Tryptophan 512 is sensitive to conformational changes in the rigid relay loop of smooth muscle myosin during the MgATPase cycle †

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Running Title: Intrinsic fluorescence during the myosin ATPase cycle

Abbreviations
AlF₄⁻, aluminum fluoride; BeFₓ, berellium fluoride; Dicty, Dictyostelium discoideum; Mant, 2ʻ(3ʻ)-O-(N-methylanthraniloyl); MDE, motor domain essential light chain; FRET, fluorescence resonance energy transfer.
Summary

To examine the structural basis of the intrinsic fluorescence changes which occur during myosin’s MgATPase cycle, we generated three mutants of smooth muscle myosin MDE (motor domain and essential light chain) containing a single conserved tryptophan residue located at Trp-441 (W441-MDE), Trp-512 (W512-MDE), or Trp-597 (W597-MDE). Although W441- and W597-MDE were insensitive to nucleotide binding, the fluorescence intensity of W512-MDE increased in the presence of MgADP-BeF₅ (31%), MgADP-AlF₄⁻ (31%), MgATP (36%), and MgADP (30%) compared to the nucleotide-free environment (rigor), which was similar to the results of wild type-MDE. Thus, Trp-512 may be the sole ATP-sensitive tryptophan residue in myosin. In addition, acrylamide quenching indicated that Trp-512 was more protected from solvent in the presence of MgATP or MgADP-AlF₄⁻ than in the presence of MgADP-BeF₅, MgADP, or in rigor. Furthermore, the degree of energy transfer from Trp-512 to (2’(3’)-O-(N-methylanthraniloyl)) mant-labeled nucleotides was greater in the presence of MgADP-BeF₅, MgATP or MgADP-AlF₄⁻ than MgADP. We conclude that the conformation of the rigid relay loop containing Trp-512 is altered upon MgATP hydrolysis and during the transition from weak to strong actin binding, establishing a communication pathway from the active site to the actin-binding and converter/lever arm regions of myosin during muscle contraction.
Introduction

It has been well established that muscle contraction is driven by structural rearrangements within myosin during its ATPase cycle and interaction with actin. The crystal structures of myosin (see Figure 1) solved in different nucleotide states have provided a framework for examining the structural basis of myosin’s ATPase cycle (1-6). In addition, solution techniques that include monitoring myosin’s intrinsic fluorescence have proven extremely valuable for examining the enzymatic and kinetic properties of the molecule (7-13). The following kinetic scheme of the myosin MgATPase cycle was developed based on observed changes in intrinsic fluorescence corresponding to structural changes within myosin, where M represents myosin and an asterisk represents enhanced protein fluorescence (14):

\[
M + ATP \leftrightarrow M\cdot ATP \leftrightarrow M^*\cdot ATP \leftrightarrow M^{**}\cdot ADP.Pi \leftrightarrow \\
M^{**}\cdot ADP.Pi \leftrightarrow M^*\cdot ADP + Pi \leftrightarrow M\cdot ADP \leftrightarrow M + ADP
\]

In this scheme myosin first forms a collision complex with ATP followed by an isomerization to the \( M^*\cdot ATP \) complex, which results in the first level of fluorescence enhancement (*). During the rapid reversible process of ATP hydrolysis, a structural change induces an additional fluorescence enhancement (**). Then, following hydrolysis of ATP the fluorescence decreases back to the first level of enhancement (*), which is thought to correspond to the rate limiting structural change resulting in phosphate-release. The phosphate-release step then shifts myosin from a weak to strong binding conformation, and is the step believed to be associated with force generation during muscle contraction. The release of ADP is a much faster two step process in which the second step results in a decrease in fluorescence to basal levels. Thus, examining the conformational changes that result in alterations in intrinsic tryptophan fluorescence may lead to important insights about the structural properties of the myosin MgATPase cycle.
Dynamic structural information about domain motions within myosin during its MgATPase cycle has been pursued extensively (reviewed in 15). Indeed, several studies have demonstrated key rearrangements in the light chain-binding region, (residues 781-820 highlighted in pink in Figure 1), also referred to as the lever-arm, providing a structural mechanism for force generation (reviewed in 16). Electron paramagnetic resonance studies, utilizing a probe on the regulatory light chain (RLC) have shown a 30° rotation of this region (17), suggesting the lever-arm region rotates relative to the catalytic domain during muscle contraction. In addition, fluorescence studies utilizing a fluorescent probe at the RLC demonstrated rotation of the lever-arm in contracting muscle fibers (15). Cryo-electron microscopy studies have revealed that smooth muscle myosin decorated on actin filaments undergoes a large structural change (30-35 Å) of the lever-arm upon ADP-release (18). Furthermore, nucleotide analogs, which trap myosin in specific nucleotide states, have been useful in elucidating the structural properties of the normally short-lived stages of the MgATPase cycle. Structural studies suggest that MgADP-BeF_x corresponds to the M·ATP state, and MgADP-AlF_4^- corresponds to the transition-state of ATP hydrolysis (19), whereas MgATP present at saturating levels at 20ºC represents primarily the M.ADP.Pi state (20). Fluorescence resonance energy transfer (FRET) experiments have shown that the lever-arm is further away from the catalytic domain in the MgATP state simulated by MgADP-BeF_x, moves closer in the MgADP.Pi state simulated by MgADP-AlF_4^- or MgADP-Vi, and further away again in the MgADP and rigor states (21, 22). While many studies have demonstrated rotation of the lever-arm relative to the catalytic domain during the contractile cycle, the structural details of how conformational changes in the catalytic domain are communicated to the lever-arm are currently unclear.
The converter region (residues 709-780, highlighted in blue in Figure 1), a flexible region connecting the lever-arm to the motor domain, may be responsible for allowing the lever-arm to rotate relative to the catalytic domain. Structural studies have demonstrated large differences in the converter region when comparing the crystal structures of myosin in different nucleotide states (5, 6, 23). Furthermore, the rigid relay loop (residues 512-518, highlighted in red in Figure 1), which provides a link between the active site and the converter domain as well as actin-binding regions, contains a conserved tryptophan residue (Trp-512 in smooth muscle myosin) that may be sensitive to nucleotide binding and ATP hydrolysis (24-26). Thus, examination of conformational changes in Trp-512 of myosin during different stages of the contractile cycle may lead to important insights into the coupling pathways that propagate through the rigid relay loop to the actin-binding and converter/lever arm regions of myosin. Also, it is still unclear if other tryptophan residues that are conserved among skeletal muscle, smooth muscle, and Dictyostelium discoideum (Dicty) myosin II (Trp-441 and Trp-597 in smooth muscle myosin) play a role in the intrinsic fluorescence changes that have been demonstrated in all three myosin isoforms (7, 11, 12).

In the current study, intrinsic tryptophan fluorescence was used to examine changes in the environment of three conserved tryptophans (441, 512, and 597) during the MgATPase cycle of smooth muscle myosin. Three different fragments of smooth muscle myosin, containing the motor domain with the essential light chain (MDE), were engineered to contain a single tryptophan located at each of these positions; 441, 512, or 597. The tryptophan fluorescence from W441-, W512-, and W597-MDE was examined in the presence and absence of nucleotides as well as nucleotide analogs to investigate the contribution of each of these tryptophans to the changes in intrinsic protein fluorescence observed in different stages of the MgATPase cycle. In
addition, we examined the movements that occur in the rigid relay loop by monitoring the amount of FRET between Trp-512 and (2'(3’)-O-(N-methylanthraniloyl)) (mant) labeled nucleotides and nucleotide analogs. We have identified structural changes that occur in the rigid relay loop upon nucleotide binding and ATP hydrolysis, providing new insights into the structural pathways that couple the active site to the actin-binding and converter/lever arm regions of myosin.
Experimental Procedures

Chemicals. Mant (2’(3’)-O-(N-methylanthraniloyl)) labeled ATP and ADP were purchased from Molecular Probes Inc. (Eugene, Oregon). Potassium chloride (KCl), sodium chloride (NaCl), ethylene glycol bis(-aminoethyl ether), beryllium chloride (BeCl₂), aluminum chloride (AlCl₃), sodium fluoride (NaF), adenosine 5’triphosphate (ATP), and adenosine 5’diphosphate (ADP) were purchased from Sigma (St. Louis, MO). All other reagents were the highest quality available.

Solutions. A stock solution of BeCl₂ (Be, atomic absorption standard solution, in 1% HCl) was adjusted to pH 5.0 by addition of 1 N NaOH. Stock solutions of 1 M NaF were freshly prepared each day. Our standard buffer was MOPS buffer at pH 7.4 and 4 ºC, (20 mM MOPS, 20 mM KCl, 1 mM EGTA, and 1 mM NaN₃), and 1 mM DTT was added when necessary. Imidazole buffer at pH 7.4 and 4 ºC, (10 mM imidazole-HCl, 90 mM NaCl, 1 mM NaN₃, and 1 mM DTT) was used during protein purification steps only (described below).

cDNA Construction. We performed site-directed mutagenesis on a clone of smooth muscle myosin heavy chain containing the motor domain and essential light chain binding regions (residues 1-819, kindly provided by Kathleen M. Trybus, University of Vermont) to produce three mutants, each containing a single tryptophan residue. We constructed these mutants containing a single conserved tryptophan residue, Trp-441, Trp-512, or Trp-597, by mutating six of the seven endogenous MDE tryptophans to phenylalanine or methionine in the case of Trp-546 (highlighted as space-filling models in Figure 1). We also constructed as a mutant containing no tryptophans (Null-MDE), and a molecule containing all seven native tryptophans (WT-MDE) described previously (27). The FLAG epitope sequence (DYKDDDDK) was attached at the C-terminus of all MDE constructs for purification purposes (28).
**Protein Expression and Purification.** The baculovirus system was used to express all MDE constructs by co-infecting Sf9 insect cells with recombinant baculoviruses encoding the desired myosin heavy chain and essential light chain sequences. Following incubation with baculovirus for three days, the Sf9 cells were lysed, fractionated between 40 and 70% saturated ammonium sulfate, and dialyzed overnight in Imidazole buffer at 4 °C. The dialyzate was bound to an anti-FLAG antibody column and eluted with the homologous peptide and dialyzed overnight into MOPS buffer with 1 mM DTT present. The degree of purity of the MDE was assessed by SDS/PAGE (29) using Coomassie-stained gels. Purified MDE concentrations were determined by the method of Bradford (30) using the BioRad microplate assay and WT-MDE as a standard. All experiments were performed on 2-3 different MDE preparations.

Actin was purified from chicken pectoralis muscle using an acetone powder method described previously (31). The concentration of purified actin was determined spectrophotometrically using an extinction coefficient of 0.62 (mg/ml)⁻¹·cm⁻¹ at 290 nm.

**Preparation of MDE-Nucleotide Analog Complexes.** MDE trapped with ADP-BeF₄ or ADP-AlF₄ was prepared as described previously (32). Briefly, 0.6 µM purified MDE in MOPS buffer at pH 7.4 was mixed with 1 mM MgCl₂, 3 µM ADP or mantADP, 2 mM NaF, and 0.2 mM AlCl₃ or BeCl₂ for 20 minutes at room temperature. The MDE/analog complexes formed with greater than 85% efficiency based on the inhibition of their ammonium (0.4 M NH₄Cl, 35 mM EDTA, 25 mM Tris, pH 8.0 at 20 °C ) ATPase activities.

**Enzymatic Assays.** Actin-activated ATPase assays were performed at 37°C in low ionic strength MOPS buffer using the method of White (33). Purified MDE was assayed at a concentration of 0.1 mg/ml in the presence of 0, 10, 30, 50, 70 and 90 µM actin and inorganic phosphate production was measured colorimetrically every 10 minutes for a 70 minute period.
The average ATPase rates [nmoles Pi·(nmoles MDE)$^{-1}$·s$^{-1}$] of two separate preparations of W441-, W512-, W597-, and WT-MDE were plotted as a function of actin concentration and fit with a non-linear least squares method using GraphPad Prism software (San Diego, CA). Values of $V_{\text{MAX}}$ and $K_{M}$ were determined using Michaelis-Menten kinetics.

**Fluorescence Measurements.** A Quantamaster fluorimeter (Photon Technology International, South Brunswick, NJ) equipped with a 75-W Xenon arc-lamp as an excitation source, excitation/emission monochrometers, and a WG—320 cut-off emission filter was used to measure steady-state fluorescence from tryptophan and mant-labeled nucleotides. Tryptophan fluorescence emission spectra were measured by exciting the sample at 295 nm and collecting the emitted fluorescence from 305-400 nm. Mant-labeled nucleotides were excited at 360 nm and the emission spectrum was measured from 380-500 nm. The excitation spectra of mant-labeled nucleotides were collected from 250 – 400 nm with an emission wavelength of 440 nm. Slit widths were set at a resolution of 2 nm. All fluorescence spectra were corrected for variations in the wavelength sensitivity of the detector system and the presence of Raman scatter and background fluorescence in the appropriate buffer solution. Quantum yields ($\Phi$) for tryptophan were calculated by a comparative method (34) using L-tryptophan as a standard ($\Phi = 0.14$) (35).

The fluorescence emission spectrum of each of the three mutants was measured in the presence of 2 mM MgADP or MgATP, as well as in the absence of nucleotide. In addition, the fluorescence spectrum of W512-MDE was measured complexed with the nucleotide analogs, MgADP-AlF$_4^{-}$ and MgADP-BeF$_{6}$. The binding of mantATP or mantADP to W512-MDE was examined by titrating increasing concentrations of mantADP or mantATP with 0.6 µM W512-
MDE and monitoring the two-fold increase in fluorescence of mant at 440 nm, which occurs upon binding to myosin (12).

Acrylamide quenching was used to determine the degree of exposure to solvent of Trp-512 in the presence of nucleotide and nucleotide analogs. The decrease in fluorescence intensity at the \( \lambda_{\text{MAX}} \) was measured as a function of increasing acrylamide concentrations ([Q]). The fluorescence in the absence of quencher (\( F_0 \)) divided by the fluorescence in the presence of quencher (\( F \)) was used to quantify the relative change in fluorescence from acrylamide quenching (\( F_0/F \)). \( F_0/F \) was plotted as a function of [Q] and fit to the Stern-Volmer relationship, taking into account both static (\( V \)) the and dynamic quenching (\( K_{SV} \)) constants: \( F_0/F = (1 + K_{SV}[Q])(\exp V[Q]) \) (36). The static quenching value, which determines the amount of upward curvature of the Stern-Volmer plot, was determined by manually varying \( V \) and found to be similar (\( V = 3.0 \text{ M}^{-1} \)) in all cases. Thus, the dynamic quenching constant was determined by fitting the Stern-Volmer plots by a non-linear least squares method using GraphPad Prism software (San Diego, CA), while \( V \) was held constant at 3.0 M\(^{-1}\).

**Fluorescence Energy Transfer Measurements.** Fluorescence resonance energy transfer (FRET) was used to measure the distance between the donor fluorophores, Trp-512, and acceptor fluorophores, mant labeled nucleotides, using the Förster energy transfer theory (37). We measured the efficiency (\( E \)) of energy transfer by examining the increase in the acceptor excitation intensity upon donor excitation using the equation:

\[
E = \left[ \frac{F_{DA285}}{F_{DA360}} - \varepsilon_{A285}/\varepsilon_{A360} \right] \cdot \varepsilon_{A360}/\varepsilon_{D285} \quad \text{(Eq. 1)}
\]

where \( F_{DA285} \) and \( F_{DA360} \) are the intensities from the excitation spectrum of the acceptor in the presence of the donor, corrected by subtracting the fluorescence from the unbound mant nucleotides, and \( \varepsilon_{A285} \), \( \varepsilon_{A360} \), and \( \varepsilon_{D285} \) are the extinction coefficients of the acceptor at 285 nm.
(3.0 x 10^2 M\textsuperscript{-1}\textperiodcentered cm\textsuperscript{-1}) and 360 nm (5.0 x 10^3 M\textsuperscript{-1}\textperiodcentered cm\textsuperscript{-1}) and the donor at 285 nm (5.5 x 10^3 M\textsuperscript{-1}\textperiodcentered cm\textsuperscript{-1}) (38), respectively.

The distance \( r \) between the donor and acceptor fluorophores can be calculated from Equation 2

\[
\begin{align*}
\text{r} &= R_0(E^{-1} - 1)^{1/6} \text{Å} \\
\end{align*}
\]  

(Eq. 2)

where \( R_0 \) is the Förster critical distance at which energy transfer is equal to 50%. The distance \( R_0 \) is calculated from Equation 3

\[
R_0 = 9.79 \times 10^3 \left(K^2Q_Dn^{-4}\right)^{1/6} \text{Å}
\]  

(Eq. 3)

where \( n \) is the refractive index of the buffer (= 1.4), \( Q_D \) is the quantum yield of W512-MDE determined in the presence of MgADP, MgADP-BeF, MgADP-AlF\textsubscript{4}\textsuperscript{-}, MgATP, and absence of nucleotide (0.31, 0.31, 0.31, 0.33, and 0.22, respectively), \( K^2 \) is the orientation factor assuming free rotation of the donor and acceptor fluorophore (= 2/3) (39), and \( J \) is the spectral overlap integral in cm\textsuperscript{3}\textperiodcentered M\textsuperscript{-1} given by the Equation 4 (40).

\[
J = \int F_D(\lambda)e_A(\lambda)\lambda^4d\lambda
\]  

(Eq. 4)

where \( F_D(\lambda) \) is the normalized total integrated intensity of the unquenched donor group and \( e_A(\lambda) \) is the extinction coefficient of the acceptor in M\textsuperscript{-1}\textperiodcentered cm\textsuperscript{-1}. \( J \) was numerically integrated at 1-nm intervals and determined to be 5.18 x 10\textsuperscript{-15} M\textsuperscript{-1}\textperiodcentered cm\textsuperscript{3} in all nucleotide states studied, where \( \lambda \) is the wavelength in centimeters. Since the presence of various nucleotides changed only the intensity and not the shape of the fluorescence emission spectra from W512-MDE, the calculated value of \( J \) was relatively insensitive to the nucleotide present at the active site of W512-MDE.
Results

Functional Assays. Functional assays were performed to examine the impact of the conservative mutations made to generate the smooth muscle myosin single tryptophan-containing mutants, W441-, W512, and W597-MDE. All MDE constructs were expressed and purified to similar levels (>90%) and yields (1-2 mg per 1 x 10^9 Sf9 cells). All three mutants displayed VMAX values that were reduced 4-5 fold, and KM values 2-4 fold lower than WT-MDE (Table 1). Previously, similar MDE mutants were shown to decorate actin with the classic arrowhead appearance as demonstrated by electron microscopy (27) and thus W441-, W512-, W597-MDE are likely to bind actin similarly. Most importantly, despite alterations in the ATPase rates, all three mutants display enhanced MgATPase rates in the presence of actin.

Tryptophan Fluorescence Enhancement During the MgATPase Cycle. We examined the fluorescence properties of W441-, W512-, and W597-MDE in the presence of nucleotides and nucleotide analogs to determine how the structural properties of these conserved tryptophans were altered throughout the MgATPase cycle. The fluorescence spectra and emission peak maxima of W441- and W597-MDE (334 and 332 nm, respectively) were unchanged in the presence of either MgATP or MgADP (data not shown). In contrast, the fluorescence properties of W512-MDE demonstrated a 31.0 ± 1.1 % increase in the peak fluorescence intensity (336 nm) in the presence of MgADP-BeF₄⁻ (i.e. the M·ATP-state), a 30.7 ± 2.5 % increase in the presence of MgADP-AlF₄⁻ (i.e. the transition state between the M·ATP and M·ADP.Pi-states), a 36.1 ± 1.1 % increase in the presence of MgATP (i.e. predominantly the M·ADP.Pi-state), and a 30.1 ± 2.1 % increase in the presence of MgADP (i.e. the M·ADP-state), when compared to the nucleotide-free or rigor environment (i.e. the M-state) (Figure 2B and Table 2). Interestingly, the fluorescence spectrum of WT-MDE demonstrated an increase in its peak fluorescence intensity
of 8.9 ± 2.1% in the presence of MgADP-BeF$_X$, 13.2 ± 1.2% in the presence of MgADP-AlF$_4^-$, 18.4 ± 0.0% in the presence of MgATP, and 12.1 ± 0.0% in the presence of MgADP compared to the absence of nucleotide (Figure 2A and Table 2). Although the fluorescence enhancement of W512-MDE was 2-3 fold greater than WT-MDE, the level of enhancement followed a similar pattern; greatest in the presence of MgATP, slightly lower in the presence of MgADP-AlF$_4^-$, MgADP-BeF$_X$, and MgADP, and lowest in the nucleotide-free state. Thus, Trp-512 appears to be the sole tryptophan of those conserved between skeletal muscle, smooth muscle, and Dicty myosin II that is sensitive to nucleotide binding and hydrolysis, and its enhancement profile was similar to previous studies on smooth muscle myosin (8, 12).

**Acrylamide quenching of W512-MDE.** In order to examine the solvent exposure of Trp-512 in different nucleotide states we performed acrylamide quenching experiments on W512-MDE in the presence of MgADP-BeF$_X$, MgADP-AlF$_4^-$, MgATP, MgADP, and in the absence of nucleotide (rigor) (Figure 3 and Table 2). The Stern-Volmer relationship was used to quantitatively determine the amount of acrylamide quenching (see Experimental Procedures). The dynamic quenching constants, indicative of the quenching that occurs by interaction of acrylamide with the excited state of tryptophan, of W512-MDE were calculated to be 2.81 ± 0.04 M$^{-1}$, 1.96 ± 0.05 M$^{-1}$, 1.93 ± 0.09 M$^{-1}$, 2.96 ± 0.07 M$^{-1}$, and 3.05 ± 0.03 M$^{-1}$ in the presence of MgADP-BeF$_X$, MgADP-AlF$_4^-$, MgATP, MgADP, and rigor, respectively (Table 2). These data suggest that Trp-512 remains in a solvent exposed conformation upon MgATP binding, becomes more protected from solvent during the process of and following ATP hydrolysis, adopts a more solvent exposed conformation following Pi-release, and remains in a similar environment upon ADP-release.
Energy transfer from Trp-512 to mant labeled nucleotides/analogs. We examined the amount of FRET from Trp-512 to mant-labeled nucleotides and nucleotide analogs (MgATP, MgADP, MgADP-BeFx, MgADP-AlF₄⁻), to monitor changes in the distance between Trp-512 and the active site of myosin in different nucleotide states. Binding of mantATP or mantADP to W512-MDE was examined by titrating 0.6 µM W512-MDE with mant labeled nucleotides (0.5 – 5.0 µM) and monitoring the enhanced fluorescence of mantATP or mantADP known to occur upon myosin binding. This analysis demonstrated that there was a two-fold increase in mant fluorescence upon binding myosin, similar to previous results (12), and allowed us to determine the concentration of mantADP (1.5 µM) or mantATP (1.8 µM) at which W512-MDE was fully saturated with nucleotide (data not shown). The excitation spectra of mant-labeled nucleotides/analogs (3.0 µM) in the presence of W512-MDE (0.6 µM) shown in Figure 4 was corrected by subtracting the fluorescence from the unbound mant-labeled nucleotides assuming W512-MDE was 100% saturated with nucleotide. The efficiency of energy transfer, quantified by monitoring the change in the acceptor excitation spectrum at 285 nm, the peak excitation wavelength of Trp-512 (see Experimental Procedures), is highest when W512-MDE is complexed with mantADP-BeFx (43.5 ± 3.3 %), somewhat lower when complexed with mantADP-AlF₄⁻ (30.3 ± 6.7 %) or mantATP (35.4 ± 5.5 %), and much lower in the presence of mantADP (13.0 ± 1.3 %) (Table 3). R₀ was calculated to be 25 Å for the (Trp-512)/(mant-labeled nucleotide) donor/acceptor pair in the presence of mantADP, mantATP, mantADP-BeFx, and mantADP-AlF₄⁻ from Eqs. 3 and 4 in Experimental Procedures. The calculated distances based on the measured efficiencies, R₀, and the Förster equation (see Eq. 2 in Experimental Procedures) were 26.1 ± 0.6 Å, 28.7 ± 1.6 Å, 27.6 ± 1.1 Å, and 34.3 ± 0.7 Å for W512-MDE complexed with mantADP-BeFx, mantADP-AlF₄⁻, mantATP, and mantADP, respectively (Table
3). Thus, Trp-512 is the closest to the active site in the MgATP state, moves ~1-3 Å away in the MgADP.Pi state, and upon Pi-release from the active site and formation of the strongly bound state Trp-512 moves an additional ~5-7 Å further away from the active site.
**Discussion**

Determining how myosin converts chemical energy, via ATP hydrolysis, into mechanical work and force generation is critical for understanding how motor proteins such as myosin function. The intrinsic fluorescence of myosin is sensitive to nucleotide binding and MgATP hydrolysis (7-13), although the structural changes that lead to these alterations in intrinsic fluorescence have not been clearly elucidated. We have developed an extremely sensitive method utilizing mutants of smooth muscle myosin containing only one of the three conserved tryptophans among smooth muscle, skeletal muscle, and Dicty myosin II (Trp-441, Trp-512, Trp-597) to examine which of these residues is responsible for the observed changes in intrinsic fluorescence observed in all three myosin isoforms. In the current study, only Trp-512 appears to be sensitive to nucleotide binding and hydrolysis. Because Trp-512 is located on the rigid relay loop connecting the converter and actin-binding regions to the active site of myosin, we have exploited the fluorescence changes observed in Trp-512 to determine the direction of movement and alterations in environment of the rigid relay loop during the MgATPase cycle of smooth muscle myosin. Therefore, we have identified key structural rearrangements in the rigid relay loop that allow coupling from the active site to the actin-binding and converter/lever-arm regions of myosin.

**Functional properties of W441-, W512, and W597-MDE**

Enzymatic assays were used to determine the extent to which the conservative mutations made in the W441-, W512-, and W597-MDE constructs affected the native properties of myosin by comparing them to WT-MDE. The actin-activated ATPase properties of W441, W512-, and W597-MDE demonstrated a 4-5 fold reduction in the maximal ATPase rate, while their $K_M$ values were comparable to that of WT-MDE (Table 1). Despite the relatively modest effect on
enzymatic function, we suggest that these mutants are extremely valuable in examining the structural basis of myosin’s MgATPase cycle, as their ability to hydrolyze ATP in an actin-dependent manner was maintained.

**Tryptophan fluorescence enhancement in myosin**

We observed no changes in the fluorescence of W441- and W597-MDE in the presence of MgATP or MgADP. However, the W512-MDE fluorescence was enhanced ~31% in the presence of MgADP-BeF$_X$ (i.e. the M·ATP-state) and MgADP-AlF$_4^-$ (i.e. the transition state between the M·ATP- and M·ADP.Pi-states), increased ~5% further in the presence of MgATP (i.e. predominantly the M·ADP.Pi-state), and was reduced ~6% upon Pi-release (i.e. the M·ADP-state). We obtained similar results, although lower in magnitude, for the relative changes in the intrinsic tryptophan fluorescence of WT-MDE. Thus, Trp-512 appears to be the only nucleotide-sensitive tryptophan in MDE, and because of the high degree of sequence conservation at this site across myosin isoforms, is probably solely responsible for the fluorescence enhancements observed in skeletal muscle and Dicty myosin as well. These results are consistent with other studies suggesting that this conserved tryptophan residue is responsible for at least some of the ATP-sensitive intrinsic fluorescence signal of myosin (24-26). Manstein and colleagues have demonstrated that when Trp-501, the equivalent residue in Dicty myosin, is substituted with tyrosine (W501Y) no fluorescence enhancement was observed in the presence of MgATP (41). Hence, the other tryptophans in the W501Y construct did not appear to contribute to the ATP-sensitivity of Dicty myosin. However, Dicty myosin only displays enhanced fluorescence upon the hydrolysis of ATP (42), whereas smooth and skeletal muscle myosins demonstrate an increase upon ATP-binding and a further increase upon the hydrolysis of ATP (see the ATPase scheme in the *Introduction*). Thus, the fluorescence enhancement demonstrated in both smooth
and skeletal muscle myosin upon ATP-binding may result from structural changes in Trp-512, but these changes may be unique to vertebrate muscle myosin. Our results suggest the structural changes that occur in the rigid relay loop and lead to the fluorescence changes in Trp-512 during the hydrolysis step, may be conserved among all three myosin isoforms. It is also possible that other non-conserved tryptophans in MDE are sensitive to nucleotide binding and hydrolysis, especially in the ATP-bound state (i.e. MgADP-BeFx) since the fluorescence enhancement of W512-MDE is 3.5 fold higher than WT-MDE, instead of 2.3-2.5 fold in the other nucleotide states. One other tryptophan that could be nucleotide sensitive in WT-MDE is Trp-29 because it is unique to smooth muscle myosin and conformational changes around this residue are quite possible given its location near the converter region.

The fluorescence enhancement of W512-MDE was only 2-3 fold greater than WT-MDE, containing seven tryptophans and potential for a 7-fold stronger background fluorescence signal. One possible explanation for this result may be that the structure of W512-MDE is modified compared to WT-MDE, altering the normal nucleotide induced conformational changes sufficiently to decrease the fluorescence enhancement levels of W512-MDE. However, we feel this is unlikely because the fluorescence enhancement profile of W512-MDE follows a similar pattern to WT-MDE and other studies with smooth muscle myosin (8, 12). Alternatively, other tryptophan residues in WT-MDE may contain extremely low quantum yields and be essentially non-fluorescent. We previously measured the quantum yields of two other endogenous tryptophans, Trp-546 and Trp-625, in MDE (27), which were similar to the three conserved tryptophans examined in this study. This suggests that the two endogenous tryptophans not yet analyzed, Trp-29 and Trp-36, may be highly quenched in the WT-MDE construct.

**Conformational changes in Trp-512**
In order to gain a better understanding of the changes in the environment of Trp-512 in different nucleotide states we performed acrylamide quenching experiments with W512-MDE bound to MgADP-BeF₄, MgADP-AlF₄⁻, MgATP, MgADP, or in the absence of nucleotide (rigor). Our results demonstrate that Trp-512 remains in a solvent exposed conformation in the MgATP-bound state (i.e. MgADP-BeF₄), becomes more protected from solvent in the transition (i.e. MgADP-AlF₄⁻) and MgADP.Pi states (i.e. MgATP), and returns to a more exposed conformation following Pi-release (i.e. MgADP). Since we were not able to accurately determine the fluorescence lifetime of Trp-512 under all conditions studied, we did not determine bimolecular quenching constants from our acrylamide quenching data. However, we still believe that the calculated dynamic quenching constants are likely to be reflective of the overall accessibility of Trp-512 in our experiments. The quantum yields determined in the absence or presence of various nucleotides and analogs, which reflect the same excited state relaxation properties as the corresponding fluorescence lifetime values, differed by no more than 50%, and scaling the calculated dynamic quenching constants by the relative quantum yield values did not qualitatively change the interpretation of our data.

Quenching experiments performed on the corresponding residue in skeletal muscle myosin, Trp-510, demonstrated a decrease in the dynamic quenching constant and thus the solvent exposure of Trp-510 upon MgATP binding and a further decrease following the hydrolysis of ATP (26). Our dynamic quenching constants (Kₜₛ) follow a similar trend suggesting conformational changes in the rigid relay loop may be quite similar in smooth and skeletal muscle myosin. Park et al. (26) suggested that the cleft surrounding Trp-510, referred to as the “probe binding cleft”, closes as myosin binds and hydrolyzes ATP. Hence, closure of the probe binding cleft as ATP is hydrolyzed would correspond to the pre-powerstroke state and
opening of this cleft during Pi-release might be part of the structural changes in myosin responsible for the transition to the strongly bound state and the powerstroke. Interestingly, studies that have examined conformational changes in the lever-arm have revealed a relationship between the nucleotide state and the position of the lever-arm (21, 22). Thus, the rigid relay loop may communicate structural changes from the active site to the converter and lever-arm regions of myosin.

**Movement of the rigid relay loop**

We specifically examined movement of the rigid relay loop in relation to the active site of myosin by performing FRET experiments between Trp-512 and mant-labeled nucleotides. The distances calculated between Trp-512 and mant-labeled nucleotides, which ranged from 26-34 Å, were similar to the distances between Trp-512 and the nucleotide bound to the active site of myosin in various crystal structures (5, 6, 43). We assumed that the orientation factor \( K^2 \) was 2/3 (i.e. the rotational motion of our donor and acceptor probes was unrestricted during the fluorescence lifetime of the donor) and that \( K^2 \) as well as the extinction coefficients of the donor and acceptor were unchanged in the different nucleotide states, which may have introduced error into our distance and efficiency calculations. However, since our calculated distances were similar to those observed in the available crystal structures our assumptions probably are valid, or at least do not alter our qualitative interpretation of the data. Previous studies that have examined the conformation of mantADP, mantATP, and mantADP-BeFx suggest that the mant fluorophore has a similar conformation in each stage of the skeletal muscle myosin MgATPase cycle (44). Thus, the reproducible changes in the excitation spectrum of mant labeled nucleotides/analogs bound to W512-MDE most likely represent significant changes in the
relative distance between Trp-512 and mant-labeled nucleotides caused by movement of Trp-512, which can be discussed in terms of the structure of myosin.

The distance between Trp-512 and mant-labeled nucleotides was closest in the weak binding, MgATP and MgADP.Pi, states and furthest away in the strong binding MgADP state. Studies that have examined the position of the lever-arm of skeletal muscle and Dicty myosin during the MgATPase cycle suggest that it is closest to the active site in the MgADP.Pi state, farthest away in the MgADP- and rigor-states, and intermediate in the MgATP state (21, 22). Thus, the position of the lever-arm may change as a result of the position of the converter region, which is dependent on contacts with the rigid relay loop containing Trp-512 to communicate changes from the active site. Contacts between the rigid relay loop and the converter region, which have been discussed in detail in previous structural studies (5, 6), suggest the highly conserved loop moves as a rigid body during the MgATPase cycle. The coupling pathway proposed by Dominguez et al. (5) suggests that the highly conserved switch II region changes conformation, propagating structural changes from the active site to the rigid relay loop and then to the converter and lever-arm regions. Our results suggest that in solution, when MgATP binds, the rigid relay move closer to the active site while remaining exposed to solvent, perhaps due to lack of contacts with the converter region. In the MgADP.Pi state the rigid relay loop may form more contacts with the converter region causing Trp-512 to be more protected from solvent while remaining close the active site. However, upon Pi-release and formation of the strong binding state contacts of the rigid relay loop with the converter may be disrupted due to the conformational changes propagated from the switch II region. This disruption may result in the rigid relay loop and Trp-512 becoming more solvent exposed and moving further away from the
active site. The end result is that the lever arm is free to rotate around the converter region during the transition from the weakly to strongly bound acto-myosin complexes.

**Model of coupling pathways during myosin’s MgATPase cycle**

We propose a simple model, depicted in Figure 5, to explain how changes at the active site of myosin may be coupled to changes in the actin-binding region and the position of the lever-arm. First, starting in the rigor state, when MgATP binds the switch II region changes conformation, leading to closure of the actin-binding cleft and disruption of contacts with actin. The rigid relay loop moves closer to the active site while contacts between the rigid relay loop and the converter domain are minimal, resulting in Trp-512 remaining solvent exposed and the lever-arm being free to adopt multiple conformations. After ATP hydrolysis, the rigid relay loop moves further away from the active site while Trp-512 adopts a more protected conformation, due to greater contacts of the rigid relay loop with the converter and stabilization of the lever-arm in the pre-powerstroke conformation. During Pi-release, Trp-512 adopts a more solvent exposed conformation due to movement of the rigid relay loop away from active site when the switch II region is no longer stabilized by the gamma-phosphate of ATP within the active site. As a result, the rigid relay loop contacts with the converter domain are disrupted and the lever arm can proceed through its powerstroke. Also, the actin-binding cleft may shift to the open position during Pi-release allowing strong actin binding to occur. Finally, ADP-release may only cause a small local conformational change in the rigid relay loop and Trp-512, while the lever arm rotates further during formation of the rigor complex (18). Although we have suggested above that the actin-binding cleft is closed in the weak binding states (M-ATP and M-ADP.Pi) and open in the strong binding states (M and M-ADP) (5), the conformation of the actin-binding
cleft in the presence of actin is still controversial and the exact opposite model has been proposed (45).

Conclusions

In summary, we have demonstrated that Trp-512 may be the sole conserved tryptophan responsible for the changes in intrinsic tryptophan fluorescence during the MgATPase cycle of the class II myosin isoforms. Furthermore, we provide direct evidence for structural changes that occur in the rigid relay loop containing Trp-512 in different stages of MgATPase cycle. Thus, the observed alterations in the rigid relay loop are likely to be involved in coupling conformational changes from the active site to the actin-binding and converter/lever-arm regions of myosin during muscle contraction.
Acknowledgements

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References


**Figure 1:** Crystal structure of the smooth muscle myosin motor domain essential light chain construct (MDE) with the myosin heavy chain (residues 1-819) colored yellow and the essential light chain colored cyan. Also, the putative actin-binding region (purple), converter region (blue), switch II region (green), and rigid relay loop (red) are highlighted. Six of the seven endogenous tryptophans, highlighted as space-filling models, were substituted with phenylalanine, or methionine in the case of Trp-546, leaving a single endogenous tryptophan at residue 441, 512, or 597.

**Figure 2:** Fluorescence enhancement of WT- and W512-MDE. The steady-state fluorescence spectra of (A) WT- and (B) W512-MDE in the presence of nucleotides and nucleotide-analogs are compared to the nucleotide-free environment (rigor). The percent increase in peak fluorescence in the different nucleotide states relative to rigor is shown in Table 2.

**Figure 3:** Acrylamide quenching of W512-MDE in the presence of MgADP-BeF₄, MgADP-AlF₄, MgATP, MgADP, and in the absence of nucleotide (rigor). The relative change in fluorescence (F₀/F) is plotted as a function of increasing acrylamide (mM) and the data points are fit to the Stern-Volmer relationship (F₀/F = [1 + Kₛᵥ([Q])]exp(V[Q])), where the static quenching constant (V) was held constant at 3.0 M⁻¹. The results are summarized in Table 2.

**Figure 4:** Energy transfer from Trp-512 to 2’(3’)-O-(N- methylanthraniloyl) mant-labeled nucleotides/analogs (mADP, mATP, etc.). The excitation spectrum of mant-ADP (3.0 μM) free in solution is compared to mant-labeled nucleotides/analogs bound to W512-MDE (0.6 μM). The peak fluorescence excitation at 285 nm, which is the peak excitation wavelength of Trp-512,
was used to quantify the energy transfer efficiency in different nucleotide states (see Eqs. 1-4 in Experimental Procedures). The energy transfer efficiencies and the corresponding distances between the donor/acceptor pairs in each nucleotide state are summarized in Table 3.

**Figure 5:** Structural model to explain the proposed conformational changes in myosin during its MgATPase cycle. Binding of MgATP to myosin (M*⋅MgATP) may result in closure of the actin-binding cleft and dissociation of the strongly bound rigor complex, while the position of the lever-arm is not stabilized by the converter domain (Trp-512 is exposed and close to the active site). The hydrolysis of MgATP (M**⋅MgADP.Pi) locks the lever-arm into the pre-powerstroke position due to more contacts between the rigid relay loop and the converter region, while the actin-binding cleft remains closed (Trp-512 is buried and close to the active site). Upon Pi-release (M*⋅MgADP) the conformation of the converter domain and lever-arm undergo a large rotation producing the power-stroke of muscle contraction. Also, Pi-release may open the actin-binding cleft resulting in a large increase in myosin’s affinity for actin (Trp-512 is exposed and far away from the active site). The release of ADP from the active site (rigor) changes the converter/lever-arm slightly, which may be associated with the additional movement of the lever-arm (Trp-512 becomes slightly more buried, but similar to M*⋅MgADP).
Table 1

Enzymatic Analysis of MDE Mutants

<table>
<thead>
<tr>
<th>MDE Mutant</th>
<th>MgATPase Rates (s⁻¹) @ 70 uM Actin</th>
<th>V_MAX (s⁻¹)</th>
<th>K_M (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-MDE</td>
<td>0.37 ± 0.02</td>
<td>0.76</td>
<td>62</td>
</tr>
<tr>
<td>W441-MDE</td>
<td>0.15 ± 0.02</td>
<td>0.17</td>
<td>14</td>
</tr>
<tr>
<td>W512-MDE</td>
<td>0.09 ± 0.01</td>
<td>0.13</td>
<td>33</td>
</tr>
<tr>
<td>W597-MDE</td>
<td>0.10 ± 0.01</td>
<td>0.18</td>
<td>39</td>
</tr>
</tbody>
</table>

The MgATPase rates [nmoles Pi·(nmoles MDE)⁻¹·s⁻¹] in the presence of 70 µM actin after subtracting the basal rate, of all 3 mutants are compared to WT-MDE. Values are given as the mean (± std. dev.). V_MAX and K_M values were calculated by plotting actin-activated ATPase rates as a function of actin concentration and fitting the curves to Michaelis-Menton kinetics.
Table 2

Fluorescence Enhancement and Quenching of WT- and W512-MDE

<table>
<thead>
<tr>
<th>Nucleotide/Analog Complex</th>
<th>WT-MDE (% enhancement)</th>
<th>W512-MDE (% enhancement)</th>
<th>$K_{SV}$ ($M^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgADP-BeFx</td>
<td>8.9 ± 2.1</td>
<td>31.0 ± 1.1</td>
<td>2.81 ± 0.04</td>
</tr>
<tr>
<td>MgADP-AlF$_4^-$</td>
<td>13.2 ± 1.2</td>
<td>30.7 ± 2.5</td>
<td>1.96 ± 0.05</td>
</tr>
<tr>
<td>MgATP</td>
<td>18.4 ± 0.0</td>
<td>36.1 ± 1.1</td>
<td>1.93 ± 0.09</td>
</tr>
<tr>
<td>MgADP</td>
<td>12.1 ± 0.0</td>
<td>30.1 ± 2.1</td>
<td>2.96 ± 0.07</td>
</tr>
<tr>
<td>Rigor</td>
<td>-</td>
<td>-</td>
<td>3.05 ± 0.03</td>
</tr>
</tbody>
</table>

The mean (± std. dev.) enhancement in the peak fluorescence intensity of WT- and W512-MDE are expressed relative to the nucleotide-free (rigor) condition. The dynamic quenching constants ($K_{SV}$) (mean ± std. error of the fit) of W512-MDE in the presence of MgADP-BeFx, MgADP-AlF$_4^-$, MgATP, MgADP, and in rigor are compared.
Table 3

Energy Transfer Results

<table>
<thead>
<tr>
<th>Nucleotide/Analog</th>
<th>E</th>
<th>$R_0$</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex</td>
<td>(%)</td>
<td>(Å)</td>
<td>(Å)</td>
</tr>
<tr>
<td>Mant-ADP-BeF$_X$</td>
<td>43.5 ± 3.3</td>
<td>25</td>
<td>26.1 ± 0.6</td>
</tr>
<tr>
<td>Mant-ADP-AlF$_4^-$</td>
<td>30.3 ± 6.7</td>
<td>25</td>
<td>28.7 ± 1.6</td>
</tr>
<tr>
<td>Mant-ATP</td>
<td>35.4 ± 5.5</td>
<td>25</td>
<td>27.6 ± 1.1</td>
</tr>
<tr>
<td>Mant-ADP</td>
<td>13.0 ± 1.3</td>
<td>25</td>
<td>34.3 ± 0.7</td>
</tr>
</tbody>
</table>

Energy transfer efficiencies (E) (mean ± std. dev.), distance (Å) at which the efficiency of energy transfer is 50% ($R_0$), and distance (Å) between the donor and acceptor (r) (mean ± relative error) calculated using Eqs. 1-4 in *Experimental Procedures.*
Figure 1
Figure 2

A

Wavelength (nm)

Fluorescence (Arbitrary Units)

300 320 340 360 380 400

Rigor
MgADP
MgADP-AlF₄⁻
MgADP-BeFₓ⁺⁻
MgATP

B

Wavelength (nm)

Fluorescence (Arbitrary Units)

300 320 340 360 380 400

Rigor
MgADP
MgADP-AlF₄⁻
MgADP-BeFₓ⁺⁻
MgATP
Figure 3

![Graph showing the relationship between [Acrylamide] mM and F₀/F for different compounds: MgATP, MgADP-AlF₄, MgADP-BeFx, MgADP, Rigor, ADP-BeFx, ATP, ADP-AlF₄⁻.](image-url)
Figure 4
Figure 5

Rigor

Upper
50 kDa

Active-site

Rigid Relay
Loop
WS12

Converter/Lever-Arm

Lower
50 kDa

ADP

M**·ADP

M**·ADP.Pi

M*·ATP

ATP

Pi

Active-site

N-term

Lower
50 kDa

Rigid Relay
Loop
WS12

Converter/Lever-Arm

Upper
50 kDa

Active-site

N-term
Tryptophan 512 is sensitive to conformational changes in the rigid relay loop of smooth muscle myosin during the MgATPase cycle
Christopher M. Yengo, Lynn R. Chrin, Arthur S. Rovner and Christopher L. Berger

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