</tr>
<tr>
<td>Helix W (L50)</td>
<td>77.5</td>
<td>83.5 ± 2.1</td>
<td>6.0 ± 2.1</td>
<td>78.4 ± 2.1</td>
<td>1.0 ± 2.1</td>
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</table>

Values are derived using probe orientations obtained either from EPR (Experimental columns) or directly from a cryo-EM model of actomyosin (Model-based column) (32).

ADP causes a direct measurement of this internal structural change has not previously been provided for actin-bound myosin.

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Additional Labeling Sites Would Provide More Detailed Structural Information. These results demonstrate BSL’s potential for dramatically improving the accuracy of structure determination in an oriented assembly. Although BSL’s high EPR resolution permits the precise determination of helix axial tilt angles in myosin with respect to actin (\(\theta_{AH}\)), the measurements taken here are not sensitive to the orientation of the helix in the plane normal to the actin filament. This missing information means that a single labeling site on a helix cannot completely define how that helix is oriented within each myosin head, in a way that would allow direct modeling of the bend. This ambiguity should be alleviated by combining this approach with distance measurements between pairs of sites, detected by dipolar spin–spin interaction (24, 45). Such an approach would be essentially equivalent to the method used in structure determination by NMR, where both orientation and distance constraints are determined (46). The superior sensitivity of EPR makes it applicable to rapid measurements in dynamic samples, such as contracting muscle fibers; such measurements are needed to resolve crucial questions about the structural transitions that generate contractile force. The present work introduces a powerful application of EPR to intrinsically oriented biological systems in general, allowing for the derivation of accurate structural constraints not only for sarcomeric proteins, but also for proteins associated with nucleic acid chains, microtubules, and lipid bilayers.

Materials and Methods

Protein and Muscle Fiber Preparations. Mutant myosin constructs were prepared in a Cys-lite S1dC Dictyostelium myosin II background truncated at residue 758 and containing only one native (nonreactive) Cys at position 655 (47). Constructs were expressed and purified from Dictyostelium orf17 cells. Skinned rabbit psoas muscle fiber bundles were dissected and permeabilized as described previously (48).

EPR Spectroscopy. EPR spectra were recorded at X-band (9.6 GHz) using an E500 EleXsys spectrometer (Bruker Instruments). Acquisition of spectra for oriented samples was performed as described previously (21, 22). BSL spectra
were analyzed to determine the orientational distribution of the nitrooxide coordinate frame with respect to the applied magnetic field for parallel-oriented samples, using computational simulation and least-squares minimization as described previously (R, 30).

A detailed description of methods and reagents can be found in SI Materials and Methods.