Structural and Kinetic Studies of the 10 S $\leftrightarrow$ 6 S Transition in Smooth Muscle Myosin*

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Steven S. Rosenfeld‡‡, Jung Xing, Brenda Rener‡, Jacob Lebowitz**, Sambit Kar††, and Herbert C. Cheung‡

From the Departments of Neurology, Cell Biology, Microbiology, Biochemistry and Molecular Genetics, and the Graduate Program in Biophysical Sciences, University of Alabama at Birmingham, Birmingham, Alabama 35294

The conformational transitions that smooth muscle myosin undergoes after nucleotide binding have been examined using fluorescently labeled nucleotides and regulatory light chain. The 10 S conformation of smooth muscle myosin could be induced by addition of 1-N\(^{\text{2}}\)-ethanoadenosine or mant ADP plus beryllium fluoride, as well as by mant adenosine 5'-($\gamma$,\(\text{H}\))-imidotriphosphate (AMPPNP). Fluorescence lifetime studies using 1-N\(^{\text{2}}\)-ethanoadenosine and beryllium fluoride reveal two components for both (10 S) and (6 S)-myosins, with little difference in the values of these lifetimes, their fractional amplitudes, or solute accessibilities. Anisotropy decay studies of myosin-mant nucleotide complexes demonstrate that the rotational correlation time for (10 S)-myosin is nearly 4-fold longer than that for (6 S)-myosin. Qualitatively similar results were obtained with a 5-[\text{\text{2-(iodoacetyl)aminolethylamino}naphthalene-1-sulfonic acid fluorescent probe attached to the regulatory light chain. Mant AMPPNP can be trapped in the active site by (10 S)-myosin. Actin accelerates this release rate by 40-50-fold. These studies reveal: 1) reduction in nucleotide release rate by converting (6 S) to (10 S)-myosin is not due to a reduction in solute accessibility of the nucleotide, 2) the heads in (10 S)-myosin are rigidly attached to the rest of the molecule, while in (6 S)-myosin, they have segmental flexibility, 3) regulatory light chain phosphorylation mimics the effect of high salt in enhancing segmental flexibility of the myosin heads, and 4) actin can induce the unfolding of (10 S)-myosin in the absence of regulatory light chain phosphorylation.

The ability of smooth muscle and nonmuscle cells to change their shape and mechanical properties requires dynamic capabilities in their force-generating cytoskeletal elements. This is reflected in the ability of dephosphorylated myosin filaments from these sources to rapidly disassemble into soluble myosin monomers on binding of ATP (1-3). These monomers assume a compact configuration that sediments at 10 S and is produced by the folding of the \(\alpha\) helical tail into equal thirds (4-7). Phosphorylation of the regulatory light chain (RLC)\(^{1}\) induces the tail to straighten, producing a monomer that predominates at high ionic strength, sediments at 6 S, and represents an intermediate in the process of filament formation (8). Assumption of the 10 S conformation alters more than the state of the \(\alpha\) helical tail. (10 S)-myosin releases the products of ATP hydrolysis over 200 times more slowly than does (6 S)-myosin or myosin filaments (9, 10).

The structural basis for nucleotide trapping by (10 S)-myosin is not known. Trapping of nucleotide by (10 S)-myosin could be produced by a partial closure of the active site around its substrate, making it less accessible to solute. This occurs, for example, when S-1 binds nucleotide, and it leads to a 10-fold reduction in accessibility of fluorescent nucleotides bound to the active site (11). More recent crystallographic data on S-1 also suggest that binding of nucleotide is associated with partial closure of a substrate-binding pocket (12). However, other mechanisms could contribute to the increased affinity of nucleotide in (10 S)-myosin, such as local charge or hydrophobic interactions. For example, although vanadate increases the affinity of eADP for S-1 by several orders of magnitude, it reduces solvent accessibility by only approximately 2-fold (11).

Fluorescence anisotropy studies of skeletal muscle myosin have determined that the heads are capable of moving independently of the tail, reflecting segmental flexibility of the S-1/S-2 junction (13). In (10 S)-myosin, the \(\alpha\) helical tail comes into close proximity with the S-1/S-2 junction. The trapping of nucleotide by the 10 S conformation suggests that some interaction might occur between the head and tail. This could be reflected in a loss of segmental flexibility of the head or of the S-1/S-2 junction. Since structural models of the ATPase cycle of S-1 propose a bending of the carboxyl-terminal segment of S-1 relative to the active site (12), different portions of the head of (10 S)-myosin might be expected to have different degrees of segmental flexibility, even if the head/rod junction is constrained by the folding back of the tail.

While ATP hydrolysis is associated with assumption of the 10 S conformation, it is not required. This is supported by the finding that the nonhydrolyzable ATP analog, AMPPNP, can induce formation of (10 S)-myosin (3, 15). Likewise, active site trapping may not be tightly coupled to the conformation of the myosin tail. Stabilization of dephosphorylated myosin filaments by antibody at physiologic ionic strength (150 mM KCl) only accelerated product release by 10-fold. Phosphorylation of the regulatory light chain in this antibody-stabilized preparation was required to accelerate product release by an additional 5-10-fold (16). These results suggest that nucleotide trapping, hydrolysis, and folding of the tail in (10 S)-myosin may not be tightly linked.
In this study, we have investigated the interaction of smooth muscle myosin with fluorescent nucleotide derivatives as well as with AEDANS-labeled RLC in both the 10 S and 6 S conformations. We have used beryllium fluoride in the presence of fluorescent ADP to demonstrate that nucleotide trapping by (10 S)-myosin is not due to any significant change in the solvent accessibility of the catalytic site. We have used fluorescence anisotropy decay measurements to show that formation of (10 S)-myosin leads to a significant loss of segmental flexibility of the head. Finally, we have found that the addition of actin to (10 S)-myosin accelerates the rate of nucleotide release to that seen in (6 S)-myosin in a manner consistent with a model in which unfolding of myosin goes hand-in-hand with acceleration of nucleotide release.

**Materials and Methods**

**Reagents**—The N-[methylanthraniloyl] derivatives of ATP, ADP, and AMPPNP (mant ATP, mant ADP, and mant AMPPNP) were synthesized and purified as described (17, 36). cADP and 5,5′-IADANS were obtained from Molecular Probes (Eugene, OR). Protease inhibitors, antibiotics, and chemicals used for buffers and agarose gel electrophoresis were obtained from Sigma. Q-Sepharose FF and S-Sepharose FF and prepored Sephadex G-25 columns were obtained from Pharmacia Bio- tech Inc. Oligonucleotides used in polymerase chain reaction amplification of the RLC-encoding insert were synthesized by Oligos Etc. (Guilford, CT). Restriction enzymes and modifying enzymes were obtained from Stratagene, Inc. (La Jolla, CA). T4 polymerase was supplied by Perkin-Elmer Corp. Nucleotides used in polymerase chain reaction were from Life Technologies, Inc. Media components were obtained from Difco.

**Protein Isolation**—Dephosphorylated smooth muscle myosin was prepared from chicken gizzards (18). Urea gel electrophoresis of the myosin preparations revealed >95% of the RLC was in the dephosphorylated state (19). Actin was prepared from rabbit skeletal muscle acto-ne powder (20). HMM and S-1 were prepared by digestion of myosin prepared from chicken gizzards (18). Urea gel electrophoresis of the HMM and S-1 was performed by digestion of myosin by chymotryptic digestion, as described (21). The regulatory light chain of myosin was phosphorylated by myosin light chain kinase (kindly supplied by Dr. J. T. Stull) as described (18). Urea gel electrophoresis (19) of phosphorylated HMM revealed less than 5% unphosphorylated regulatory light chain (data not shown). EDTA, pH 7.5, was labeled with 1,5-IAEDANS as described (24). Stoichiometry of labeling was 0.95–0.98 mol of AEDANS/mol of RLC, using an extinction coefficient of AEDANS of 5700 M−1 cm−1 at 336 nm. The change of labeled RLC onto myosin was performed as described (24). The final preparation of AEDANS-RLC/myosin contained >92% exchange as determined by the AEDANS absorbance.

**Fluorescence Methodologies**—Steady state fluorescence measurements were made on an SLM/Aminco 8000C fluorescence spectrometer with sample holder thermostated at 20 °C. Fluorescence lifetimes and anisotropy decays were measured in a thermostated photon counting PRA 2000 pulsed nanosecond fluorescence spectrometer, as described (25). For K+, for nucleotides, a Ditric C-9-cavity 334-nm interference filter was used for excitation and a KV 380-nm cut-off filter was used to isolate the emission. For mant nucleotides, a 350-nm interference filter was used for excitation and a Corning 3–73 cut-off filter was used to isolate the emission. For AEDANS-labeled myosin, a 350-nm interference filter was used for excitation and a Corning 3–73 cut-off filter was used to isolate the emission. Measurements of intensity decay were made under rotation-free optical conditions, and the data were analyzed by a nonlinear least squares iterative reconvolution procedure based on the Marquardt search algorithm. The decay curves were fitted to a sum of exponential terms, and the weighted residuals, the autocorrelation function of the weighted residuals, the reduced χ square ratio, and the Durbin-Watson parameters were used to judge the goodness of fit. Anisotropy decays were analyzed by a biexponential function. The standard errors of the individual lifetime fits were usually in the range of 1–3% of the fitted values, and the standard errors of the long correlation times from the fits were usually less than 5% of the fitted values. Individual lifetimes recovered from anisotropy measurements were in good agreement with the values determined from intensity decay methods.

**Kinetic Methodologies**—Measurement of the rate of mant AMPPNP release from 10 S and (6 S)-myosin was performed by mixing a complex of mant AMPPNP in either 150 mM KC1 (for (10 S)-myosin) or 600 mM KC1 (for (6 S)-myosin), 20 mM HEPES, 5 mM MgCl₂, 1 mM DTT, pH 7.50, 20 °C with an equal volume of the same buffer to which 2 mM ATP had been added. The fluorescence intensity decrease at λₘₐₓ = 450 nm, associated with mant AMPPNP release from the active site, was monitored either by exciting the mant fluorophor directly (λₑₓᵣ = 350 nm) or through energy transfer from a tryptophan residue in the active site (λₑₓᵣ = 295 nm). Identical results were obtained by both methods. Data were fit to a sum of exponentials. The effect of actin on the rate of mant AMPPNP release from (10 S)-myosin was measured by mixing 2 μM (10 S)-myosin/mant AMPPNP in 150 mM KC1, 20 mM HEPES, 5 mM MgCl₂, 1 mM DTT, pH 7.50, 20 °C, with an equal volume of actin in the same buffer to which 2 mM ATP had been added. The resulting fluorescence transient was monitored by direct excitation at λₑₓᵣ = 350 nm, as described above.

Data from these experiments was found to be satisfactorily described by the Scheme I.

**Scheme I**

Where the asterisk denotes a state of enhanced fluorescence emission of the bound nucleotide, A is actin, M₅ₐₓ is (10 S)-myosin, M₆ₐₓ is (6 S)-myosin, T is nucleotide, and where K₁, K₂, and K₃ are rapid equilibria for the dissociation. The model assumes that one of the two heads of myosin interact with actin independently. A similar kinetic scheme has been found to apply for the dissociation of ADP from skeletal muscle actomyosin (27). The fluorescence intensity decrease fit an Equation 1 of the form:

F = F₀ exp(−k₁t) + F₀ exp(−k₂t)

(1)

Where F is the normalized fluorescence (F = 1 at t = 0), α₀ is negative, and λ₁ and λ₂ are the apparent rate constants. The first preexponential term predicts a lag in the fluorescence intensity decrease, which is due to the delay in populating the MT₆ₐₓ + AMTP₆ₐₓ states. The average rate constants are as follows.

k₂ > k₁ exp(λ₂) = (k₁/K₄) + k₃/(K₅/K₆ + 1)

(2)

or

k₂ > k₁ exp(λ₂) = (k₁/K₄) + k₃/(k₄/K₅ + 1)

The apparent rate constants are as follows.
Fig. 1. Double reciprocal plot of the rate of myosin solubilization, monitored by the light scattering decrease at 500 nm, versus beryllium concentration. 1/\(k'_{\text{d}}\), reciprocal of apparent rate constant for the decrease in light scattering, 1/(\([\text{Be}^{2+}]\)), reciprocal of beryllium concentration. The conditions were as follows: 10 \(\mu\)g myosin in 150 mM KCl, 20 mM HEPES, 5 mM MgCl\(_2\), 1 mM DTT, 5 mM NaF, 50 mM ADP, pH 7.5, 20 °C. The straight line defines a maximum rate of 1.5 \(\times 10^{-2}\) s\(^{-1}\) and binding constant 4.4 \(\times 10^3\) M\(^{-1}\).

\[
(\lambda_1, \lambda_2) = 1/2(S^2 - 4C^2) / (E1%\text{app})
\]

Where \(S = <k_+ + k_+^* + k_-^* + k_2^* + k_-^*>\) and \(C = <k^+_{} + k_-^*\). At high actin concentrations, the maximum values of \(\lambda_1\) and \(\lambda_2\) approach \(k_+\) and \(k_-^*\).

Protein Purification and Concentration Assays—Proteins were run on SDS-polyacrylamide gel electrophoresis as described (28). Protein concentrations were determined colorimetrically (Bio-Rad Protein Assay, Bio-Rad). In addition, the following extinction coefficients were used for determination of specific protein concentrations: myosin, 4.5; actin, 1.0.

Sedimentation Methods—Myosin at a concentration of 20 \(\mu\)g in 150 mM KCl, 20 mM HEPES, 5 mM MgCl\(_2\), 1 mM DTT, pH 7.5, was sedimented in the Beckman Airfuge at 30 psi at room temperature for 20 min in the presence or absence of nucleotide. The concentrations of protein in the supernatant and pellet were determined colorimetrically (Bio-Rad Protein Assay).

Myosin/ADP/beryllium fluoride at a concentration of 1.0 mg/ml in 150 mM KCl, 20 mM HEPES, 5 mM MgCl\(_2\), 1 mM DTT, 0.2 mM BeSO\(_4\), 5 mM NaF, pH 7.5, 20 °C was sedimented at 40,000 rpm in a Beckman Model E analytical ultracentrifuge, using boundary sedimentation velocity techniques and absorbance optics at 293 nm. The standard error for these measurements is \(\pm 5\%\).

RESULTS

Formation of (10 S)-Myosin by Nucleotide Analogs—Previous studies have demonstrated that dephosphorylated myosin filaments could be solubilized by a variety of nucleotide triphosphates, as well as by ADP + vanadate and AMPPNP (3, 6, 9, 15, 42). Solubilization of smooth muscle myosin filaments could also be accomplished by the addition of 0.2 mM BeSO\(_4\) to samples preincubated for 24 h with 150 mM KCl, 20 mM HEPES, 5 mM MgCl\(_2\), 1 mM DTT, 5 mM NaF, 50 mM ADP, pH 7.5. Over 99% of sedimentable myosin was solubilized by this method, as determined by the Airfuge assay, and this correlated with a 10-12-fold reduction in light scattering intensity at 500 nm. Identical results were obtained with eADP or mant ADP. The kinetics of myosin filament disassembly by beryllium fluoride + ADP were monitored by light scattering at 500 nm as a function of beryllium concentration. As shown in Fig. 1, the apparent rate constant demonstrates hyperbolic dependence on beryllium concentration, saturates at a value of 1.5 \(\times 10^{-2}\) s\(^{-1}\), and is characterized by an apparent binding constant of 4.4 \(\times 10^3\) M\(^{-1}\). In a velocity sedimentation experiment, myosin solubilized by beryllium fluoride + ADP was found to sediment as a single component with sedimentation coefficient of 11 S, confirming that myosin solubilized by this method behaves identically to that solubilized by ATP.

Solute Quenching of Fluorescent Nucleotide Bound to (10 S)-Myosin, (6 S)-Myosin, and HMM + RLC Phosphorylation—Information about the accessibility of a fluorophor to solvent can be obtained by measuring the effect of a solute quencher, such as acrylamide, on the emission properties of the probe. The equation that relates fluorescence quenching, \(F/ F_0\), to quencher concentration, \([Q]\), is as follows (29).

\[
F_0/F = (S_0/ S + K [Q]^{1/2})^{1/2} (\text{Eq. 3})
\]

Where \(F_0\) and \(F\) are the fluorescence intensities in the absence and presence of quencher, \(Q\) is the fractional emission of each component, \(S_0\) is the Stern-Volmer quenching constant, and \(V\) is the static quenching parameter. The presence of multiple components causes downward curvature in a plot of \(F_0/F\) versus \([Q]\), while static quenching causes upward curvature. As Fig. 2 shows, both (10 S)-myosin/eADP/beryllium fluoride and (6 S)-myosin/eADP/beryllium fluoride could be adequately fit to a two-component model in which \(V = 0\) (no static quenching). For both (10 S)- and (6 S)-myosins, the two states have fractional emissions of 0.8 and 0.2. The values of \(K\) and \(V\) for (6 S)- and (10 S)-myosin are summarized in Table 1. By contrast, a similar plot of (10 S)-myosin/mant ADP/beryllium fluoride and (6 S)-myosin/mant ADP/beryllium fluoride reveals a single state, with identical values of \(K = 1.1 \text{ M}^{-1}\), while for smooth muscle S-1/mant ADP/beryllium fluoride, \(K = 0.8 \text{ M}^{-1}\) (data not shown).

Examination of the effect of acrylamide on the fluorescence lifetimes can give less ambiguous information about solute accessibility of a fluorophor, since this measurement is not affected by static quenching. According to the Stern-Volmer equation (30)

\[
1/\tau = 1/\tau_0 + k_1[Q]
\]

Where \(\tau_0\) is the singlet lifetime in the absence of quencher, \(\tau\) is
Table I

<table>
<thead>
<tr>
<th>Parameter</th>
<th>(10 S)-myosin</th>
<th>(6 S)-myosin</th>
<th>HMM</th>
<th>HMM</th>
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<tr>
<td>[KCl] (mM)</td>
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<td>690 mm</td>
<td>150 mm</td>
<td>150 mm</td>
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<td>12.3, 1.2</td>
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<td>0.96</td>
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<td>f₂</td>
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<td>11.3</td>
<td>4.9</td>
<td>9.1</td>
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<tr>
<td>k₄₁ (m⁻¹ s⁻¹)</td>
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<td>4.3 × 10⁹</td>
<td>4.0 × 10⁹</td>
<td>4.0 × 10⁹</td>
</tr>
<tr>
<td>k₄₂ (m⁻¹ s⁻¹)</td>
<td>1.6 × 10⁹</td>
<td>1.2 × 10⁹</td>
<td>8.8 × 10⁹</td>
<td>7.0 × 10⁹</td>
</tr>
</tbody>
</table>

the lifetime at quencher concentration (Q), and k₄ is the bimolecular Stern-Volmer quenching rate constant. As Table I indicates, the intensity decays of both (10 S)-myosin/ADP/beryllium fluoride and (6 S)-myosin/ADP/beryllium fluoride consist of two components, with the longer lifetime component constituting approximately 90% of the total for both (10 S)- and (6 S)-myosins. The dependence of 1/τ on acrylamide concentration is depicted in Fig. 3 for (6 S)- and (10 S)-myosin/ADP/beryllium fluoride. The slopes of these plots define values of k₄ for (10 S)- and (6 S)-myosin/ADP/beryllium fluoride that are summarized in Table I. For (6 S)-myosin, the calculated values of K and k₄ (derived from the relationship K = k₄τ₀) are 1.2 × 10⁻¹ and 13.6 × 10⁻¹, while for (10 S)-myosin, they are 0.6 × 10⁻¹ and 24.0 × 10⁻¹. Both sets of calculated values are in excellent agreement with the steady-state quenching measurements. Thus, for both (10 S)- and (6 S)-myosins, the two components of the fluorescence decay correspond to states with markedly different solute accessibilities, as had been previously reported for skeletal muscle S-1 (11). Nevertheless, the relative accessibilities of (10 S)-myosin/nucleotide states are nearly identical to the corresponding (6 S)-myosin/nucleotide states. Quantitatively similar results were obtained with complexes of ADP + beryllium fluoride with HMM in 150 mM KCl. Two components in the fluorescence decay were observed (Fig. 3B and Table I), with the vast majority corresponding to a species of very limited solute accessibility. Phosphorylation of the RLC marginally increased the population of the minority species even further, and shortened its lifetime. However, RLC phosphorylation had essentially no effect on solute accessibility of either species.

Anisotropy Decay of Smooth Muscle Myosin, HMM, and S-1:myosin Nucleotide Complexes—Soluble complexes of myosin with mact ADP and beryllium fluoride were formed in 150 mM KCl (for (10 S)-myosin) and 600 mM KCl (for (6 S)-myosin). Similar complexes were formed with HMM and S-1. Lifetime and anisotropy decay studies revealed a single component for all of these complexes. Data are summarized in Table II. A typical anisotropy decay curve for (10 S)-myosin/mact ADP/beryllium fluoride is demonstrated in Fig. 4. The lifetimes of the mant fluorophor are essentially identical in (6 S)-myosin, (10 S)-myosin, HMM, and S-1 in both low and high ionic strength. There is nearly a 4-fold increase in the rotational correlation time in the transition from (6 S)- to (10 S)-myosin. The shorter correlation time for (6 S)-myosin is consistent with previous work on skeletal muscle myosin, which demonstrated evidence of segmental flexibility at the S-1/S-2 junction (13).

Anisotropy decay studies with HMM/mact ADP/beryllium fluoride reveal similar results to myosin at 600 mM KCl. Reducing the ionic strength of HMM/mact ADP/beryllium fluoride produced a small but statistically significant increase (37%, p = 0.0012) in the rotational correlation time. By contrast, similar complexes with S-1 showed no ionic strength dependence. Phosphorylation of the regulatory light chain of HMM/mact ADP/beryllium fluoride significantly reduced the rotational correlation time when compared with unphosphoryl-
HEPES, 5 mM MgCl₂, 1 mM DTT, pH 7.5, 20 °C. Myosin: ADP:BeF was formed by adding 0.2 mM BeSO₄ and 5 mM NaF to a 1:1 complex of myosin: ADP. Symbols: τ, singlet lifetime, A₀, limiting anisotropy, φ, rotational correlation time. HMM-P, heavy meromyosin phosphorylated on the regulatory light chain by myosin light chain kinase.

Previous work had demonstrated that the value of the rotational correlation time of S-1-bound ADP was shortened by the addition of vanadate (25). This was interpreted to mean that while S-1:ADP is ellipsoidal, the addition of vanadate causes it to fold into a compact partially spherical conformation. In order to determine if smooth muscle S-1 behaves in a similar manner, rotational correlation times were calculated from the anisotropy decays of complexes with mant ADP and mant AMPPNP, and compared with that for mant ADP + beryllium fluoride.

Results were also compared with similar studies with skeletal muscle S-1. As shown in Table II, complexes of either smooth or skeletal muscle S-1 with mant AMPPNP or mant ADP + beryllium fluoride had correlation times considerably shorter than that for mant ADP. Differences in correlation times between either S-1:mant AMPPNP or S-1:mant ADP + beryllium fluoride versus S-1:mant ADP were statistically significant (Table II).

**Anisotropy Decay of AEDANS-Labeled Regulatory Light Chain**—The regulatory light chain is essential for formation of 10 S-myosin (14), and this suggests that attachment of a fluorescent probe on the RLC should provide information complementary to the results discussed above. AEDANS-labeled RLC was found to readily label myosin, and data on the anisotropy decay for AEDANS-labeled RLC alone and combined with (6 S)- and (10 S)-myosin are summarized in Table III. Two lifetimes and two rotational correlation times were seen for both free AEDANS-RLC and that complexed to (6 S)- and (10 S)-myosins. The shorter rotational correlation time presumably represents local motions by the fluorescent probe. The longer rotational correlation time for free AEDANS-RLC, at 13.3 ns, is compatible with a molecule of M₀, 29,000, which assumes an elongated shape. This increases 9-fold on complex formation with (6 S)-myosin, and increases again by nearly 2-fold on formation of (10 S)-myosin (Table III).

**Kinetic Studies of mant AMPPNP Release from (6 S)- and (10 S)-Myosin ± Actin**—Data on the kinetics of mant AMPPNP release from (6 S)- and (6 S)-myosins fit a single exponential process. The apparent rate constant for release from (6 S)-myosin was 1.7 x 10⁻³ s⁻¹, while the corresponding rate for (10 S)-myosin was 5.0 x 10⁻³ s⁻¹. Release of unlabeled AMPPNP from (10 S)- and (6 S)-myosin was performed by mixing with a 10-fold molar excess of mant ATP and monitoring the fluorescence increase. Data for both (10 S)- and (6 S)-myosins also fit a single exponential process. For (10 S)-myosin, the apparent rate constant was 6.7 x 10⁻³ s⁻¹, while for (6 S)-myosin, it was 1.1 x 10⁻² s⁻¹.

The rate of formation of (10 S)-myosin by AMPPNP was estimated by mixing filamentous myosin in 150 mM KCl, 20 mM HEPES, 5 mM MgCl₂, 1 mM DTT, pH 7.5, with AMPPNP at a final concentration of 0.5 mM. The resultant decrease in light scattering at 500 nm fit a single exponential process with an apparent rate constant of 8.0 x 10⁻³ s⁻¹ at 20 °C.
FIG. 5. Fluorescence transients for the actin-accelerated release of mant AMPPNP from (10S)-myosin. The conditions were as follows: 2 μM myosin:mant AMPPNP in 150 mM KCl, 20 mM HEPES, 5 mM MgCl₂, 1 mM DPT, pH 7.50, was mixed with an equal volume of buffer containing actin + 2 μM ATP. The resulting fluorescence decrease was monitored by direct excitation with \( \lambda_{\text{exc}} = 350 \text{ nm} \) and \( \lambda_{\text{em}} = 450 \text{ nm} \). Final actin concentrations are: 7.5 μM (curve 1), 20 μM (curve 2), and 30 μM (curve 3).

FIG. 6. Dependence of the two apparent first order rate constants for mant AMPPNP release from (10S)-myosin on actin concentration. Conditions as in Fig. 5. Data for \( \lambda_1 \) (closed circles), the apparent first order rate constant associated with the lag phase, showed a hyperbolic dependence on actin concentration, with \( K_a = 1/K_{\text{app}} = 5 \text{ μM} \) and \( \lambda_{\text{app}} = 6.4 \times 10^{-2} \text{ s}^{-1} \). By contrast, \( \lambda_2 \) (open circles), the apparent rate constant for the fluorescence decay, showed no actin concentration dependence over a nearly 10-fold range, remaining at \( 1.6 \times 10^{-3} \text{ s}^{-1} \).

By contrast, \( \lambda_1 \) (closed circles), the apparent first order rate constant associated with the lag phase, showed a hyperbolic dependence on actin concentration, with \( K_a = 1/K_{\text{app}} = 5 \text{ μM} \) and \( \lambda_{\text{app}} = 6.4 \times 10^{-2} \text{ s}^{-1} \). By contrast, \( \lambda_2 \) (open circles), the apparent rate constant for the fluorescence decay, showed no actin concentration dependence over a nearly 10-fold range, remaining at \( 1.6 \times 10^{-3} \text{ s}^{-1} \).

**DISCUSSION**

The equilibrium constant for the 10 S ↔ 6 S transition under physiologic conditions must be very favorable for formation of (10S)-myosin, as little (6 S)-myosin can be detected by sedimentation velocity, gel filtration, or kinetic techniques (7; this study). That the equilibrium constant for the hydrolytic step of the myosin ATPase is \( 1.5 \) (11) implies that the free energy that drives formation of (10 S)-myosin must be produced by the binding of nucleotide. It also implies that the conformations of myosin:ATP (mimicked by myosin:AMPPNP) and myosin:ADP: P₈ (mimicked by myosin:ADP-beryllium fluoride) are very similar. This is supported by our finding that the rotational correlation times of mant AMPPNP and mant ADP:beryllium fluoride-labeled S-1 are very similar to each other, and much shorter than that for mant ADP (Table II). The observation that AMPPNP (3), ADP + beryllium fluoride (this study), and ADP + vanadate (42) induce formation of (10 S)-myosin implies that it is the conformational state of the head and not the specific nucleotide occupying the active site, that determines the position of the 10 S ↔ 6 S equilibrium. The kinetics of solubilization of myosin:ADP by beryllium fluoride support a mechanism in which formation of an initial collisional complex is followed by a rate-limiting isomerization:

\[
(M:D) \rightarrow (M:D)_\text{lag} + \text{Be} + F \rightarrow (M:D:Be:F)_{\text{lag}} \rightarrow (M:D:Be:F)_{\text{slow}}
\]

Where \( M = \) myosin, \( D = \) ADP, and the subscript \( n \) reflects the finding that several fluoroberyllate species can occupy the active site in stable ternary complexes with ADP and myosin (32). Formation of (10 S)-myosin would occur after dissociation of (6 S)-myosin from a filament. This dissociation step must not be rate limiting, since filament disassembly by AMPPNP and ATP is at least 5 times faster than by ADP + beryllium fluoride. Monitoring formation of the \( (M:D:Be:F)_{\text{slow}} \) state by the light scattering transient is an indirect method, but its use was necessary since none of the luminescent probes showed any change in steady state emission during the 6 S ↔ 10 S transition (Figs. 2 and 3). The validity of using light scattering to measure the formation of (10 S)-myosin is supported by the calculated values of \( k_1 \) (Scheme II) and \( K_{\text{app}} \). These values are nearly identical to those for the M-ADP-BeF₃⁻ → M⁺-ADP-BeF₃⁻ transition described for skeletal muscle S-1 (33). This supports the conclusion that it is the conformational change produced by binding of ADP + beryllium fluoride to the active site that leads to formation of the 10 S state.

Structural models of the interactions of S-1 with nucleotide, based on fluorescence, NMR, and crystallographic studies (11, 12, 34) suggest that S-1/nucleotide exists as an equilibrium mixture of two states that differ in their affinities for and solute accessibilities of bound nucleotide. During the S-1 ATPase
cycle, the nucleotide binding pocket has been proposed to alternate between "open," solute accessible, and "closed," solute inaccessible configurations. The structural changes in the head associated with this cycle would then lead to mechanoechemical transduction. By contrast, nucleotide trapping by (10 S)-myosin does not lead to a further loss of solute accessibility. This suggests that the structural changes in the head that lead to nucleotide trapping in (10 S)-myosin are different than those utilized by myosin during its chemomechanical transduction cycle. Although both (6 S) and (10 S)-myosin: eADP:BeF₆⁻ consist of two states with markedly different solute accessibilities, the relative proportions of these two states are essentially identical in these two myosin configurations (Table I). This finding is not likely to be due to a fortuitous effect of the high ionic strength used to form (6 S)-myosin, since studies with HMM at a constant, physiologic ionic strength show that RLC phosphorylation, which by anisotropy studies generates a 6 S-like state (see below), has no effect on nucleotide accessibilities (Table I). The heterogeneous nature of myosin-bound fluoroberyllates (32), is not responsible for the two lifetime components, as similar results were obtained with AIF₃ (data not shown). Solute quenching studies of mant ADP:BeF₆⁻ revealed a Stern-Volmer quenching constant of 1.1 M⁻¹ for both (6 S)- and (10 S)-myosins. It suggests that although the N-methylanthraniloyl fluorophor, located at the 2' or 3' ribose hydroxyl, report on a different environment than that of the fluorescent purine derivative in eADP, it also detects no difference in solute accessibility of nucleotide bound to (6 S)- and (10 S)-myosins. Although the lifetime of smooth muscle S-1: mant ADP:BeF₆⁻ is similar to that reported previously for skeletal muscle S-1: mant ADP:vanadate (9.4 ns versus 8.6 ns), the Stern-Volmer constant for the latter is 0.12 M⁻¹, nearly 7-fold lower (41). The finding that acrylamide is a much weaker quencher for skeletal muscle S-1 suggests that significant differences in the geometries of the active site exist between skeletal and smooth muscle myosins. This is supported by a recent study using the photoaffinity label mant-S-N₂₃-ATP, which demonstrated different labeling patterns in smooth and skeletal muscle myosin (42).

Assumption of the 10 S state leads to a close approximation of the α helical tail with the S-1/S-2 junction. Since the S-1/S-2 junction is the site of a flexible "hinge" between the head and tail (13), it might be expected that the association of the folded tail with this region in (10 S)-myosin would lead to a loss of flexibility of the head and tail (23). The results of fluorescence anisotropy experiments reported in this study support this conclusion (Tables II and III). Formation of (10 S)-myosin is associated with a 4-fold increase in the rotational correlation time of a mant ADP probe in the active site. In (6 S)-myosin, by contrast, the heads retain significant rotational mobility. The value of the correlation time for (10 S)-myosin is within the range expected for rotation of a molecule of this size and shape. This suggests that in this state, the catalytic site is highly constrained by the α helical tail and rotates as a unit with the rest of the molecule. It appears that the amino-terminal third of the α helical tail contributes to this process, as the correlation time of HMM still shows a modest salt dependence, which is absent in S-1 (Table II). These results are consistent with spin echo ¹H NMR studies of smooth muscle HMM and S-1 (35). Phosphorylation by myosin light chain kinase reduces the rotational correlation time of HMM: mant ADP: beryllium fluoride to a value comparable with that seen in 600 mM KCl. This argues that the 6 S ↔ 10 S transition, which is generally examined in vitro by altering the ionic strength, is modulated under physiologic conditions by RLC phosphorylation. It further argues that the degree of flexibility of the head of myosin: ADP-P, in high ionic strength is similar to what is seen under physiologic conditions when the RLC is phosphorylated.

Data from Table II also clarify the effect of the specific nucleotide on the overall shape of the myosin head. Our finding that S-1: mant AMPPNP and S-1: mant ADP:BeF₆⁻ appear to behave as more compact and spherical than S-1: mant ADP is consistent with earlier work on skeletal muscle S-1 (25). If the heads in (10 S)-myosin: mant ADP:BeF₆⁻ are as compact and spherical as the corresponding S-1 species, then the much longer correlation time for (10 S)-myosin must be due to an interaction between the tail and head that is not present in (6 S)-myosin, and not due to some unusually elongated configuration that these myosin heads assume under physiologic ionic strength. The rotational correlation times for the smooth muscle S-1: mant nucleotide species were found to be longer than the corresponding skeletal muscle S-1 species. This may be due to the fact that chymotryptic skeletal S-1 lacks the regulatory light chain, while S. aureus V8 protease-prepared smooth muscle S-1 retains it.

Not all portions of (10 S)-myosin, however, are equally rigid. Complex formation of AEDANS-labeled RLC to (6 S)-myosin increased the long rotational correlation time 10-fold, to 13.5 ns. This is nearly 2-fold lower than the correlation time measured using mant ADP and suggests that some rotation of the RLC persists on complex formation. This is even more apparent in the transition to (10 S)-myosin. The AEDANS-RLC probe becomes even more immobilized (φ = 233 ns) than in (6 S)-myosin, consistent with data from the use of mant nucleotides (Table II) and supporting the idea that folding back of the tail constrains flexibility of the head. However, this is still 3.5-fold shorter than the rotational correlation time measured with the mant ADP fluorophor. These results suggest that the folding back of the C-terminal tail has more of an effect on the N-terminal portion of the head, containing the active site, than the C-terminal portion, containing the RLC. Previous work by Morita et al. (24) demonstrated that converting (10 S)-myosin to (6 S)-myosin decreased the calculated rotational correlation time (φ) for an AEDANS probe on the RLC from 37.8 to 20.0 ns. These authors utilized Perrin-Weber plots, which rely on steady state fluorescence measurements and provide a single correlation time that represents the harmonic mean of the various rotational modes of the AEDANS probe. Deconvolution of the various correlation times from such measurements is not possible, and consequently, conclusions about global versus segmental mobility cannot be made. By contrast, our study utilized time-correlated photon counting measurements. These allow determination of the various correlation times that reflect the multiple rotational modes of the AEDANS-RLC probe. Thus, although the long correlation time for AEDANS-RLC (Table III), at 13.3 ns, is 53% larger than that of Morita et al. (24), the weighted mean value derived from our data, at 9.2 ± 1.6 ns, is essentially identical to that measured by these authors (8.7 ± 0.5 ns). Likewise, while the long correlation time for (6 S)-myosin (Table III), at 113 ± 41 ns, is significantly larger than that of Morita et al. (24) (20.0 ± 1.7), the weighted mean value from our data, at 65 ± 23 ns, is much closer. Because we have utilized time-correlated fluorescence measurements and have placed fluorescent probes in both the active site and on the RLC, we can conclude that in (10 S)-myosin, most of the head, including that portion that contains the active site, is rigidly attached to the rest of the tail. By contrast, the S1-S2 junction, containing the RLC binding site, retains some degree of flexibility, although even this flexibility is reduced compared with (6 S)-myosin.

The kinetic studies of mant AMPPNP dissociation from (10 S)-myosin are in close agreement with those using unlabeled
Addition of actin to (10 S)-myosin:mant AMPPNP produces a biphasic fluorescence decay. As Fig. 6 demonstrates, only the apparent rate constant associated with the lag showed a dependence on the concentration of actin, with an apparent dissociation constant of 5 μM. The apparent rate constant for the second phase, at 1.6 x 10^-3 s^-1, showed no actin concentration dependence and is essentially identical to that for mant AMPPNP release from (6 S)-myosin. The data could be reasonably fit to a model (Scheme I) in which Kd = Kdp and Ks = Kdp and are rapid equilibrium relative to other rates in the system. According to this scheme, actin accelerates AMPPNP release by accelerating the 10 S ↔ 6 S transition (k_d > k_p). While actin can accelerate the release of substrate (e.g. ATP) from S-1 (37), it would have no direct effect on the release of AMPNP from the active site of (6 S)-myosin (e.g. k_p = k_p), since the regulatory light chain is not phosphorylated.

Previous studies, employing light scattering and electron microscopy (3) established that (10 S)-myosin:AMPPNP could be induced to assemble into filaments by addition of actin. These authors proposed that actin binds only to (6 S)-myosin, and drives the 10 S ↔ 6 S equilibrium to the 6 S state. In contrast, our data are best fit to a model (Scheme I) that assumes that (10 S)-myosin can bind directly to actin, and that this interaction accelerates the unfolding to a 6 S state. Under relaxing conditions, with intracellular ATP concentrations in the millimolar range, the myosin heavy chain would be expected to assume the 10 S configuration as soon as it is synthesized and associated with its light chains. Our results with a mant nucleotide probe trapped in the active site clearly establish for the first time that (10 S)-myosin behaves as a rigid unit. As such, (10 S)-myosin would be more diffusible and less likely to become enmeshed in the cytoplasmic matrix than the segmentally flexible, elongated 6 S conformation. The use of mant nucleotide has also enabled us to examine the kinetics of nucleotide release from (10 S)-myosin. Modelling of this data allows us to predict that even in the absence of regulatory light chain phosphorylation, (10 S)-myosin unfolds and assembles when it comes into contact with free actin filaments. The values of the apparent dissociation constant for this process, at 5 μM, and the intracellular actin concentration, at 100-250 μM (39), predict that little (10 S)-myosin should normally be present, even under conditions favoring regulatory light chain dephosphorylation. This is supported by previous work that has shown that even under relaxing conditions, nearly all smooth muscle myosin is filamentous (39). Whether this process of actin-induced myosin assembly is controlled by such regulatory proteins as caldesmon or tropomyosin will need to be determined in future studies.

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