Structural and Kinetic Studies of Phosphorylation-dependent Regulation in Smooth Muscle Myosin*

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In this study, we have examined the mechanism of phosphorylation-dependent regulation in smooth muscle myosin through the use of structural and kinetic methodologies applied to several myosin fragments. Fluorescence anisotropy decay measurements demonstrate that regulatory light chain phosphorylation significantly reduces the rotational correlation time of regulatable myosin preparations, whereas minimally regulated ones show little effect in this assay. Sedimentation equilibrium studies show that the regulatory domain can dimerize with a dissociation constant that is unaffected by regulatory light chain phosphorylation. Finally, kinetic studies on the interactions of myosin-ADP constructs with actin are also consistent with a model in which interactions occur between the two heads, which are lost with regulatory light chain phosphorylation. We propose that in the absence of regulatory light chain phosphorylation, the two heads of myosin interact with each other, due to a weak intrinsic dimerization of the regulatory domains that is significantly stabilized by the proximal rod. Regulatory light chain phosphorylation abolishes the stabilizing effect of the proximal rod, leading to a loss of this interaction.

Although myosin II isoforms from skeletal, cardiac, and smooth muscle and from non-muscle sources are all characterized by common structural features, they differ from each other in several important respects. One of these is in the mechanism of calcium-dependent regulation. Myosin II from smooth muscle and non-muscle sources is regulated by phosphorylation of serine 19 on the regulatory light chain (RLC)1 (1–6). Phosphorylation by the calcium-dependent enzyme myosin light chain kinase enables the myosin to interact with actin in a force-productive manner, activates the myosin MgATPase, and induces myosin II to unfold and form filaments under physiologic conditions (7).

The crystallographic model of the myosin II head demonstrates that it consists of two domains as follows: the amino-terminal “catalytic” domain that contains the nucleotide and actin-binding sites, and the carboxyl-terminal “regulatory” domain, which contains the ELC and RLC. The RLC is located at the extreme carboxyl-terminal end of this domain (8). Thus, the crystallographic model does not provide a ready explanation of how phosphorylation of the RLC alters the behavior of the catalytic and actin-binding sites, located over 80–100 Å away. One possible explanation, supported by crystallographic studies of the regulatory domain from scallop myosin, is that phosphorylation-induced conformational changes in the RLC are communicated to the motor domain by the more amino-terminal ELC (9). However, this is not supported by more recent studies of recombinant smooth muscle myosins lacking the ELC, which demonstrated that although the ELC is necessary for efficient chemo-mechanical transduction, its presence is not necessary for regulation (10, 11). Several lines of evidence suggest an alternative mechanism. 1) Regulation requires two heads. Single-headed soluble fragments (S-1) or single-headed myosins are unregulated (e.g. are fully active regardless of the state of phosphorylation of the RLC), whereas double-headed fragments (HMM) are fully regulated (12–14, 20). 2) Regulation requires specific sequences in the carboxyl-terminal half of the RLC. Substitution with skeletal myosin RLC produces a myosin II hybrid that is locked in an off-state, suggesting that phosphorylation actively disinhibits myosin II (15–18). 3) Dimerization is not enough to ensure regulation. Recombinant HMMs that lack a minimum length of tail (>15 heptads) are not completely inhibited when unphosphorylated, even under conditions where they are guaranteed to be dimeric. Furthermore, the degree of regulation increases with increasing tail length, approaching that for conventional HMM when the tail is of the same length as the head (19). Taken together, these findings suggest that interactions between the two heads, mediated in some way through the regulatory domain and facilitated by the presence of the proximal portion of the S-2 segment, are a central component of the regulatory process. In this study, we have examined the process of phosphorylation-dependent regulation by both structural and kinetic means. Results of these studies are consistent with a model in interactions between the regulatory domain and the amino-terminal portion of the S-2 segment lead to a coupling of the motor domains that inhibits their interaction with actin. Phosphorylation of the RLC would block this interaction and allow for force generation and actin activation of the myosin MgATPase.

EXPERIMENTAL PROCEDURES

Reagents—The N-methylanthraniloyl derivatives of ADP, 2’-deoxy-ADP, and ATP were synthesized as described (21). 1.5 IAEDANS and 1,5 IAEDANS and...
Steady-state fluorescence measurements were made on an SLM/Aminco 8000C fluorescence spectrophotometer, with sample holder thermostated at 20 °C. Fluorescence lifetimes and anisotropies were measured using a picosecond laser as excitation source, as described (33) in samples that had been equilibrated in 150 mM KCl, 25 mM Hepes, 1 mM MgCl₂, 1 mM DTT, and 5 mM NaF. Calculation of the bound mant concentration, utilizing the published extinction coefficient at 358 nm (5800 μM⁻¹ cm⁻¹, see Ref. 21), demonstrated that for proteolytic HMM, and 2HepZip, the fractional labeling was 88, 93, and 90%, respectively. Single turnover studies were performed by mixing recombinant HMM (phosphorylated or unphosphorylated) with a 5-fold molar excess of mant ATP, followed by mixing with a 1000-fold excess of actin. These revealed a small amplitude (approximately 5% of the total amplitude) phase with rate of 0.9–2.1 s⁻¹, followed by the main phase with rate of 0.04–0.10 s⁻¹.

Kinetic Methodologies—Measurements of the rates of mant nucleotide binding to and release from HMM, S-1, and recombinant myosin constructs were made using a Hit-Tech Scientific stopped-flow spectrometer equipped with a 150 Watt Xenon lamp. The instrument dead-time was determined to be 1.8 ms. Fluorescence was excited using a 295-nm input from a monochromator, and a 400-nm cut-off filter was used to measure the fluorescence emission at a right angle from the incident beam. Data were fit to a sum of exponentials. For studies using pyrene actin fluorescence, the excitation wavelength was 365 nm, and a 400 nm interference filter was used to collect the fluorescence emission (Omega Optical). The dependence of rate constant on actin concentration was fit to a series of rectangular hyperbolas using a curve fitting procedure (Delta Graph Pro3) to get initial estimates of maximum rate and apparent affinity. Final fitting was performed with the NLIN procedure on PC SAS version 6.12.

RESULTS

Anisotropy Decay of Native and Recombinant Myosin Fragments Labeled with Mant Nucleotide—Our previous studies (31) demonstrated that addition of beryllium fluoride to complexes of S-1 or HMM with mant ADP significantly shortened the rotational correlation times. This suggests that formation of a pre-hydrolytic state, mimicked by the complex myosin-mant ADP-beryllium fluoride, is associated with assumption of either a more compact configuration or greater segmental flexibility (Table I). In this study, we have examined the effect of RLC phosphorylation on the anisotropy decays of fluorescently labeled HMM, S-1, and of two recombinant constructs containing shorter tail segments (2HepZip and 7HepZip) (19). These latter two constructs consist of the motor domain, the regulatory domain, and either two or seven heptads of the S-2 segment, followed by a leucine zipper sequence (Fig. 1). The 2HepZip construct shows minimal regulation (less than a 2-fold change in actin-activated activity upon phosphorylation), and the 7HepZip construct shows partial regulation (approximately a 5-fold increase in actin-activated activity upon phosphorylation, Fig. 3 of Ref. 19). Anisotropy decay studies of these preparations labeled in the absence of ADP or beryllium fluoride could be fit to decays characterized by a long rotational correlation time, reflecting global motions of the protein, and a short correlation time (0.5–2.5 ns), reflecting local probe motions. Several observations are evident on examining the data summarized in Table I. First, phosphorylation reduces the rotational correlation times for proteolytic and recombinant
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**Table I**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fluorescent probe</th>
<th>RLC phos.</th>
<th>A(0)</th>
<th>(\tau) (ns)</th>
<th>(\phi) (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteolytic HMM</td>
<td>Mant ADP</td>
<td>–</td>
<td>0.35</td>
<td>9.5 ± 0.5</td>
<td>369 ± 17</td>
</tr>
<tr>
<td>Proteolytic HMM</td>
<td>Mant ADP</td>
<td>+</td>
<td>0.35</td>
<td>9.3 ± 0.5</td>
<td>386 ± 21</td>
</tr>
<tr>
<td>Proteolytic HMM</td>
<td>Mant ADP + BeF</td>
<td>–</td>
<td>0.35</td>
<td>9.3 ± 0.5</td>
<td>261 ± 21</td>
</tr>
<tr>
<td>Proteolytic HMM</td>
<td>Mant ADP + BeF</td>
<td>+</td>
<td>0.35</td>
<td>9.3 ± 0.5</td>
<td>160 ± 4</td>
</tr>
<tr>
<td>Recombinant HMM</td>
<td>Mant ADP + BeF</td>
<td>–</td>
<td>0.27</td>
<td>8.8 ± 0.01</td>
<td>244 ± 12</td>
</tr>
<tr>
<td>Recombinant HMM</td>
<td>Mant ADP + BeF</td>
<td>+</td>
<td>0.27</td>
<td>8.8 ± 0.01</td>
<td>194 ± 8</td>
</tr>
<tr>
<td>7HepZip</td>
<td>Mant ADP + BeF</td>
<td>–</td>
<td>0.26</td>
<td>8.7 ± 0.01</td>
<td>178 ± 9</td>
</tr>
<tr>
<td>7HepZip</td>
<td>Mant ADP + BeF</td>
<td>+</td>
<td>0.25</td>
<td>9.7 ± 0.03</td>
<td>143 ± 5</td>
</tr>
<tr>
<td>2HepZip</td>
<td>Mant ADP + BeF</td>
<td>–</td>
<td>0.28</td>
<td>8.9 ± 0.02</td>
<td>149 ± 6</td>
</tr>
<tr>
<td>2HepZip</td>
<td>Mant ADP + BeF</td>
<td>+</td>
<td>0.25</td>
<td>8.6 ± 0.01</td>
<td>137 ± 5</td>
</tr>
<tr>
<td>S-1*</td>
<td>Mant ADP + BeF</td>
<td>–</td>
<td>0.36</td>
<td>9.1 ± 0.02</td>
<td>140 ± 5</td>
</tr>
<tr>
<td>Motorless</td>
<td>AEDANS-RLC</td>
<td>–</td>
<td>0.20</td>
<td>16.3 ± 0.04</td>
<td>214 ± 14</td>
</tr>
<tr>
<td>Motorless</td>
<td>AEDANS-RLC</td>
<td>+</td>
<td>0.20</td>
<td>16.2 ± 0.03</td>
<td>155 ± 9</td>
</tr>
</tbody>
</table>

*From ref. 31.

**Fig. 1.** Schematic of recombinant myosin fragments examined in this study. Wild type HMM consists of the catalytic domain (hatched ellipse) and regulatory domain (checkered string of ovals) followed by 37 heptads from the S-2 segment (solid string of ovals). The 2HepZip and 7HepZip constructs consist of truncated HMM with 2 or 7 heptads, respectively, followed by the leucine zipper domain from GCN4. Motorless consists of two regulatory domains, followed by 37 heptads from the S-2 segment. Regulatory domain consists of heavy chain residues 788–873 and the essential and regulatory light chains.

HMM and 7HepZip when the active site is labeled with mant ADP + beryllium fluoride to a statistically significant degree (\(p < 0.01\)) but not when it is labeled with mant ADP. Second, the correlation times for maximally regulated HMM preparations (proteolytic and recombinant) are significantly greater than for moderately or minimally regulated HMM (7HepZip and 2HepZip) in the absence of RLC phosphorylation. Third, phosphorylation reduces the correlation times of maximally regulated HMM preparations to values similar to those for unphosphorylated 7HepZip and 2HepZip HMM. These results suggest that in the absence of RLC phosphorylation, the two heads of maximally regulated HMM preparations are less mobile, as might be expected if the heads interact with each other.

A previous study (31) demonstrated that the rotational correlation time of mant ADP-labeled myosin preparations varied with the nature of the ligand in the active site, with longer correlation times observed for ADP-bound states. Thus, comparisons of correlation times to previously reported values need to take into account the state of the myosin preparation. In this regard, the value of the long correlation time for RLC-phosphorylated, mant ADP-labeled proteolytic HMM is remarkably close to that reported for AEDANS-labeled skeletal HMM in the absence of nucleotide (386 ± 20 ns; Ref. 37). Likewise, the correlation time of phosphorylated proteolytic HMM-mant ADP•BeF is very similar to that for S-1-mant ADP•BeF (140 ± 5 ns, Table I and reference 31).

**Anisotropy Decay of AEDANS-labeled Regulatory Light Chain Reconstituted onto Recombinant Dimeric Regulatory Domains**—The results with mant ADP-labeled myosin constructs suggest that RLC phosphorylation increases the segmental flexibility of the motor domains, where the mant probe is located. It is not clear from these results, however, if the presence of the motor domain is necessary for this effect. In order to test this, anisotropy decay studies were performed on a recombinant construct called motorless, consisting of the two regulatory domains and 37 heptads from the proximal S-2 segment (Fig. 1). Recombinant, AEDANS-labeled RLC was exchanged onto motorless, purified from unbound AEDANS-RLC by gel filtration chromatography on Superose 6 FPLC, and the anisotropy decay of the labeled complex was measured with and without RLC phosphorylation. RLC phosphorylation reduced the long correlation time by 38%, from 214 ± 14 to 155 ± 9 ns (Table I).

**Sedimentation Equilibrium Studies**—The data presented above suggest that interactions occur between the two heads of myosin in the dephosphorylated state that reduce the segmental flexibility of the heads and that are disrupted by RLC phosphorylation. In order to further identify the portions of the myosin molecule that may contribute to these interactions, sedimentation equilibrium studies were performed on a recombinant myosin fragment consisting of the ELC and RLC complexed to residues 788–873 from the chicken gizzard smooth muscle.
muscle myosin heavy chain. This complex, referred to as the regulatory domain (Fig. 1), was studied in the ultracentrifuge in the presence and absence of RLC phosphorylation. Since the binding of the RLC and ELC to the recombinant heavy chain segment remains stoichiometric at these concentrations, essentially all of the light chain would remain bound to the heavy chain peptide in this study. This conclusion is also consistent with the measured monomer molecular weight, which closely matches the calculated molecular weight (see below).

Fig. 2 presents the absorbance at 280 nm as a function of radial distance for the unphosphorylated and phosphorylated regulatory domain. The upper panels in Fig. 2 represent the residuals for the fit. The data were found to be adequately described by a monomer-dimer self-associating equilibrium, characterized by a dissociation constant of 7.9 μM for the unphosphorylated sample and 6.0 μM for the phosphorylated sample. Higher orders of association could not be detected in the data. The monomer molecular masses for each fit were 45,354 and 47,220 daltons for unphosphorylated and phosphorylated species, respectively, which are within 4% of the calculated monomer molecular mass of 47,143 daltons. Thus, whereas regulatory domain-regulatory domain interactions may contribute to the structural basis of regulation, other portions of the myosin molecule must modulate this process to provide for phosphorylation-dependent control.

Kinetic Studies of Binding of Native and Recombinant Myosin Fragments to Pyrene-labeled Actin—The results from fluorescence and sedimentation studies suggest that at least in ATP-like states, regulation is mediated by reversible interactions between the two heads. However, previous work on regulated skeletal muscle actomysin has indicated that the kinetics of ADP release is also regulated, albeit to a lesser degree (38). In order to see if head-head interactions occur in the myosin-ADP state, we have examined the kinetics of binding of the various myosin fragments with pyrenyl-labeled actin in the presence of ADP. Binding of myosin to actin, with the subsequent formation of the “strong” binding state, is associated with quenching of pyrene fluorescence when the pyrenyl probe is attached to cysteine 374 of actin. This has been used in kinetic studies of binding of skeletal S-1-ligand complexes to pyrene-labeled actin (32, 39). We utilized this quenching to examine the kinetics of binding of the various myosin constructs to actin in the presence of ADP and with actin in large excess over myosin. Proteolytic HMM and S-1, as well as the 2HepZip and 7HepZip constructs in the presence of 40 μM ADP, were mixed with a 10-fold molar excess of pyrene-labeled actin. The dissociation constant of ADP binding to acto-HMM under these conditions is approximately 60 μM (40). The ADP concentration used in this experiment was thus a compromise between the need to saturate the catalytic site of HMM and the need to prevent substantial rebinding of dissociated ligand to the acto-HMM complex. For S-1, phosphorylated HMM, and unphosphorylated 2HepZip and 7HepZip constructs, binding to pyrenyl-labeled actin was associated with a single exponential process which followed a 3–5-ms lag. A plot of the rate constant of the major exponential process versus actin concentration fit a rectangular hyperbola for each myosin preparation (Fig. 3), defining maximum rates (λ_{max}) and apparent dissociation constants (K_p) that are summarized in Table II. By contrast, the fluorescence transient produced by binding of HMM-ADP to pyrenyl-labeled actin remained biphasic at all actin concentrations, with relative amplitudes of the fast and slow phase of 1.0:1.5. The rates of both processes demonstrated an actin concentration dependence, and data could be fit to rectangular hyperbolas that, as in the case of S-1, define maximum rates and apparent binding constants (Table II and Fig. 4).

As Table II shows, the maximum rate of the first phase for HMM-ADP is similar to that for phosphorylated HMM-ADP, whereas the second phase is nearly 10-fold slower. Furthermore, the apparent dissociation constants for unphosphoryl-
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FIG. 3. Rate of binding of myosin fragment:ADP to pyrene-labeled actin. Conditions are as follows: 20 mM KCl, 25 mM Hepes, 1 mM MgCl₂, 1 mM DTT, pH 7.50, 20 °C. Myosin fragment + 40 μM ADP was mixed with a 10-fold molar excess of pyrene-labeled actin. The resulting fluorescence decrease could be fitted to a single exponential process, characterized by rate constant λ. The dependence of λ on pyrene actin concentration was adequately described by a series of rectangular hyperbolas, defining maximum rates (λₘₐₓ) and apparent dissociation constants (Kₐₕ₋(app)). Closed squares, S-1; closed circles, unphosphorylated 2HepZip; open triangles, RLC phosphorylated proteolytically prepared HMM; open diamonds, unphosphorylated 7HepZip. Values of λₘᵢₓ and Kₙₐ₋(app) are summarized in Table II.

TABLE II Kinetic parameters for binding of myosin fragment:ADP to pyrene-labeled actin

<table>
<thead>
<tr>
<th>Reaction</th>
<th>RLC Phos.</th>
<th>λₘₐₓ</th>
<th>Kₐ₋(app)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteolytic HMM:ADP × actin</td>
<td>−</td>
<td>5.7 ± 3.8</td>
<td>54.0 ± 21.0</td>
</tr>
<tr>
<td>Proteolytic HMM:ADP × actin</td>
<td>+</td>
<td>9.0 ± 0.9</td>
<td>16.2 ± 4.3</td>
</tr>
<tr>
<td>S-1:ADP × actin</td>
<td>−</td>
<td>9.4 ± 0.9</td>
<td>12.1 ± 3.7</td>
</tr>
<tr>
<td>2HepZip:ADP × actin</td>
<td>−</td>
<td>12.1 ± 1.2</td>
<td>14.4 ± 3.6</td>
</tr>
<tr>
<td>7HepZip:ADP × actin</td>
<td>−</td>
<td>10.8 ± 0.5</td>
<td>5.1 ± 1.0</td>
</tr>
</tbody>
</table>

Recombinant Myosin Fragments—In order to examine the actin-myosin interaction from the point of view of the nucleotide, the kinetics of mant ADP release from S-1, HMM, and the various recombinant constructs were studied. This was accomplished by pre-mixing myosin constructs at a concentration of 1–5 mM with 20 μM mant ADP, and then mixing this complex in the stopped-flow with an equal volume of 1 mM ADP or with 1 mM ADP + a 10–15-fold molar excess of actin. The dissociation of bound mant ADP was monitored by exciting the mant fluorophore through energy transfer from one of the myosin tryptophans, as has been described for skeletal S-1 (41). Release of mant ADP from all the myosin fragments could be described by a single exponential process in the absence of actin, although several preparations of proteolytic S-1 and HMM did produce transients which included a faster phase that accounted for 10–15% of the signal amplitude. Mixing mant ADP-labeled S-1, RLC-phosphorylated HMM, and the unphosphorylated 2HepZip with actin produced single exponential fluorescence transients (Figs. 5-7). By contrast when unphosphorylated, both recombinant and proteolytically prepared HMM demonstrate two rate processes at all actin concentrations (Figs. 5 and 6). The actin activation of this release could be fitted to rectangular hyperbolas, defining values of λₘᵢₓ and Kₙₐ₋(app) summarized in Table III. Two exponential decays were also observed for experiments using unphosphorylated proteolytic HMM complexed to 2’-deoxy-mant ADP (data not shown). A consistent difference that was noted in these studies is that the maximum rates of proteolytically prepared HMM are 7–10-fold slower than the corresponding rates for recombinant HMM. This may be due to internal cleavages that occur when HMM is prepared by proteolysis (24).

Kinetic Studies of Nucleotide Binding to and Dissociation from acto-S-1 and acto-HMM—The rate of ADP dissociation from a complex of acto-HMM was measured in two ways. The first was by mixing 7 μM proteolytically prepared HMM + 70 μM pyrenyl-labeled actin + 100 μM ADP with 4 mM ATP in the stopped-flow. The resulting fluorescence enhancement, associated with dissociation of ADP and subsequent binding of ATP to acto-HMM, was monophasic both in the absence and presence of RLC phosphorylation. The rate constant was 40 ± 8 s⁻¹ in the absence of RLC phosphorylation and 52 ± 20 s⁻¹ in its presence. Similar studies were performed with recombinant HMM, which revealed rates of 65 ± 4 s⁻¹ in the absence of RLC phosphorylation and 63 ± 8 s⁻¹ in its presence. The second method was by mixing mixing 7 μM proteolytically prepared HMM + 70 μM actin + 50 μM mant ADP with 4 mM ATP. This revealed rates of 32 ± 4 s⁻¹ in the absence of RLC phosphorylation and 43 ± 3 s⁻¹ in its presence. Data are summarized in Table IV.

The kinetics of ATP-induced dissociation of rigor complexes of acto-HMM and acto-S-1 were examined by mixing a complex of 1 mM HMM or S-1 + 10 μM pyrenyl-labeled actin with a range of ATP concentrations from 20 to 400 μM. For both acto-S-1 and acto-HMM, the resulting fluorescence enhancement followed a single exponential process. Acto-HMM and phosphorylated acto-HMM demonstrated a hyperbolic dependence on ligand concentration (Fig. 8, Table V), defining maximum rates of 107 s⁻¹.
transients at all actin concentrations. Values of 
whereas phosphorylated HMM demonstrated a biphasic process, whereas phos-
phorylated HMM and S-1 produced monoexponential fluorescence tran-
sients at all actin concentrations. Values of \( \lambda_{\text{max}} \) and \( K_d(\text{app}) \) are summarized in Table III.

for unphosphorylated acto-HMM and 135 s\(^{-1}\) for phosphorylated acto-HMM, with corresponding dissociation constants for ATP of 127 \( \mu M \) for unphosphorylated acto-HMM, and 87 \( \mu M \) for phosphorylated acto-HMM. Whereas a maximum rate could not be observed for acto-S-1, the apparent second order rate constants for acto-S-1 and acto-HMM are very similar to each other (Fig. 8, Table V).

**DISCUSSION**

An explanation of regulation in smooth muscle must take into account the fact that there is no obvious connection between the RLC and the catalytic domain. A model that involves direct interactions between the RLC and the catalytic domain of the S-1 molecule has been proposed for scallop myosin and requires that these interactions be mediated at least in part by the ELC, which is juxtaposed between these two regions of the molecule (9). Applying such a model to smooth and non-muscle myosin II, however, is inconsistent with the finding ELC-de-
plicated myosin II, however, is inconsistent with the finding ELC-de-

**Fig. 5.** Rate of actin-activated mant ADP release from proteo-

cythetically prepared HMM and S-1. Conditions are as follows: 20 mM KCl, 25 mM Hepes, 1 mM MgCl\(_2\), 1 mM DTT, pH 7.50, 20 °C. Myosin fragment + 20 \( \mu M \) mant ADP was mixed with a 10–15-fold molar excess of actin + 1 mM ADP. Data were fit to a series of rectangular hyperbolas, defining maximum rates (\( \lambda_{\text{max}} \)) and apparent dissociation constants (\( K_d(\text{app}) \)). Open squares, S-1; open circles, proteolytically-prepared HMM; closed diamonds, phosphorylated HMM. Dephosphorylated HMM demonstrated a biphasic process, whereas phosphorylated HMM and S-1 produced monoexponential fluorescence transients at all actin concentrations. Values of \( \lambda_{\text{max}} \) and \( K_d(\text{app}) \) are summarized in Table III.

**Fig. 6.** Rate of actin-activated mant ADP release from recom-
bibinant HMM ± RLC phosphorylation. Conditions are as in Fig. 5. Open triangles, phosphorylated HMM; open squares and open circles, dephosphorylated HMM. As in the case of proteolytically-prepared HMM, dephosphorylated HMM demonstrated a biphasic process, whereas phosphorylated HMM produced monoexponential fluorescence transients at all actin concentrations. Values of \( \lambda_{\text{max}} \) and \( K_d(\text{app}) \) are summarized in Table III.

**Fig. 7.** Rate of actin-activated mant ADP release from recom-
bibinant 2HepZip. Conditions are as in Fig. 5. A single exponential process was observed at all actin concentrations. Values of \( \lambda_{\text{max}} \) and \( K_d(\text{app}) \) are summarized in Table III.

**Table III**

**Kinetic parameters for actin-activated release of mant ADP from myosin fragments**

<table>
<thead>
<tr>
<th>Reaction</th>
<th>RLC Phos.</th>
<th>( \lambda_{\text{max}} )</th>
<th>( K_d(\text{app}) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recombinant HMM-mADP</td>
<td>× actin</td>
<td>10.7 ± 1.7</td>
<td>3.1 ± 1.9</td>
</tr>
<tr>
<td>Recombinant HMM-mADP</td>
<td>× actin</td>
<td>1.8 ± 0.4</td>
<td>7.7 ± 5.6</td>
</tr>
<tr>
<td>Proteolytic HMM-mADP</td>
<td>× actin</td>
<td>16.2 ± 4.4</td>
<td>16.6 ± 5.5</td>
</tr>
<tr>
<td>Proteolytic HMM-mADP</td>
<td>× actin</td>
<td>1.0 ± 0.02</td>
<td>8.7 ± 1.2</td>
</tr>
<tr>
<td>2HepZip-mADP × actin</td>
<td></td>
<td>0.1 ± 0.01</td>
<td>12.0 ± 4.0</td>
</tr>
<tr>
<td>S-1-mADP × actin</td>
<td></td>
<td>14.8 ± 3.3</td>
<td>101.0 ± 38.5</td>
</tr>
<tr>
<td>2HepZip-mADP × actin</td>
<td></td>
<td>16.5 ± 1.4</td>
<td>23.1 ± 5.2</td>
</tr>
</tbody>
</table>

Studies using fluorescence anisotropy decay (Table I) demon-
strate that the mobility of a mant ADP probe, when com-
plexed to regulatable myosin fragments in the presence of beerylum fluoride, is significantly increased by RLC phospho-
rylation. Since the limiting anisotropies of the mant ADP probe are high (0.25–0.35), the long rotational correlation times reflect rotational motions of the head to which the probe is attached. A decrease in correlation time with RLC phosphoryl-
ation suggests that phosphorylation regulates an interaction between the two heads, mediated at least in part through the regulatory light chain. Data expressed as mean ± 1 S.D.
Kinetic parameters for ADP dissociation from acto-HMM

Conditions are as follows: 20 mM KCl, 25 mM Hepes, 1 mM MgCl2, 1 mM DTT, pH 7.50, 20 °C. Rates of ADP dissociation were monitored by mixing proteins in the stopped flow either 7 μM HMM + 70 μM pyrenyl-labeled actin + 100 μM ADP or 7 μM HMM + 70 μM actin + 50 μM mant ADP with 4 μM ATP. Fluorescence transients could be fit to a single exponential process, defining a dissociation rate constant (k_off). RLC Phos., phosphorylation status of the regulatory light chain. Data expressed as mean ± 1 S.D.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>RLC Phos.</th>
<th>Probe</th>
<th>k_off s⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteolytic HMM-actin-ADP</td>
<td>−</td>
<td>Pyrene actin</td>
<td>40 ± 8</td>
</tr>
<tr>
<td>× ATP</td>
<td>+</td>
<td>Pyrene actin</td>
<td>52 ± 20</td>
</tr>
<tr>
<td>Recombinant HMM-ADP × ATP</td>
<td>−</td>
<td>Pyrene actin</td>
<td>65 ± 4</td>
</tr>
<tr>
<td>Recombinant HMM-ADP × ATP</td>
<td>+</td>
<td>Pyrene actin</td>
<td>63 ± 8</td>
</tr>
<tr>
<td>Proteolytic HMM-ADP × ATP</td>
<td>−</td>
<td>Mant ADP</td>
<td>32 ± 4</td>
</tr>
<tr>
<td>Proteolytic HMM-ADP × ATP</td>
<td>+</td>
<td>Mant ADP</td>
<td>43 ± 3</td>
</tr>
</tbody>
</table>

FIG. 8. Rate of ATP-induced dissociation of acto-HMM and acto-S-1, monitored by pyrene actin fluorescence. Conditions are as follows: 20 mM KCl, 25 mM Hepes, 1 mM MgCl2, 1 mM DTT, pH 7.50, 20 °C. 1 μM HMM or S-1 + 10 μM pyrenyl-labeled actin was mixed with a range of ATP concentrations from 20 to 400 μM. Acto-HMM (open circles) and phosphorylated acto-HMM (open triangles) demonstrated a hyperbolic dependence of rate on ligand concentration, whereas a maximum rate could not be observed for acto-S-1 (open squares). Values of λ_max and K_d(app) are summarized in Table V, along with the apparent second order rate constant for ATP binding (k_a).

Kinetic parameters for ATP-induced dissociation of acto-HMM and acto-S-1

Conditions are as follows: 20 mM KCl, 25 mM Hepes, 1 mM MgCl2, 1 mM DTT, pH 7.50, 20 °C. Rates of ATP-induced dissociation of rigor complexes were measured by mixing 1 μM HMM or S-1 + 10 μM pyrenyl-labeled actin with a range of ATP concentrations. The ATP concentration dependence of the rate was found to be adequately described by a rectangular hyperbola for HMM, defining maximum rates (λ_max(app)) and apparent dissociation constants (K_d(app)). For acto-S-1, the concentration dependence remained linear, defining a second order rate constant (k_a). Actin*, pyrene-labeled actin; RLC Phos., phosphorylation status of the regulatory light chain.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>RLC Phos.</th>
<th>λ_max s⁻¹</th>
<th>K_d(app) μM</th>
<th>k_a μM s⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteolytic HMM-actin* × ATP</td>
<td>−</td>
<td>107 ± 127</td>
<td>8.3 × 10⁶</td>
<td></td>
</tr>
<tr>
<td>Proteolytic HMM-actin* × ATP</td>
<td>+</td>
<td>135 ± 87</td>
<td>1.6 × 10⁶</td>
<td></td>
</tr>
<tr>
<td>Proteolytic S1-actin* × ATP</td>
<td>−</td>
<td></td>
<td>7.8 × 10⁶</td>
<td></td>
</tr>
</tbody>
</table>

The lack of effect of RLC phosphorylation on the correlation time of HMM-mant ADP may imply that the structural transition associated with nucleotide hydrolysis and phosphate release produces a significant elongation of the heads or loss of internal flexibility that obscures any effect of RLC phosphorylation on the correlation time. Previous studies with the microtubule motor kinesin have demonstrated that this transition is associated with loss of internal flexibility, reflected by an increase in rotational correlation time of a mant ADP probe (33). Results with the AEDANS-RLC-labeled motorless demonstrate that alterations in the segmental flexibility of the head by RLC phosphorylation do not require the presence of a motor domain (Fig. 1, Table I). This reduction in mobility with dephosphorylation is consistent with loss of internal flexibility of at least a portion of the regulatory domain, since the value of the correlation time is 8–9 times larger than that of the isolated RLC (31) and is consistent with interactions of the regulatory domains with each other and/or to the proximal S-2 segment to which they are attached.

In order to more rigorously test this head-head interaction model, as well as to identify the role of the regulatory domain in mediating this process, sedimentation equilibrium studies were performed on a recombinant regulatory domain containing the ELC and RLC complexed to a peptide from the heavy chain containing residues 788–873. These studies demonstrated that this recombinant regulatory domain dimerizes with a dissociation constant of 6–8 μM. However, RLC phosphorylation has no appreciable effect on this interaction. Thus, phosphorylation-dependent regulation must require other structural elements that further modify the strength of head-head interactions.

We propose that in the intact myosin molecule, the relatively weak dimerization capacity of the regulatory domain is stabilized by interactions between it and the proximal S-2 segment. The torsional forces produced by binding of both heads to an actin filament would ultimately pull apart the dimerized regulatory domains, but this dissociation would be rate-limiting due to the stabilization provided by the proximal S-2 segment. This would explain why phosphorylation was able to reduce the rotational correlation time of AEDANS-RLC-labeled motorless, even though this construct is devoid of the motor domain. Phosphorylation of the RLC is proposed to disrupt the interaction between the regulatory domain and the proximal S-2 segment, allowing each head to bind to actin at rates unaffected by the weak dimerization of the regulatory domain. This would explain why constructs containing less than a minimum number of heptad repeats (2HepZip and 7HepZip) demonstrate nearly complete actin activation in the absence of RLC phosphorylation (19) and show correspondingly little effect of phosphorylation on the mobility of the motor domains (Table I). This would also explain why the rate of ADP dissociation, when measured from an acto-HMM-ADP complex mixed with ATP is unaffected by RLC phosphorylation, whereas that measured from HMM-ADP mixed with actin is regulated. Formation of a stable acto-HMM-ADP complex prior to mixing in the stopped-flow would allow both heads of HMM to strongly attach to actin, due to a slow dissociation of the dimerized regulatory domain-proximal S-2 complex. This would also explain why the rate of dissociation of rigor complexes of HMM with actin by ATP is RLC phosphorylation-independent (Table V). The model also explains why at least 15 heptad repeats are needed in HMM constructs to produce complete regulation, since this
length of S-2 segment is approximately equal to the length of the regulatory domain and would therefore have the capacity for interacting with the regulatory domain throughout its entire length (19). This model is schematically depicted in Fig. 9.

In skeletal muscle myosin, the rate of ADP release is regulated by the troponin-tropomyosin complex (38). As noted above, spectroscopic probes of myosin-mant ADP complexes were not able to detect any change in rotational correlation time with RLC phosphorylation. Consequently, kinetic studies of the interaction of myosin-ADP with actin were undertaken to see if regulation could be detected and if the pattern of regulation is consistent with a model of head-head interactions. The pyrenyl fluorophor was used to monitor the kinetics of the weak-to-strong transition using myosin-ADP, whereas the mant ADP probe was used to monitor the kinetics of nucleotide dissociation. For S-1, the fluorescence transients using both mant ADP and pyrenyl-labeled actin could be described by the following kinetic Model A:

\[
(MD + A)^* \xleftrightarrow{k_1} (MDA)^* \xleftrightarrow{k_2/k_1} MDA \xleftrightarrow{k_3/k_2} MA + D
\]

where the asterisk represents a state of enhanced fluorescence, either mant ADP bound to myosin or pyrenyl-labeled actin that is unquenched; MD indicates myosin-ADP; A indicates actin; and MDA indicates myosin-ADP-actin. The kinetic equations that describe this model have been discussed previously (32) and predict a single exponential decay, following a lag, that was observed for studies with pyrene actin fluorescence. The rate constant for the major exponential term, \( \lambda \), approaches \( k_2 + k_3 \) at high actin concentrations. A similar conclusion applies to experiments with mant ADP, since the large excess of ADP in the syringe containing actin ensures that \( K_d \) is irreversable. According to Equation 2, \( K_1 \) and \( K_2 \) are rapid equilibria in relation to the second step, and \( k_1 \gg k_2 \), so:

\[
\lambda = \frac{(k_3 + k_2)K_1[A]}{K_2[A] + 1} \quad \text{(Eq. 2)}
\]

A single exponential decay with a hyperbolic dependence on actin concentration for binding of S-1:ADP to actin was observed using both pyrene actin and mant ADP probes, as expected (Tables II and III). Comparison of the data from Tables II and III reveals that the maximum rates for S-1 are comparable, but there is nearly a 10-fold difference in the apparent dissociation constant. This may reflect differences in the affinities of S-1 for pyrene actin versus unmodified actin (39).

For unphosphorylated HMM, a different result was observed. For the interaction with actin followed a biphasic process with amplitudes that were of similar magnitude, whereas phosphorylated HMM or minimally regulated myosin fragments demonstrated a single phase, much like S-1. We propose that the two phases in the fluorescence transients represent the sequential interaction of each head with actin. A head-head interaction model of regulation would predict the following: 1) attachment of the first head to actin should be somewhat slower than for S-1 or phosphorylated HMM, since cross-linking might be expected to impede the diffusion-mediated stereospecific interaction of the first head with actin, and 2) attachment of the second head would require a rate-limiting “melting” of the cross-link interaction and it would therefore be much slower (Fig. 9). This is consistent with the finding that the rate constant for the faster phase of the binding of HMM:ADP to actin is approximately 50% that for phosphorylated HMM or S-1, whereas the second phase is nearly 10-fold slower (Tables II and III). If attachment of the second head were exclusively to the same actin filament as that of the first, then the rate constant for the second phase would show no actin concentration dependence. The finding that there is a small actin concentration dependence on the second rate constant implies that the second head is capable of binding to a second actin filament. If RLC phosphorylation disrupts head-head interactions, it might be expected that binding of the second head to actin should occur more rapidly than the first, since the unattached second head would experience a high local concentration of actin due to its being tethered by the attached head. This would make attachment of the first head rate-limiting, and binding to actin would be associated with a single exponential transient. This in fact was observed for phosphorylated HMM, as well as for the unregulatable constructs 2HepZip and 7HepZip.

Both mant ADP and pyrene actin probes give qualitatively the same results, viz. a single process for unregulated or phosphorylated species and two phases for unphosphorylated HMM. However, the rate constants derived for these two probes differ (Tables II and III). This may reflect differences in
affinities of unmodified versus mant modified nucleotides for myosin (41). Finally, although two exponentials in the fluorescence transients for unphosphorylated HMM:ADP are interpreted to reflect sequential binding of the two heads, another possibility is that they reflect a slow isomerization between two different HMM:ADP states. This is not favored, however, since the release of mant ADP from unphosphorylated HMM in the absence of actin is monoeXponential, and it seems unlikely that binding to actin would slow this isomerization. However, the data do not exclude the possibility that HMM:ADP is a mixture of two states in rapid equilibrium. This is in fact supported by the finding that the rate of ADP dissociation from a pre-formed, phosphorylated acto-HMM complex was 5–7 times faster than that measured by mixing phosphorylated HMM:ADP with actin. Results from our previous study (30), which established that smooth muscle S-1:ADP is an equilibrium mixture of two states, support this conclusion.

Finally, the data in Tables IV and V suggest that once both heads of HMM are attached to actin, they become indistinguishable and behave kinetically in an identical manner, regardless of the phosphorylation status of the RLCs. The kinetic asymmetry of the two heads of smooth muscle myosin in the absence of RLC phosphorylation may have relevance to the phenomenon of the “latch state,” viz. where maximally contracted smooth muscle can continue to produce force even after its myosin is dephosphorylated but with slower steady state kinetics (41–45). Determining the relationship between our findings and the latch state will require measuring the number of force-generating states in the myosin ATPase cycle, which is currently underway in our laboratories.

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REFERENCES
Structural and Kinetic Studies of Phosphorylation-dependent Regulation in Smooth Muscle Myosin
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