Combining EPR with Fluorescence Spectroscopy to Monitor Conformational Changes at the Myosin Nucleotide Pocket

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We used spin-labeled nucleotide analogs and fluorescence spectroscopy to monitor conformational changes at the nucleotide-binding site of wild-type Dictyostelium discoideum (WT) myosin and a construct containing a single tryptophan at position F239 near the switch 1 loop. Electron paramagnetic resonance (EPR) spectroscopy and tryptophan fluorescence have been used previously to investigate changes at the myosin nucleotide site. A limitation of fluorescence spectroscopy is that it must be done on mutated myosins containing only a single tryptophan. A limitation of EPR spectroscopy is that one infers protein conformational changes from alterations in the mobility of an attached probe. These limitations have led to controversies regarding conclusions reached by the two approaches. For the first time, the data presented here allow direct correlations to be made between the results from the two spectroscopic approaches on the same proteins and extend our previous EPR studies to a nonmuscle myosin. EPR probe mobility indicates that the conformation of the nucleotide pocket of the WT-SLADP (spin-labeled ADP) complex is similar to that of skeletal myosin. The pocket is closed in the absence of actin for both diphosphate and triphosphate nucleotide states. In the actin-myosin-diphosphate state, the pocket is in equilibrium between closed and open conformations, with the open conformation slightly more favorable than that seen for fast skeletal actomyosin. The EPR spectra for the mutant show similar conformations to skeletal myosin, with one exception: in the absence of actin, the nucleotide pocket of the mutant displays an open component that was approximately 4–5 kJ/mol more favorable than in skeletal or WT myosin. These observations resolve the controversies between the two techniques. The data from both techniques confirm that binding of myosin to actin alters the conformation of the myosin nucleotide pocket with similar but not identical energetics in both muscle and nonmuscle myosins.

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Introduction

A fundamental goal of myosin biophysics is to define an atomic-level model for the generation of force and motion by myosin via an interaction with actin and the hydrolysis of the substrate ATP. Achieving this goal will require defining the conformational changes that occur during the myosin working cycle and their relationship to the interaction with substrate and polymer. This is particularly important at the nucleotide site where
the actin-modulated chemomechanical coupling of the free energy of hydrolysis to force and motion is initiated.

The initial X-ray structures of myosin isoforms all showed a closed triphosphate-binding domain. The triphosphate moiety was enclosed in a tunnel-like structure termed the phosphate tube composed of the P-loop and switches 1 and 2. The latter switches are structurally homologous to the switch domains seen in the G-proteins. Indeed, structural homology points to myosin, the G-proteins and kinesin-family motors having evolved from a common ancestor protein. However, unlike the myosin X-ray structures, the X-ray structures of kinesin-family motors showed an open phosphate tube with switch 1 displaced away from the nucleotide triphosphate moiety. As structural similarity and functional similarity frequently go hand in hand, these X-ray structures led us to initially hypothesize that the closed phosphate tube of myosin might also open during the motor cycle. The hypothesis was supported by demonstrating the functionally competent binding of several γ-phosphate-modified nucleotide analogs at the nucleotide site of myosin and actomyosin. These included a γ-phosphate-modified electron paramagnetic resonance (EPR) spin probe (ATP-γSL) and PiP3d[adenosine-5’] pentaphosphate (Ap3’A). The ATP-γSL spin probe moiety has a van der Waals surface diameter of 9.0 Å, and Ap3’A is terminated by two adenosine moieties. These γ-phosphate moieties are too bulky to thread through the closed phosphate tube seen in the X-ray structures of myosin. The same argument applies to caged ATP, which is widely accepted to bind to the nucleotide site before photolysis. The photoreactive leaving group has a van der Waals surface that is 8.5 Å across.

The X-ray structure of Dictyostelium discoideum myosin complexed with dynamin and the subsequent structure of myosin V both showed an open nucleotide site. These were interpreted to be actin-bound states. Actin was not present in the X-ray structures; however, Holmes et al. used cryo-electron microscopy of myosin decorating actin filaments to suggest that the closing of the 50-kDa cleft and the opening of the nucleotide pocket were coupled. Other data have been brought into question whether the coupling is obligatory.

All experimental techniques have strengths and limitations. A limitation of X-ray diffraction and cryo-electron microscopy is that the proteins are fixed in space and only a single state can be visualized at a time. This makes understanding the thermodynamics associated with transitions between multiple states difficult, leading others to employ spectroscopic studies. Here, the proteins can be studied in an aqueous environment, with and without actin, under conditions that simulate physiological conditions. Robertson et al. have shown an opening of the nucleotide site of smooth muscle myosin upon the transition from bound ATP to ADP. Sun et al., using myosin V, employed fluorescence resonance energy transfer between a fluorescent nucleotide analog and a biarsenical compound to measure the distance between the switch 1/upper 50-kDa regions and the nucleotide site. Their results indicated that actin binding did not cause opening of the nucleotide pocket until after phosphate release and prior to the release of the nucleotide.

Another approach is to place a single reporter group directly at the active site of myosin. Zeng et al. and Kintses et al. have engineered a D. discoideum myosin (W239+) with a single tryptophan (F239W) in switch 1 at the myosin nucleotide site to monitor conformational changes in the presence and in the absence of actin. We have likewise used nucleotide-analog EPR spectroscopy probes to monitor conformational changes at the nucleotide site of myosin. Both approaches have demonstrated an opening of the nucleotide site upon binding to actin. Additionally, multiple open and closed nucleotide-state-dependent conformations have been detected. This indicates that the opening and closing of the nucleotide site are not all-or-none events but instead represent an equilibrium between available states. This has significant implications for models that posit that there is an obligatory coupling between opening (closing) of the nucleotide-binding site and the closing (opening) of the 50-kDa cleft in the strong-to-weak binding of myosin to actin.

As noted previously, any experimental protocol has strengths and limitations. Tryptophan fluorescence monitors a change in the local environment of the amino acid in question, with global implications inferred by a comparison between fluorescence intensity and structures with known nucleotide pocket conformations (e.g., myosin-ATP is closed and actin-myosin is open). EPR spectroscopy monitors the mobility of a spin probe moiety, from which the more or less restrictive nature of the adjacent protein surface is likewise inferred. EPR spectroscopy has the advantage that both the wild-type D. discoideum (WT) and W239+ myosins can be investigated in the absence of any confounding fluorescence signal from actin when present. The fluorescence signal from spin-labeled analogs and that from the native nucleotides can be compared. Multiple protein conformations in equilibrium can be easily quantified in a single EPR spectrum. Tryptophan fluorescence requires mutated proteins and the attendant complication of perturbed function, while EPR studies require addition of the spin probe to either the protein or the nucleotide. This is particularly relevant for the current studies since the W239+ myosin appears to have altered kinetics and structural coupling. Fluorescence spectroscopy has greater temporal sensitivity, allowing kinetic studies. With these individual restrictions on interpretation, questions regarding the validity of conclusions drawn using fluorescence or EPR spectroscopic approaches to monitor the nucleotide pocket have been raised. Additional confidence in the conclusions drawn can be gained by applying the two protocols to the same proteins. That is the approach we took in this study. We used EPR probes to monitor conformational changes in WT myosin.
and W239+ myosins in the presence and in the absence of actin. We then compared the EPR spectroscopy results with those previously obtained via tryptophan spectroscopy. We also used tryptophan fluorescence to compare the conformational changes induced by the native nucleotides with those induced by their spin-labeled analogs. We show that despite subtle differences in behavior, tryptophan fluorescence and EPR spectroscopy are in agreement regarding the open-to-closed transition in the myosin nucleotide pocket and the presence of multiple states in equilibrium.

Results

Our goal was to compare the results from tryptophan fluorescence and EPR spectroscopy using the nucleotide-analog spin probes for both approaches to monitor conformational changes at the nucleotide site of a tryptophan-free Dictyostelium myosin motor domain construct into which the F239W tryptophan has been introduced (W239+). Figure 1 shows the structures of the EPR probes used. Figure 2a shows our basic experimental observation using EPR spectroscopy. The spectra are from 2'-SLADP (spin-labeled ADP) bound to WT myosin motor domain in the presence (red) and in the absence (blue) of actin at 25 °C. The spectra show a mixture of states with different probe mobilities. The sharp central peaks, P2, P3 and P4, arise from unbound spin probe tumbling rapidly in solution. When the probe binds at the active site of myosin, the adjacent protein surface determines the region in space that the probe can explore in the presence of thermal fluctuations. The restriction on the available space for motion now results in a broadening of the spectrum. The resulting low-field peak, P1, and high-field dip, P5, are from a probe bound at the active site of WT myosin. The P1 and P5 arrows are colored to denote the different spectra. (b) Spectra as in panel (a) but at 2 °C. (c) Detail of the low-field, P1, peak in the absence of actin as a function of temperature for the spectra in panels (a) and (b) plus intermediate temperatures. (d) Detail of the low-field, P1, peak in the presence of actin as a function of temperature for spectra in panels (a) and (b) plus intermediate temperatures.

![Fig. 1. Structures of the EPR probes used in our studies. The nitroxide bond in the spin ring moiety contains an unpaired electron, denoted by a dot. For 2',3'-SLADP, the label is attached to only one of the hydroxyls on the ribose at a time but can readily isomerize between the 2'- and 3'-positions. For 2'-SLADP, the spin moiety, R, is at the 2'-position and the 3'-position is a hydrogen (no oxygen).](image)

![Fig. 2. (a) Spectra of 2'-SLADP bound to WT myosin in the absence (blue spectrum) and in the presence (red spectrum) of actin at 25 °C. Peaks P2, P3 and P4 are from a probe that is not bound to myosin but is instead tumbling rapidly in solution. Peaks P2–P4 are truncated to enhance detail. The low-field peak, P1, and high-field dip, P5, are from a probe bound at the active site of WT myosin. The P1 and P5 arrows are colored to denote the different spectra. (b) Spectra as in panel (a) but at 2 °C. (c) Detail of the low-field, P1, peak in the absence of actin as a function of temperature for the spectra in panels (a) and (b) plus intermediate temperatures. (d) Detail of the low-field, P1, peak in the presence of actin as a function of temperature for spectra in panels (a) and (b) plus intermediate temperatures.](image)
tein surface is most certainly not a true cone of revolution, the analysis can still serve as a useful first approximation to relate the change in the magnetic field variable to the physical magnitude of the conformational change we are observing. The change in splitting in Fig. 2a corresponds to a change in the vertex angle of the cone of mobility from 82° in the absence of actin to a cone angle of 105° in the presence of actin, implying a significant opening of the nucleotide pocket. Low-field-to-high-field splittings and effective cone angles for all data collected are summarized in Table S1.

Figure 2b shows the results obtained at 2 °C. As in Fig. 2a, the spectra indicate more mobile probes in the presence of actin. However, the spectrum in the presence of actin (red) is clearly more complicated. There are two components in the high-field dip, P5, component denoted by the colored arrows. The low-field peak, P1, is significantly broader when compared with the spectrum in the absence of actin (blue) due to a shoulder on the low-field side of the P1 peak, again marked by a red arrow. The additional shoulder and P5 dip indicate that there are at least two states present in the presence of actin: a major state with greater probe mobility showing that the nucleotide site has opened and a minor state with more restricted probe mobility. The P5 dip of the more mobile component shows it has a degree of mobility that lies between the more restricted mobility of the actin-bound state and the mobility of myosin in the absence of actin. Thus, it cannot simply be from myosin that is not bound to actin in the sedimented pellet used for spectral accumulation. Figure 2c and d provides greater detail of the change in the low-field peak as a function of temperature in the absence and in the presence of actin. Figure 2d emphasizes the low-field shoulder in the spectrum at 2 °C (blue) relative to the spectrum at 25 °C (red). A similar presence of two conformations of the nucleotide site in the actin-bound state has previously been observed for rabbit skeletal myosin. However, the fraction of the more immobile conformation was significantly larger for skeletal myosin.

With any spectroscopic study, additional confidence in the results can be obtained by considering multiple probes. We have additionally used the probe 2′,3′-SLADP (Fig. 1). Qualitatively similar results were obtained with this alternative probe at the active site. However, the spectra are more complicated due to the presence of multiple spectral components, potentially arising from the two enantiomers that are present with this analog. Spectra for 2′,3′-SLADP bound to WT myosin at 2 and 25 °C are given in Figs. S1 and S2.

As demonstrated above, nucleotide-analog EPR probes bound to WT myosin report a closed pocket for myosin with an opening of the nucleotide pocket in the presence of actin, as has been seen in other myosin isoforms. The tryptophan fluorescence data have likewise suggested an opening of the nucleotide pocket of the W239+ mutant upon binding to actin. However, the fluorescence data also implied the presence of a more open conformation of the nucleotide pocket in the W239+·ADP complex in the absence of actin. Thus, the nature of the W239+·ADP state was ambiguous, with EPR probes suggesting a closed nucleotide pocket for WT myosin and tryptophan fluorescence suggesting a more open nucleotide pocket for W239+ myosin. A major motivation for the present study was to resolve this ambiguity in the data. This question was resolved by spectra of nucleotide-analog EPR spin probes bound to W239+ myosin as described below.

Figure 3 shows the spectra of 2′-SLADP bound to W239+ myosin in the presence and in the absence of actin at 2 °C (Fig. 3a) and 25 °C (Fig. 3b). In the absence of actin at 2 °C, there are two components in equilibrium in the spectrum as demonstrated by the high-field shoulder on the P1 component and the two distinct dips in the high-field P5 component of the spectrum. The more immobile component is similar to the closed pocket seen in other myosin-ADP complexes. However, there is also a component with mobility similar to the open pocket. This component
is very prominent at 25 °C, diminishing as the temperature is lowered. The equilibrium between the more open and more closed conformations is detailed in Fig. 3c, showing the low-field, P1, component in the spectra in the absence of actin as a function of temperature. Thus, the EPR spectra agree with the conclusions drawn from tryptophan fluorescence. In the absence of actin, the pocket of W239+ myosin has an open component, with a shift to a closed component as the temperature is lowered.

The ambiguity described in the paragraph above is explained because the energetics of the conformational changes of the nucleotide pocket of the mutant protein in the myosin⋅ADP complex are different from those seen previously for other myosins and for those seen here for WT myosin.

In the actin-W239+-SLADP complex, there is a single dominant component of the spectrum with a splitting of 5.4 mT (cone angle of 99°), showing an open pocket. This spectrum is essentially identical with that obtained with WT myosin as described above. Figure 3d shows the P1 component in the presence of actin as a function of temperature, demonstrating a shift to the more immobilized spectral component (closed nucleotide pocket) as the temperature is lowered. In the presence of actin, the energetics of the transition from closed nucleotide pocket to open nucleotide pocket is essentially identical for W239+ and WT myosins as a function of temperature. Results obtained with 2',3'-SLADP at the active site were qualitatively similar to those obtained with 2'-SLADP (see Figs. S1–S4). Thus, the fluorescence spectra and the EPR probes agree that the nucleotide pocket of W239+ myosin is open in the actin-bound complex.

Tryptophan fluorescence has suggested that the nucleotide pocket closes in the myosin-ADP state. EPR spectroscopy is too slow to resolve the triphosphate state in the competing presence of hydrolysis to the diphosphate state. The triphosphate state was instead modeled using beryllium fluoride as a phosphate analog. When bound at the active site in the presence of ADP, the ADP-BeFx complex is a stably trapped analog of the ATP triphosphate state. Panels (a) and (b) of Fig. 4 show spectra for the WT-2'-SLADP-BeFx and W239+-2'-SLADP-BeFx complexes, respectively, as a function of temperature. For both WT and W239+ proteins, the spectra show that the nucleotide pocket is closed with little change with temperature. A similar result was obtained with 2',3'-SLADP at the active site (Fig. S5). We conclude that both EPR and fluorescence spectroscopy agree that the nucleotide site is closed in the triphosphate state at all temperatures. Attempts to collect spectra in the presence of actin and myosin-ADP-BeFx were unsuccessful because actin binding releases BeFx.

The experiments above involved the Mg2+ complex of the nucleotide. The tryptophan fluorescence data from W239+ myosin showed that the nucleotide pocket of W239+-ADP closes in the absence of Mg2+. Figure 5 shows the spectra of WT and W239+ myosins in the Mg2+-free ethylenediaminetetraacetic acid (EDTA) state at 2 and 25 °C. In the absence of Mg2+, the pocket is in the closed conformation, with a slight opening of the nucleotide pocket at higher temperature. Fig. S6 gives the same result for 2',3'-SLADP at 25 °C. Thus, both EPR and fluorescence spectra again agree that the nucleotide pocket closes in the absence of Mg2+.

In the studies described above, we used EPR spectroscopy to compare the results obtained with WT and W239+ proteins in order to determine if the
W239+ mutation alters the results obtained. Conversely, one can use fluorescence spectroscopy to determine whether the spin probes alter the interactions between the nucleotide and the protein. Control experiments showed no contribution to the fluorescence signal from the SLADP alone in solution under the conditions used. Figure 6 shows the tryptophan fluorescence of W239+ myosin as a function of temperature comparing 2′,3′-SLADP with ADP. An increase in fluorescence is interpreted as an opening of the nucleotide pocket; a decrease, as a closing.21 The results for the apo state, the ADP state and the ADP-BeFx triphosphate analog state are similar to those obtained previously.21 Most importantly, the data for ADP/SLADP and ADP-BeFx/SLADP-BeFx closely parallel each other as a function of temperature, indicating that the spin probe moiety is functioning as the physiological species at the nucleotide site. The data show a closed nucleotide pocket in the W239+SLADP complex and a partially closed nucleotide pocket in the W239+SLADP complex. The opening and closing of the nucleotide pocket are summarized in Table 1.

### Discussion

Our goal was to use EPR spectroscopy to monitor conformational changes at the nucleotide site of myosin and compare the results with those obtained from tryptophan fluorescence studies using the SLADP probes. As detailed previously, EPR and fluorescence spectroscopy individually have their distinct strengths. Agreement between two experimental techniques provides stronger support for the conclusions drawn by each. In the previous studies of myosin fluorescence,21 lower fluorescence intensity was interpreted as a more closed nucleotide pocket via a correlation with known structures. In previous studies of spin-labeled nucleotides, a more immobilized probe was interpreted as a more closed pocket based on molecular dynamics (MD) calculations and on correlations between probe mobility and the topography of the adjacent protein surface. While reasonable, the interpretation of each spectroscopic method involves some assumptions. This has led to criticisms of the results. A goal of the present study was to apply for the first time the two techniques to the same samples and see how well the results agreed. As described below, the agreement is excellent.

### Comparison of WT myosin and W239+ myosin in the presence and in the absence of actin

Figures 2 and 3 summarize our basic observations. The spectra in Fig. 2 for WT myosin show a major immobilized component in the absence of actin for all temperatures examined (2–25 °C). There is a minor component with greater mobility. The magnitude of this component increases with temperature, showing a fraction of myosin pockets in the open conformation. This is similar to observations previously obtained for skeletal myosin II.15 The situation is very different for W239+ myosin in the absence of actin. The modifications involved in eliminating all endogenous tryptophans, coupled with the additional mutation F239W, have resulted in myosin that displays two components in the absence of actin. The modifications involved in eliminating all endogenous tryptophans, coupled with the additional mutation F239W, have resulted in myosin that displays two components in the absence of actin. The modifications involved in eliminating all endogenous tryptophans, coupled with the additional mutation F239W, have resulted in myosin that displays two components in the absence of actin. The modifications involved in eliminating all endogenous tryptophans, coupled with the additional mutation F239W, have resulted in myosin that displays two components in the absence of actin. The modifications involved in eliminating all endogenous tryptophans, coupled with the additional mutation F239W, have resulted in myosin that displays two components in the absence of actin. The modifications involved in eliminating all endogenous tryptophans, coupled with the additional mutation F239W, have resulted in myosin that displays two components in the absence of actin. The modifications involved in eliminating all endogenous tryptophans, coupled with the additional mutation F239W, have resulted in myosin that displays two components in the absence of actin. The modifications involved in eliminating all endogenous tryptophans, coupled with the additional mutation F239W, have resulted in myosin that displays two components in the absence of actin.

### Table 1. Comparison of the results on the conformation of the nucleotide site from EPR spectroscopy and tryptophan fluorescence at high and low temperatures

<table>
<thead>
<tr>
<th>Ligand</th>
<th>EPR (WT) Low temperature</th>
<th>EPR (WT) High temperature</th>
<th>EPR (W239+) Low temperature</th>
<th>EPR (W239+) High temperature</th>
<th>Fluorescence (W239+) Low temperature</th>
<th>Fluorescence (W239+) High temperature</th>
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<td>ADP/Actin</td>
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opening of the nucleotide pocket. The spectra for W239+ and WT myosins are essentially identical at all temperatures in the presence of actin. Thus, WT myosin and mutant *Dictyostelium* myosin have similar energetics for the conformational changes in the nucleotide pocket in the presence of actin. Spectra obtained in the presence of actin are similar to previous observations on skeletal myosin. However, the free energy involved in opening of the pocket is more similar to slow myosin than fast myosin, with the equilibrium biased toward the open conformation. That WT myosin resembles slow skeletal myosin is not unexpected since both proteins are slow. ADP release is thought to be the rate-limiting state for sliding velocity, and thus one might expect a more closed pocket in the slower myosins. However, we note that this unexpected correlation between energetics of the pocket and myosin speed also extends to other myosin isoforms. The relationship between structure and ligand affinity in motor proteins is clearly complex. For example, the X-ray structures of kinesin and ncd bound to ADP also show very open nucleotide pockets despite the fact that ADP binding in the complex is very tight.

The thermodynamics of the closed-to-open transition can be made quantitative by deconvoluting the spectra into their mobile and immobile components. Figure 7 (top) shows the experimentally obtained spectrum of WT myosin in the presence of actin, 2 °C (red). The spectra of mobile probes at the nucleotide site (blue) and immobilized probes combined with the spectrum of unbound probe (green) used for the deconvolution are also shown. The model blue and green spectra were determined as described in Materials and Methods. The experimental spectrum can be deconvoluted into a linear sum of the blue and green basis spectra as shown by the black spectrum (middle panel). The lower trace gives the residual error spectrum showing the quality of the fit. From the relative fractions of probes, an equilibrium constant $K = \frac{[\text{Mobile Fraction}]}{[\text{Immobile Fraction}]}$ from the deconvolution fits. $\Delta S$ (J/K/mol) and $\Delta H$ (kJ/mol) from the least-squares linear fits to the data are given.

![Fig. 7](top) Red, EPR spectrum experimentally recorded for WT myosin bound to actin at 2 °C; blue, mobile component spectrum for deconvolution of experimental EPR spectral components; green, immobile component plus free probe spectrum for deconvolution of experimental EPR spectral components. (Middle) Red, experimental spectrum; black, fitted spectrum using the blue and green model spectra. (Bottom) Residual error in the fit.

![Fig. 8](van’t Hoff plot of $-R\ln(K)$ versus 1/temperature (K) for the deconvolution fits to the spectra as a function of temperature. R is the Boltzmann constant. $K = \frac{[\text{Mobile Fraction}]}{[\text{Immobile Fraction}]}$ from the deconvolution fits.)

Kintses et al. interpreted an increase (decrease) in fluorescence with an opening (closing) of the nucleotide pocket. They identified two kinetically distinct W239+·diphosphate states in equilibrium at room temperature and only a single state at low temperature, with fluorescence similar to that of WT myosin. However, the value of $\Delta H$ is similar to that of WT myosin. The plot also shows that the thermodynamics of the open-to-closed transition in the presence of actin are similar for WT and W239+ myosins (red and blue data). The thermodynamics of the transition for W239+ myosin in the absence of actin (black data) is more similar to the transition in the presence of actin than to WT myosin in the absence of actin (green data).

Kintses et al. interpreted an increase (decrease) in fluorescence with an opening (closing) of the nucleotide pocket. They identified two kinetically distinct W239+·diphosphate states in equilibrium at room temperature and only a single state at low temperature, with fluorescence similar to that of W239+·ATP. In the presence of actin, tryptophan fluorescence again argued for an open nucleotide pocket. The crucial observation is that two distinct reporter signals, tryptophan fluorescence and EPR spectroscopy, are in agreement, indicating a largely closed nucleotide pocket at low temperatures, an
opening of the nucleotide pocket with increasing temperature in the absence of actin and an opening of the nucleotide pocket in the presence of actin. This agreement of the conformational changes at the active site provides additional confidence in the interpretation of each observation. The opening of the nucleotide pocket upon binding to actin is expected as the binding of actin leads to the release of nucleotides and vice versa.

### Comparison with X-ray structures

To understand better the experimental results in terms of myosin X-ray structures, we have done MD simulations of WT and modeled W239+ myosins using the X-ray structure of the original Dictyostelium myosin motor domain with bound ADP-BeFx\(^2\) (phosphate tube in a closed conformation) and the X-ray structure of Dictyostelium myosin from the myosin-dynamin complex\(^1\) (phosphate tube in an open conformation). The open conformation has been proposed to represent an actin-bound state of myosin.\(^{12,27}\)

Figure 9a shows a ribbon diagram of the nucleotide site of the closed phosphate tube X-ray structure of Dictyostelium myosin\(^2\) with the mutation F239W and 2'-SLADP docked at the active site, after MD simulation. Compared with the original phenylalanine ring, the tryptophan ring is in approximately the same plane as the WT phenylalanine ring in a hydrophobic pocket formed by the side chains of L222, I258, I243 and F430 and the butyl moiety in the side chain of K241. The tryptophan ring is actually pulled slightly deeper into this hydrophobic pocket, resulting in a 2.1-Å displacement of the Cα backbone atom of the W239+ myosin when compared with the WT myosin. W239 is at the junction of the core β-sheet of myosin and switch 1. We note that the displacement of the protein backbone in the W239+ simulation propagates N-terminally into the switch 1 domain. This results in the Oα of the side chain of S236 forming a hydrogen bond with a β-phosphate oxygen. Additionally, E223 forms a 1.8-Å hydrogen bond with Mg\(^++\). Neither is seen in the WT simulation. All other coordination mechanisms between MgADP and the protein are identical in the two simulations. When W239 is changed back to F239, creating a tryptophan-free construct, WT coordination with the nucleotide is seen in the simulation. These observations provide at least a structural indication that the W239+ protein may interact differently with the substrate and may have the altered kinetics as observed experimentally.\(^{21}\)

Figure 9b shows a ribbon diagram superposition of the results from the MD simulations of the closed phosphate tube X-ray structure\(^6\) (coral) and the open phosphate tube X-ray structure\(^1\) (light blue), with 2'-SLADP docked at the active site (ADP in red). The spin moiety ring (green with the nitroxide in purple) points out into the solvent phase. As is evident, the P-loops and N-4 loops on one side of the nucleotide pocket superimpose. The N-4 loop interacts with the adenine ring. On the opposite side of the nucleotide site, amino acids N233–N235 in switch 1 (black) and amino acids K316–V318 (dark blue, located in a surface loop) are also able to interact with the spin moiety, restricting mobility. In the transition between the closed and open conformations, there is a flexing of the core β-sheet of myosin that results in a 13.9-Å displacement of K316–V318 (dark blue) away from the spin probe ring. There is a concurrent 9.6-Å displacement of N233–N235 (black) in switch 1. This opens the nucleotide pocket in the proposed actin-bound state, and one would expect a more mobile EPR probe due to the much larger volume for the spin moiety to explore due to thermal motion. This is consistent with our experimental observations of a more mobile EPR probe in actin-bound states. W239 is at the junction of switch 1 and the myosin core β-sheet. Thus, a displacement of switch 1 would be anticipated to alter the local environment of the tryptophan and its intrinsic fluorescence.

### Comparison of WT myosin and W239+ myosin in the triphosphate and Mg\(^++\)-free states

EPR spectroscopy shows that when bound to the ATP analog, 2'-SLADP·BeFx, the nucleotide sites of WT myosin and W239+ myosin show only a single immobilized component to the spectra with a splitting representing a closed conformation of the nucleotide site. The triphosphate state is slightly more immobile than the WT-2'-SLADP state or the more immobilized component of the W239+-2'-SLADP state. Tryptophan fluorescence studies of W239+ myosin with either ATP or AMPPNP, the nonhydrolyzable ATP analog, at the active site (Fig. 3; Ref. 21)

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**Fig. 9.** (a) Ribbon diagram of the active site of Dictyostelium myosin with a closed phosphate tube with the mutation F239W (red) and 2’-SLADP docked at the active site after MD simulation. The P-loop (blue), switch 1 (magenta) and switch 2 (green) form the phosphate tube surrounding the diphosphate moiety of ADP. The spin probe moiety points out into the solvent phase. Standard colors are used for the atoms in 2’-SLADP, except for the hydrogens, which are shown in green. (b) Ribbon diagram of the nucleotide-binding sites of Dictyostelium myosin following MD simulation with a closed phosphate tube (coral) and an open phosphate tube (light blue) with 2’-SLADP at the nucleotide site after MD simulation. The structures have been superimposed based on least-squares distance minimization of the P-loop Cα atoms. The ADP moiety of 2’-SLADP in red; the spin moiety is in green, with the nitroxide in purple. The P-loop and the N-4 loop superimpose. The N-4 loop interacts with the adenine ring and the spin moiety. On the opposite side of the nucleotide site, amino acids N233–N235 in switch 1 (black) and amino acids K316–V318 (dark blue) located in a surface loop are also able to interact with the spin moiety, restricting mobility. In the transition between the closed and open phosphate tube conformations, there is a displacement of K316–V318 and N233–N235 away from the spin probe ring, which would increase probe mobility.
Fig. 9 (legend on previous page)
or SLADP–BeFx at the active site (Fig. 4) also report that the active site is closed. Thus, EPR spectroscopy and tryptophan fluorescence are again in agreement that the nucleotide site is closed in the triphosphate state. EPR spectroscopy does indicate a very slight trend toward a more open pocket with increasing temperature. Fluorescence indicates tighter closing with increasing temperature. However, this could be due to the fact that different triphosphate-state analogs were used. Nonetheless, the two experimental approaches agree on a closed nucleotide pocket.

Agafonov et al. have also used EPR spectroscopy to compare skeletal muscle and D. discoideum myosins using spin probes attached at the reactive sulphydryl, SH1, in the force-generating domain. As we have in the present study, they found that the two myosins showed essentially identical structural states but different distributions between the two states. Similar to the results we present here, they found that the energetics of the structural transition in going in the direction from weak to strong actin binding, obtained from a van’t Hoff plot, showed a large favorable entropy increase that is balanced almost exactly by a large unfavorable enthalpy increase, resulting in a very small free energy change. One explanation of these observations is that the force-generating domain is coupled to the nucleotide pocket, with both sites showing two resolved conformational states that differ only slightly in free energy. An informative point of difference from that study at the Dictyostelium SH1 site is that the ATP analog produced from ADP and beryllium fluoride showed both states present, while the present study at the nucleotide site shows a single closed state. This shows that there is not always a one-to-one correspondence between the probe spectra obtained at the two sites.

The EPR spectra of 2′-SLADP bound to WT myosin and W239+ myasin in the absence of Mg2+ were also examined. The spectra again show a single component at all temperatures, with P1–P5 splittings indicating a more closed nucleotide pocket. Tryptophan fluorescence also indicated a closed nucleotide site conformation. The P1–P5 splittings were comparable for WT myosin, with or without Mg2+, as the pocket is closed for both states. For W239+ myosin, the P1–P5 splittings in the absence of Mg2+ indicated a more closed nucleotide site in the absence of Mg2+. A similar conclusion was reached with tryptophan fluorescence as the reporter group. Thus, in the presence and in the absence of Mg2+, EPR spectroscopy and tryptophan fluorescence again reach the same basic conclusions regarding the conformation of the nucleotide pocket.

In summary, we have compared the results from EPR spectroscopy and tryptophan fluorescence spectroscopy on the conformational changes occurring at the nucleotide site of WT myosin and W239+ myosin. In the absence of actin with bound Mg-diphosphate, the pocket is mostly open at high temperatures and closes on cooling. Removal of Mg2+ results in a closing of the pocket. With bound Mg-triphosphate, the pocket is closed at all temperatures. In the presence of actin, the pocket is mostly in the open state. Similar results are found for WT myosin and W239+ myosin, with the exception that the mutant is more open in the W239+ diphosphate complex. An opening of the nucleotide pocket upon binding to actin is also seen in skeletal myosins, with the open form more favorable in the Dictyostelium proteins than in fast skeletal actomyosin. In addition, spin-labeled nucleotides yielded similar changes in protein fluorescence, showing that they were reasonable analogs of the corresponding nucleotides.

Materials and Methods

Protein preparations

The D. discoideum myosin W239F mutation was introduced into the tryptophan-free M761 motor domain cDNA fragment, expressed in Dd AX2-ORF+ cells and isolated as described previously. F-actin was purified from rabbit skeletal muscle.

Solutions

The basic rigor experimental buffer consisted of 50 mM KOAc, 5 mM Mg(OAc)2, 1 mM ethylene glycol bis(2-aminoethyl ether)N,N′-tетрацетат and 40 mM Mops, pH 7.0. Experiments performed in the presence of phosphate analogs were done in the rigor buffer with 10 mM NaF and 2 mM BeCl2 added, producing a 2 mM concentration of the fluoride complex. For experiments in the absence of Mg2+, the Mg(OAc)2 was reduced to 0.5 mM. The protein solution was allowed to sit for 30 min to allow all nucleotide analogs to be hydrolyzed to the diphosphate form, and 2 mM Na2EDTA was then added.

EPR spectroscopy

Dictyostelium myosin motor domain was exchanged into the rigor buffer, concentrated to 100 μM using a centrifuge concentrator and then labeled by the addition of 75 μM 2′-SLATP (spin-labeled ATP) or 2′,3′-SLATP. The triphosphate species is hydrolyzed by myosin to the diphosphate species. The labeled protein was then run through a Micro Bio-Spin P-30 (Bio-Rad) spin column to eliminate free probe. EPR spectra were obtained in a 50-μl glass capillary. For experiments with actin, the labeled myosin was added to F-actin at a stoichiometry of 1:10 myosin heads/actin monomers in the rigor buffer. This mixture was centrifuged at 70,000 g for 20 min. The supernatant was analyzed to determine the amount of myosin-SLADP remaining in solution and discarded. The pellet containing the actomyosin complex was transferred to a quartz flat cell. The actin concentration in these pellets was estimated at 600 μM. The pellet was covered with a glass cover slip, sealed with vacuum grease to prevent dehydration and placed in the EPR cavity. Spin-labeled nucleotides (Fig. 1) were synthesized using slight modifications of previously published methods. EPR measurements were performed with a Bruker EMX EPR spectrometer (Billerica, MA). First derivative X-band spectra were recorded in a high-sensitivity microwave cavity using 50-s, 10-mT-wide magnetic field sweeps. The
Fluorescence experiments

The experimental buffer contained 20 mM Hepes, 40 mM NaCl, 2 mM MgCl₂, 2 mM 2′,3′-SLADP or ADP and 3 μM W239+ myosin. Steady-state fluorescence measurements were done using a Fluoromax-3 fluorimeter (JY Horiba, Edison, NJ) with a 150-W Xe lamp and 10-mm path-length cell. Tryptophan was excited at 296 nm using 1-nm excitation and 6-nm emission slits. The fluorescence emission was detected at 345 nm. The temperature dependence was measured at 2 °C intervals by heating the sample from 9 to 23 °C and then cooling the sample back down to 9 °C. Readings from the two cycles were averaged. For fluorescence measurements of the triphosphate analog state, 2 mM βBeF₃ and 10 mM NaF were added to the experimental buffer.

Deconvolution of spectral components

Experimental spectra were sorted into two groups based upon the strongest component, either mostly closed (28 spectra representing 1075 total scans) or mostly open (42 spectra representing 1242 total scans). Each group was summed into a representative spectrum. The mostly open representative spectrum was subtracted from the mostly closed representative spectrum to yield a spectrum that had only closed and unbound components. The inverse spectrum used in data analysis was an average of 5–30 sweeps from an individual experimental preparation. Temperature was controlled by blowing dry air (warm or cool) into the cavity and monitored using a thermistor placed close to the experimental sample.

Effective cone angles of mobility can be approximated using the order parameter 

\[ S = \frac{3}{2} \left( \frac{1}{T_0} - \frac{1}{T'} \right) \]

as a measure of probe mobility. Here, \( 2T_0 \) is the observed splitting, \( 2T' \) is the splitting for an immobilized probe and \( T_0 \) is the isotropic hyperfine splitting for freely tumbling SLADP in solution. The cone angle is then given by \( \cos \theta = -0.5 \pm 0.5(1 + 8S) \), where \( 2\theta \) is the vertex angle of the cone of mobility. Additional details are in Refs. 24 and 25.

Structural modeling

Modeling of the nucleotide site used the X-ray structure of the Dictyostelium ADP-BeF₃ motor domain with ADP-BeF₃ at the nucleotide site and a closed phosphate tube (Protein Data Bank ID 1MMD2) or the structure of the closed phosphate tube myosin structure via least-squares distance minimization of the P-loop Cα backbone atoms of myosin from the myosin-dynamin complex and the closed phosphate tube myosin structure containing the previously docked 2′-SLADP. The structures were charge neutralized by addition of Na⁺ ions and solvated in a box of explicit TIP3P water molecules extending a minimum of 10 Å from the protein–nucleotide surface. The structures were then energy minimized using 1500 steps of the steepest descents algorithm, followed by 1500 steps of conjugate gradient algorithm. Root-mean-square values for the gradient vector were less than 0.2 at the end of 3000 steps. Charges for 2′-SLADP were derived by first performing a single-point energy calculation at the Hartree–Fock level of theory with a 6-31G* basis set to obtain the electrostatic charges using Gaussian 03. These were fit to the molecule using the RESP procedure. MD simulations were then done at 300 K for 1 ns of total simulation time using the energy-minimized structures as the initial states. Temperature was maintained via coupling to an external bath using the Berendsen algorithm. The SHAKE algorithm was employed for numerical integration in time with a 1-fs time step.

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2009.12.035

References


