Kinetic and Spectroscopic Evidence for Three Actomyosin:ADP States in Smooth Muscle*

Received for publication, March 29, 2000, and in revised form, May 23, 2000
Published, JBC Papers in Press, May 24, 2000, DOI 10.1074/jbc.M002685200

Steven S. Rosenfeld‡§, Jun Xing‡, Michael Whitaker§, Herbert C. Cheung‡, Fred Brown**,
Amber Wells***, Ron A. Milligan‡ and H. Lee Sweeney**

From the Departments of §Neurology and Biochemistry and Molecular Genetics, the University of Alabama at Birmingham, Birmingham, Alabama 35294, the §Department of Cell Biology, Scripps Research Institute, La Jolla, California 92037, and the **Department of Physiology, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Smooth muscle myosin II undergoes an additional movement of the regulatory domain with ADP release that is not seen with fast skeletal muscle myosin II. In this study, we have examined the interactions of smooth muscle myosin subfragment 1 with ADP to see if this additional movement corresponds to an identifiable state change. These studies indicate that for this myosin:ADP, both the catalytic site and the actin-binding site can each assume one of two conformations. Relatively loose coupling between these two binding sites leads to three discrete actin-associated ADP states. Following an initial, weakly bound state, binding of myosin:ADP to actin shifts the equilibrium toward a mixture of two states that each bind actin strongly but differ in the conformation of their catalytic sites. By contrast, fast myosins, includingDictyostelium myosin II, have reciprocal coupling between the actin- and ADP-binding sites, so that either actin or nucleotide, but not both, can be tightly bound. This uncoupling, which generates a second strongly bound actomyosin ADP state in smooth muscle, would prolong the fraction of the ATPase cycle time that this actomyosin spends in a force-generating conformation and may be central to explaining the physiologic differences between this and other myosins.

Although all myosins share basic structural and enzymatic features, they differ widely in their physiologic behavior. Comparing myosin II and myosin V provides an illustrative example (1, 2). Myosin V is designed to work as a single, processive motor, whereas myosin II is designed to work in a large, filamentous ensemble. Even within the myosin II family there are major differences in shortening velocity, economy of force production, and in vitro motility between fast myosins, such as those from vertebrate skeletal muscle, and slow myosins, including vertebrate smooth muscle and non-muscle myosin II (3). Because all myosins appear to use the same general kinetic scheme for their ATPase cycles, differences in their properties must be reflected in corresponding differences in the rate and equilibrium constants that describe the individual kinetic schemes.

During the course of its ATPase cycle, myosin alternates between conformations that bind actin weakly and those that bind actin strongly (4, 5). The strong binding conformations generate force, and the lifetime of the strong binding state limits the rate of actin-myosin filament sliding. One of the major differences between myosin isoforms is in the duty ratio, which is defined as the fraction of the total cycle time that a myosin isoform spends in a strong binding conformation (6, 7). Such differences have been described between fast skeletal and smooth muscle myosin II and include an increased duty ratio, as well as slower rates of hydrolysis (8) and ADP release (9) for smooth muscle myosin II.

An increase in the duty ratio of smooth muscle myosin II implies a longer lifetime of the strong binding state, which under physiologic conditions means a longer lifetime of ADP-bound state(s) (4). This could be accomplished either by adding an additional, unique actomyosin:ADP state for smooth muscle myosin or by slowing the ADP release step. Information relevant to this issue has been obtained from structural studies of gizzard S1. Whitaker et al. (10) produced three-dimensional density maps of nucleotide-free and ADP-bound chicken gizzard acto-S1, generated by cryo-electron microscopy and helical image reconstruction. These maps demonstrated that ADP release induces an additional 23° tilt of the regulatory domain. Similar conclusions were also reached from studies utilizing EPR spectroscopy and x-ray fiber diffraction (11, 12), as well as from studies with brush border myosin I (13). Interestingly, this movement is not seen in the corresponding skeletal muscle isoform (10). These findings have been interpreted to mean that ADP release contributes to the power stroke in a subset of myosin II isoforms, as well as in some of the unconventional myosins (13, 14), perhaps through the presence of an additional structural transition not present in skeletal muscle myosin II.

However, several other lines of evidence call into question the significance of this extra tilt. Cremo and Geeves (15) examined the equilibrium binding and kinetic properties of smooth muscle S1:ADP with actin. They concluded that the tight affinity of ADP for acto-S1 ($K_d = 5 \mu M$) would make ADP release energetically unfavorable under physiologic conditions and therefore could not provide free energy to drive rotation of the regulatory domain. Furthermore, equilibrium binding data were used to argue that the thermodynamic coupling between ADP and actin binding to S1 was appreciably lower in smooth

* This work was supported by NINDS Grants NS34856 from National Institutes of Health (to S. S. R.), by NIAMS Grant AR32899 from the National Institutes of Health (to H. C. C.), and by National Institutes of Health (to S. S. R.), by NIAMS Grant AR31239 from the National Institutes of Health (to H. L. Sweeney).
TABLE I  
Smooth Muscle Myosin:ADP States  

| Sample          | Source          | °C | ns    | f1   | f2   | f3   | K (= f2/f1) |
|-----------------|-----------------|----|-------|------|------|------|-------------|
| S1:2′dmD        | Proteolytic     | 10 | 8.45 ± 0.32 | 0.98 | 0.47 ± 0.13 | 0.011 | 0.011       |
| S1:2′dmD        | Proteolytic     | 20 | 7.95 ± 0.35 | 0.95 | 0.81 ± 0.18 | 0.043 | 0.045       |
| S1:2′dmD        | Recombinant     | 20 | 8.73 ± 0.20 | 0.93 | 1.8 ± 0.10  | 0.07  | 0.075       |
| Acto-S1:2′dmD   | Proteolytic     | 10 | 6.98 ± 0.29 | 0.64 | 0.30 ± 0.11 | 0.36  | 0.56        |
| Acto-S1:2′dmD   | Proteolytic     | 20 | 7.97 ± 0.40 | 0.43 | 0.28 ± 0.10 | 0.57  | 1.32        |
| Acto-S1:2′dmD   | Recombinant     | 10 | 7.6 ± 0.30  | 0.83 | 0.67 ± 0.20 | 0.17  | 0.20        |
| Acto-S1:2′dmD   | Recombinant     | 20 | 7.8 ± 0.30  | 0.61 | 0.72 ± 0.20 | 0.39  | 0.61        |

The conditions were 20 mM KCl, 25 mM Hepes, 1 mM MgCl2, 1 mM dithiothreitol, pH 7.5.

**RESULTS**

**Transient Fluorescence Measurements of Myosin:2′dmD**

We had previously shown that the fluorescence of recombinant smooth muscle S1 complexed with the fluorescent ADP analogue 2′dmD could be characterized by two lifetimes, suggesting that smooth S1:ADP consists of an equilibrium mixture of two states (30). In order to examine this more closely, we prepared proteolytic gizzard S1, and we measured the time-dependent fluorescence decays of complexes with 2′dmD as a function of temperature. The large concentrations of protein required for these measurements precluded our performing this experiment with recombinant S1. At all temperatures, the decay data could be adequately fitted to the sum of two exponentials, yielding two lifetimes. At 20 °C, the lifetimes of these components were 7.95 ± 0.30 and 0.81 ± 0.10 ns, with fractional amplitudes of 0.72 and 0.28, respectively (Table I). Neither of these lifetimes represents unbound nucleotide, since the lifetime of unbound mant-ADP is 3.8 ± 0.2 ns (27), and at the concentration of S1 used in this study (110 μM), less than 1% of muscle than in skeletal muscle myosin, which suggested a strain-dependent mechanism would be required to drive ADP release. Dantzig et al. (16) examined the effects of MgADP on rigor tension in gizzard smooth muscle and found that, contrary to expectation, its addition did not reduce tension. They postulated that the additional movement of the regulatory domain may contribute to the latch state, by slowing release of ADP from attached heads.

The presence of an additional tilt in the mechanism of smooth muscle myosin II implies that there is an additional actomyosin ADP intermediate in the pathway of nucleotide dissociation. If this is so, the rate of transition through this intermediate might contribute to economy of force production by smooth and non-muscle myosin II isoforms by controlling the rate of ADP release. In order to evaluate this, we have examined the interactions of ADP with smooth muscle S1 and acto-S1 with transient fluorescent and kinetic methodologies. We have also examined myosin II from Dictyostelium, which has been the subject of extensive structural and biochemical characterization (17–19). Although we find evidence for only one myosin:ADP state for myosin II from Dictyostelium, corresponding studies on smooth muscle myosin II clearly demonstrate the presence of two such states, both of which are on the pathway to ADP release. The relevance of these results to models of strain-dependent release will be discussed.

**EXPERIMENTAL PROCEDURES**

*Reagents*—The N-methylanthraniloyl derivative of 2′ deoxy-ADP was synthesized as described (20). N-1-pyrenyl iodoacetamide was obtained from Molecular Probes (Portland, OR). Protease inhibitors and chemicals used for buffers were obtained from Sigma. Sephacyr S-300-HR and prepoured Sephadex G-25 columns were obtained from Amersham Pharmacia Biotech.

*Proteins*—Dephosphorylated smooth muscle myosin was obtained from chicken gizzards (21). Actin was prepared at cysteine 374 with -1-pyrenyl iodoacetamide as described (26).

*Fluorescent Methodologies*—Steady state fluorescence measurements were made on an SLM/Aminco 8000C spectrophotometer, with sample holder thermostat at 20 °C. Fluorescence lifetimes were measured using a picosecond laser as excitation source, as described (27) in samples that had been equilibrated in 20 mM KCl, 25 mM Hepes, 1 mM MgCl2, 1 mM dithiothreitol, pH 7.5.

*Kinetic Methodologies*—Measurements of the rates of man nucleotide and pyrenyl actin binding to and release from S1 were made using a Hi-Tech Scientific stopped-flow spectrometer equipped with a 150-watt xenon lamp. The instrument dead time was determined to be 1.4 ns. For studies with mant nucleotide, fluorescence was excited by energy transfer from protein tryptophan, using a 295 nm input from a monochromer, and a 400 nm cut 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 405 nm interference filter was used to collect the fluorescence emission (OmegA Optical).

*Protein Purification and Concentration Assays*—Proteins were run on SDS-polyacrylamide electrophoresis as described (28). Protein concentrations were determined colorimetrically (Bio-Rad Protein Assay).

*Electron Microscopy and Image Analysis—Recombinant S1, derived from Dictyostelium and smooth myosin II, at 2–5 mg/ml in 10 mM imidazole, pH 7.0, 50 mM KCl, 2 mM MgCl2, 0.5 mM EDTA, 0.1 mM EGTA, 0.5 mM dithiothreitol was used to decorate rabbit skeletal muscle actin filaments adsorbed to holey carbon support films on EM grids. For the nucleotide-free condition, apyrase was added to the solution (final concentration ~10 IU/ml) on the grid just prior to blotting and freezing in liquid ethane slush. For the ADP condition, the myosin buffer was supplemented with 5 mM MgADP. A Gatan cold stage operating at ~180 °C, was used to examine the grids in a Philips CM200 FEG electron microscope. Images of filaments spanning holes in the support film were recorded at 40,000× at 1.1–2.0 μm under focus. Selected images of well ordered filaments were digitized with spot and step sizes equivalent to 4.97 Å at the specimen. Image analysis, averaging, and calculation of three-dimensional maps were carried out essentially as described previously (29). The three-dimensional maps were visualized with Volvis.
the nucleotide should be unbound. In order to determine the equilibrium constant between these two states, it is necessary to determine the fractional intensity of each component. For a two component system, the fractional intensities ($f_1$ and $f_2$) are given by Equation 1,

$$f_1 = \frac{\alpha_1 \tau_1}{\alpha_1 \tau_1 + \alpha_2 \tau_2}$$

$$f_2 = \frac{\alpha_2 \tau_2}{\alpha_1 \tau_1 + \alpha_2 \tau_2}$$

(Eq. 1)

where $\tau_1$ and $\tau_2$ are the two lifetimes with two associated fractional amplitudes $\alpha_1$ and $\alpha_2$ (31). The two fractional intensities provide a measure of the mole fractions of the two components. Thus, the ratio of $f_2/f_1$ is an equilibrium constant describing the equilibrium between these two $\text{S1:ADP}$ states.

We measured the fluorescence decay parameters of proteolytic $\text{S1:2'dM}$ as a function of temperature, and Fig. 1 shows a $\text{van't Hoff}$ plot depicting $\ln(f_2/f_1)$ versus the reciprocal of temperature, in degrees Kelvin. The component with longer lifetime is favored at all temperatures. The data fit a two-state equilibrium with $\Delta H^0 = 10.6 \text{ kcal/mol}$ and $\Delta S^0 = 29.3 \text{ cal/(degrees mol)}$. Conditions are as follows: 20 mM KCl, 25 mM Hepes, 1 mM MgCl$_2$, 1 mM dithiothreitol, 1 mM MgADP, pH 7.50.

![Fig. 1. van't Hoff plot of proteolytic S12'deoxymant-ADP. The data fit a two-state equilibrium with $\Delta H^0 = 10.6 \text{ kcal/mol}$ and $\Delta S^0 = 29.3 \text{ cal/(degrees mol)}$. Conditions as follows: 20 mM KCl, 25 mM Hepes, 1 mM MgCl$_2$, 1 mM dithiothreitol, 1 mM MgADP, pH 7.50.](image)

We next repeated these measurements for a complex of $\text{S1:2'dM}$:actin likewise consisting of two components, with lifetimes of 8.73 ± 0.2 ns and 1.8 ± 0.1 ns and fractional components of 0.70 and 0.30, respectively (30). This would correspond to $f_2/f_1 = 0.075$ at 20 °C (Table I).

Similar studies using recombinant S1 from Dictyostelium myosin II, a fast myosin, also revealed two components. However, one of these had a lifetime of 3.82 ± 0.20 ns, identical to free nucleotide, and its fractional emission of 0.30 was nearly identical to the fraction of free nucleotide determined by equilibrium dialysis (0.27). The other, corresponding to bound nucleotide, had a lifetime of 8.31 ± 0.30 ns (Table I). We therefore conclude that the short lifetime component seen with smooth muscle myosin represents an additional myosin ADP state not seen in fast myosin II isoforms.

We next repeated these measurements for a complex of 12.4 µM proteolytically prepared smooth muscle S1, 12.4 µM 2’dmD, and 25 µM phalloidin-stabilized actin. The lower concentration of S1 was used to reduce the turbidity of the acto-S1 mixture. Given the dissociation constant of S1:ADP for actin in the steady state (15), essentially all of the S1 should remain bound. We measured the concentration of 2’dM not bound to the acto-S1 complex by sedimenting the complex in an Airfuge and measuring the absorbance of the supernatant at 356 nm. This yielded an unbound nucleotide concentration of 6.8 µM, corresponding to an unbound fraction of 0.48 and a dissociation constant of 5.7 µM. The fluorescence decay of this complex at 20 °C could be described by three exponential terms with lifetimes of 7.97 ± 0.3, 3.97 ± 0.2, and 0.28 ± 0.1 ns and fractional intensities of 0.17, 0.60, and 0.23, respectively (Table I). The fractional intensity of 0.60 is similar to the fraction of unbound nucleotide, and the lifetime for this component is likewise very close to that for free nucleotide. Furthermore, the fluorescence decay of a mixture of 12 µM 2’dM and 20 µM phalloidin-stabilized actin yielded a single lifetime of 3.87 ± 0.2 ns, and we therefore conclude that the component of the acto:S1 complex with lifetime of 3.97 ns is free nucleotide. The other two lifetime components, representing two acto:S1:2’dM states, are related by an equilibrium constant $f_2/f_1 = 1.32$. Repeating this experiment at 10 °C again produced three components, with lifetimes of 6.98 ± 0.3, 4.23 ± 0.2, and 0.30 ± 0.1 ns and fractional intensities of 0.25, 0.61, and 0.14 (Table I). This corresponds to an equilibrium constant of 0.56 between the two acto:S1:2’dM states. These results thus demonstrate that actin binding shifts the equilibrium distribution of the two S1:ADP states toward the shorter lifetime component by a factor of 30–50.

Repeating these experiments with a complex of 6.0 µM recombinant smooth S1 + 6.0 µM 2’dM, and 40.0 µM phalloidinstabilized actin also revealed the presence of three components in the fluorescence decay. At 20 °C, the lifetimes were 7.82 ± 0.3, 3.70 ± 0.2, and 0.72 ± 0.2 ns with fractional intensities of 0.23, 0.63, and 0.14, respectively. The corresponding lifetimes and fractional intensities at 10 °C were 7.58 ± 0.3, 3.99 ± 0.2, and 0.67 ± 0.2 ns and 0.25, 0.70, and 0.05. The component with lifetime of 3.70 ± 0.2 ns with unbound fraction at 20 °C of 0.63, is considered free nucleotide, since its fractional intensity is in good agreement with direct measurements of unbound nucleotide that were obtained by sedimenting the complex in an Airfuge. This revealed a free nucleotide concentration of 4.1 µM, corresponding to an unbound fraction of 0.68 and dissociation constant of 8.3 µM. Measurement of actin-bound S1:ADP was performed with a sedimentation assay using H-labeled S1, as described (8), and revealed an unbound fraction of <0.07. Thus, lifetime studies of recombinant S1:2’dM:actin also reveal evidence of two states that are related by an equilibrium constant of 0.20 at 10 °C and 0.61 at 20 °C (Table I).

These results demonstrate the presence of two S1:ADP and two acto-S1:2’dM states in smooth muscle, as defined by a probe in the nucleotide-binding site. In order to determine how these states relate to actin binding affinity, we next performed a series of transient state kinetic measurements with pyrene-labeled actin.

**Kinetic Studies of Recombinant S1:ADP Binding to Pyrene Actin**—The formation of a strongly bound actomyosin state can be monitored by using a pyrenyl probe on actin, since strong binding of S1 to actin quenches the pyrene fluorescence (32). Several investigators have utilized this to demonstrate that binding of ADP to acto:S1 has no effect on the pyrene fluorescence (32).

The formation of a strongly bound actomyosin state can be monitored by using a pyrenyl probe on actin, since strong binding of S1 to actin quenches the pyrene fluorescence (32). Several investigators have utilized this to demonstrate that binding of ADP to acto:S1 has no effect on the pyrene fluorescence (32). Several investigators have utilized this to demonstrate that binding of ADP to acto:S1 has no effect on the pyrene fluorescence (32). Several investigators have utilized this to demonstrate that binding of ADP to acto:S1 has no effect on the pyrene fluorescence (32).
was mixed with recombinant S1 + 2 mM ADP at 20 °C in the stopped flow. The quenching of the pyrenyl probe followed a single exponential process following a lag phase (Fig. 2, inset), as has been described with skeletal muscle S1 (32). The rate of the quenching varied hyperbolically with actin concentration, with an initial slope defining an apparent second order rate constant of 3.4 μM⁻¹ s⁻¹. Fitting to a hyperbolic dependence on actin concentration yielded an apparent binding constant of 2.4 μM and a maximum rate of 6.9 s⁻¹. Inset, fluorescence transient produced by mixing 1 μM S1:ADP with 10 μM actin, demonstrating the lag and subsequent fluorescence decay. Smooth curve is a fit to a single exponential decay following a lag phase.

**REACTION 1**

\[
\text{M-D + A}^* \rightleftharpoons \text{M-D-A}^* \rightleftharpoons \text{M-D-A}
\]

where A* is pyrene actin with unquenched fluorescence; A is quenched pyrene actin; D is ADP; and M is myosin. This implies that, as in the case of skeletal muscle S1, the initial M-D complex is in a weak actin binding conformation. Formation of the M-D-A* complex, associated with the lag phase, is then followed by an isomerization to strong binding complex (M-D-A) whose fluorescence is quenched.

We next examined the kinetics of ADP dissociation from acto-S1 by mixing pyrene actin + recombinant S1 + ADP in the stopped flow with 4 mM ATP. At 20 °C, the resulting fluorescence enhancement occurred as a single exponential process whose rate constant showed a small concentration dependence, and over an ADP concentration range of 20–200 μM was between 61 and 77 s⁻¹ (data not shown). Repeating the experiment at 10 °C produced a fluorescence transient that was best fit by two exponential terms. The ratio of the amplitudes of these two phases remained constant at 0.20–0.25 in favor of the slower phase, over an ADP concentration range of 30–300 μM. The rate constant of the faster process demonstrated an inverse relationship with [ADP], whereas that for the second phase showed little dependence on [ADP] and was in the range of 6–10 s⁻¹ (Fig. 3A). The dependence of the rate of the faster phase on [ADP] could be described by Equation 2:

\[
λ_{obs} = \frac{K_{ATP} \cdot k_d \cdot [ATP]}{1 + K_{ATP} \cdot [ATP] + [ADP]} \quad \text{(Eq. 2)}
\]

where \(λ_{obs}\) is the observed rate constant; \(K_{ATP}\) is the apparent binding constant of ATP to the acto-S1 complex; \(k_d\) is the rate constant of acto-S1 dissociation produced by mixing with ATP at concentration [ATP]; and \(K_{ADP}\) is the dissociation constant for ADP binding to the acto-S1 complex. These values are summarized in Table II. A similar result had been reported for brush border myosin I at room temperature (14).

We had previously reported that loop 1, a surface-exposed segment of random coil bridging the catalytic site and connecting the 25- and 50-kDa domains, modulates the rate of ADP release and that its deletion reduced the rate of ADP release by nearly 10-fold (30). We repeated the above experiments with a mutant of smooth muscle S1 in which this loop had been deleted (Δ25/50 S1), and we found that at 20 °C, it produced a biphasic fluorescence transient with roughly equal amplitudes. Furthermore, the rates for the two phases demonstrated an ADP concentration dependence very similar to wild type S1 at 10 °C (Fig. 2B). The rate of the faster phase of the transient for Δ25/50 S1 could likewise be fit to Equation 2, and results are summarized in Table II. A double exponential decay would be expected if release of ADP occurs subsequent to an isomerization reaction, as has been described for brush border myosin I (14). For smooth muscle S1, this isomerization can be detected by either reducing the temperature or by eliminating loop 1, both of which appear to slow this isomerization. The fluorescence lifetime data discussed above indicate that both acto-S1:ADP states are populated at 20 °C. Thus, the presence of only a single phase in the fluorescence transient for native S1 at 20 °C implies that at...
this temperature, these two states equilibrate at least as rapidly as the rate of ADP release. In order to evaluate this further, we next examined the kinetics of 2‘dmd release from a preformed acto-S1 complex at 10 and 20 °C. In this experiment 3 μM recombinant S1 + 10 μM phallloidin-stabilized actin + 30 μM 2‘dmd were mixed in the stopped flow with 2 mM ATP. Given our measured value for the dissociation constant under steady state conditions, essentially all of the S1 should remain bound to actin. The resulting fluorescence transient, monitored by energy transfer, demonstrated a single exponential process at both temperatures. At 10 °C, the rate constant was 26.2 ± 3.7 s⁻¹, nearly identical to the faster rate produced by mixing S1:2‘dmd with actin. At 20 °C, the rate was 96.4 ± 10.7 s⁻¹. This experiment was repeated with 20 mM phosphate added to the acto-S1:2‘dmd-containing syringe, and the rate at 10 °C, 25.2 ± 4.6 s⁻¹, was essentially the same as in the absence of phosphate.

Binding of 2‘dmd to a rigor acto-S1 complex was examined at 10 °C in the stopped flow by mixing varying concentrations of 2‘dmd with 3 μM recombinant S1 + 12 μM actin + 1.0 unit/ml apyrase, the latter to ensure the acto:S1 complex is

---

**FIG. 3: Rates of the two phases of the fluorescence transient produced by mixing pyrene actin + S1 + ADP with 4 mM MgATP, plotted as a function of [ADP], for wild type S1 at 10 °C (A) or Δ25/50 S1 at 20 °C (B). A, the rate constant of the faster phase (closed squares) could be fit to Equation 2, yielding values for $K_{ATP}$, $k_o$, and $K_{ADP}$, summarized in Table II, whereas the slower phase (open squares) showed little ADP concentration dependence and remained in the range of 6–10 s⁻¹. B, the rate constant for the faster phase (closed squares) could be fit to Equation 2, and values of $K_{ATP}$, $k_o$, and $K_{ADP}$ are summarized in Table II. The rate of the slower phase remained relatively constant at 5–7 s⁻¹. Conditions are as in Fig. 1.**

**TABLE II**

**Kinetic parameters for the release of 2’deoxy mant-ADP from recombinant acto-S1**

| Reaction                  | Temperature | $K_{ATP}$ | $k_o$ | $K_{ADP}$ |
|---------------------------|-------------|-----------|-------|-----------|
| Δ25/50 S1:actinⁿ + ADP × ATP | 20°C        | 20        | 189   | 1.8       |
| S1:actinⁿ + ADP × ATP     | 10°C        | 1186      | 227   | 6.1       |

*a Actin, pyrene-labeled actin.

**TABLE III**

**Kinetic parameters for the release of 2‘dmd from recombinant acto-S1**

| Reaction                  | Temperature | $K_{ATP}$ | $k_o$ | $K_{ADP}$ |
|---------------------------|-------------|-----------|-------|-----------|
| S1:2‘dmd × actin + ATP    | 20°C        | 3.0       | 10.7  | 6.1       |
| S1:2‘dmd × actin + ATP    | 10°C        | 3.0       | 23.8  | 26.2      |
| Acto-S1:2‘dmd × ATP       | 20°C        | 96.4      |       |           |
| Acto-S1:2‘dmd × ATP       | 10°C        | 26.2      |       |           |

* From Ref. 30.
nucleotide-free. The rate of the fluorescence enhancement produced by excitation at 295 nm was monophasic, and a plot of rate versus nucleotide concentration was linear in the 0–15 mM range (Fig. 5). This defines an apparent second order rate constant of 2.9 mM⁻¹ s⁻¹, a dissociation rate constant of 12.1 s⁻¹, and a dissociation constant of 4.2 µM. This compares to a calculated value of 6.1 µM at 10 °C from results with pyrene actin (Fig. 3A and Table II).

As noted above, the fluorescence decay of complexes of 2'dmD with S1 from Dictyostelium myosin II is monoexponential, indicating the presence of only one S1:ADP state. This is consistent as well with the kinetics of 2’dmD release, measured by mixing Dictyostelium myosin II:2’dmD with an excess of actin + 4 mM ATP in the stopped flow. Release of nucleotide at both 10 and 20 °C occurred in a single phase, with rate constants demonstrating a linear dependence on actin concentration (Fig. 6).

Cryo-EM Maps of Dictyostelium Myosin II—To test further the assertion that the second strongly bound ADP state in smooth muscle is linked to the movement observed in cryo-electron microscopy, we calculated three-dimensional maps of actin decorated with recombinant Dictyostelium myosin II, with and without MgADP present. The resulting maps are shown in Fig. 7 and are compared with previously generated maps for smooth muscle myosin II (10). Consistent with the kinetic data, there is no movement of the light chain domain of Dictyostelium myosin II upon ADP release. The label motor is adjacent to the myosin II motor domain, into which has been inserted the α-carbon backbone (yellow) derived from the crystal structure (17). ELC denotes the essential light chain and RLC the regulatory light chain of myosin II, which together constitute the effective “lever arm.” Note that the RLC density is weak, especially in A, which may be indicative of light chain mobility.

**DISCUSSION**

Previous studies of the interaction of smooth muscle S1 with ADP and actin have been interpreted in terms of a kinetic scheme that invokes the existence of one S1:ADP state (15). However, several results in the literature suggest that the properties of smooth muscle myosin:ADP can only be explained by assuming the existence of several such states. First, there is an apparent discrepancy between the binding constant of smooth muscle S1:ADP for actin, measured by equilibrium techniques (15), and that determined from transient kinetic measurements (30). This could be most readily explained by the presence of an additional S1:ADP state, not seen in skeletal...
or other fast myosins, and could also explain the additional movement of the regulatory domain seen with ADP release (10, 11). In this study, we have examined this issue by investigating the interaction of S1:ADP with actin using both spectroscopic and kinetic probes.

Fluorescence lifetime studies of complexes of 2' dmD with S1 demonstrate the existence of two S1:ADP states. When bound to actin, the fluorescence decay of S1:2' dmD also demonstrates the presence of two states, and the finding that adding ADP to pyrene actin:S1 does not alter the fluorescence of the pyrenyl probe indicates that both of these states bind actin strongly. Nevertheless, the hyperbolic dependence of the rate of binding of S1:ADP to pyrene actin (Fig. 2) suggests that when dissociated from actin, the vast majority of S1:ADP is in a conformer that had relatively low thermodynamic coupling. In such a loosely coupled system, the affinity of a myosin for actin would solely be determined by the state of its actin-binding sites. Hence, species for actin would solely be determined by the state of its binding sites. We propose that these two sites can each assume indicated by the subscript next to the symbol for ADP. Tight binding sites would produce two actomyosin states, such as described previously for skeletal muscle S1:ADP (9). Since K°' > 1, the AMwD8 state would not be populated and would not con-

\[
\begin{align*}
\text{AM}_s\text{D}_8 & \rightarrow \text{AM}_w\text{D}_8 & k_{1f}/k_{1} & k_{2f}/k_{2} & \text{AM} \\
\text{M}_s\text{D}_8 & \rightarrow \text{M}_w\text{D}_8 & k_{1f}/k_{1} & k_{2f}/k_{2} & \text{M} \\
\text{K}_s & \rightarrow \text{K}_w & & & \text{K}_w \\
\text{K}_o & \rightarrow \text{K}_o & & & \text{K}_o \\
\text{K}_a & \rightarrow \text{K}_a & & & \text{K}_a \\
\end{align*}
\]

\text{REACTION 2}

\[
\begin{align*}
\text{AM}_s\text{D}_8 & \rightarrow \text{AM}_w\text{D}_8 & k_{1f}/k_{1} & k_{2f}/k_{2} & \text{AM} \\
\text{M}_s\text{D}_8 & \rightarrow \text{M}_w\text{D}_8 & k_{1f}/k_{1} & k_{2f}/k_{2} & \text{M} \\
\text{K}_s & \rightarrow \text{K}_w & & & \text{K}_w \\
\text{K}_o & \rightarrow \text{K}_o & & & \text{K}_o \\
\text{K}_a & \rightarrow \text{K}_a & & & \text{K}_a \\
\end{align*}
\]

where A is actin, M is myosin, D is ADP, K represents an equilibrium constant, k is a rate constant, and where K_1 = k_{1f}/k_{1} = M_s\text{D}_8/M_w\text{D}_8, etc. The species highlighted in boldface would be the predominant ones at equilibrium, based on the calculated values of the various equilibrium constants (see below). In Reaction 2, each myosin and actomyosin state can be characterized by the conformations of the nucleotide and actin-binding sites. We propose that these two sites can each assume one of two conformations, “strong” binding and “weak” binding. The state of the actin-binding site (S indicates strong, and W indicates weak) is indicated by the subscript next to the symbol for myosin, while the state of the nucleotide-binding site is indicated by the subscript next to the symbol for ADP. Tight coupling between the conformations of the nucleotide and actin-binding sites would produce two actomyosin states, such as is seen in skeletal muscle, whereas looser or no coupling could produce up to four such states. This is reminiscent of the model of Cremo and Geeves (15), in which strain-dependent nucleotide release was proposed to drive ADP dissociation from a system that had relatively low thermodynamic coupling.

In such a loosely coupled system, the affinity of a myosin species for actin would solely be determined by the state of its actin-binding site. Hence, K_s = K_w and therefore K_1 = K_2. As noted above, the lack of a fluorescence change when nucleotide-free pyrene actin:S1 is mixed with ADP indicates that K_1' > 1 and AMwD8 is not populated in the steady state. This is also consistent with the finding that added phosphate did not affect the kinetics of 2' dmD dissociation from acto:S1. Hence, the equilibrium constant measured from the fluorescence decay of 2' dmD bound to acto:S1 (Table 1) is a measure of K_1 and K_2'. The fluorescence lifetime-dependent equilibrium constant, f_p/f_0 (Table 1), is a measure of M_s\text{D}_8(M_w\text{D}_8 - M_w\text{D}_8). Hence, the value of this equilibrium constant can be used to solve for K_1 and K_2. These are 0.14 and 0.61, respectively, for recombinant S1:ADP at 20 °C and 0.03 and 1.3 for proteolytic S1:ADP at the same temperature. Thus, for smooth muscle myosin:ADP, the predominant state would be a weak actin-binding, strong nucleotide-binding state (M_wD8) that would be equiva-

\[
\begin{align*}
\text{A}^+ + \text{M}_w\text{D}_8 & \rightarrow \text{A}^+ \text{M}_w\text{D}_8 \rightarrow \text{AM}_w\text{D}_8 \\
\text{K}_o & \rightarrow \text{K}_m \\
\end{align*}
\]

\text{REACTION 3}

The apparent binding constant for the hyperbolic dependence of rate on actin concentration provides a measure of K_0 of 0.42 μM−1 at 20 °C (Fig. 2). Given that K_o = 0.14 and that K_0 = K_o/K_w/K_0, this would predict K_0 = 33 nM, which is remarkably close to the value measured by Cremo and Geeves (24 nM, Ref. 15).

Two rate processes were seen in the release of 2' dmD from S1 at 10 °C, whereas we had previously reported only one process was seen at 20 °C (30). In neither case was an appreciable lag phase seen. These findings could be explained by assuming that K_o is a rapid equilibrium relative to the subsequent steps and by assigning the MD state to that with the longer lifetime component. The resulting fluorescence transient for 2' dmD dissociation would be predicted to fit a double exponential decay. The apparent rate constants, λ_{1,2} are described by Equations 3 and 4 (9),

\[
\lambda_{1,2} = \frac{S + \sqrt{S^2 - 4C}}{2}
\]

where

\[
S = k_1 + k_{-1} + k_2
\]

\[
C = k_1 \cdot k_2
\]

The solution of the rate equations must reasonably agree with the calculated value of K_1 = k_{1f}/k_{1}, as well as explain the observed rates. These requirements can be satisfied for recombinant S1 by assigning values of k_1 = 0.3 s−1, k_{-1} = 0.8 s−1, and k_2 = 4 s−1 at 10 °C; and k_1 = 3 s−1, k_{-1} = 2 s−1, and k_2 = 4 s−1 at 20 °C. At the lower temperature, this would produce a biphasic transient with rates of approximately 5 and 0.6 s−1. Two rate processes would theoretically also be observed at 20 °C, although the rate constants would be approximately 2.5 and 6 s−1 and could appear as a single exponential process. These results thus suggest a strong temperature dependence on k_{1f}, which is consistent as well with the effect of temperature on K_1 (Fig. 1 and Table 1). This implies that this transition represents a concerted reaction, such as would be seen with a large conformational change (see below).

Solution of the rate equations for 2' dmD release from actomyosin takes the same form as that for myosin, although each of the rate constants now represents an averaged rate constant whose value depends hyperbolically on actin concentration, as described previously for skeletal muscle S1 (9). Since K_0' > 1, the AMwD8 state would not be populated and would not con-
of movement upon ADP dissociation seen in the cryo-electron does not exist in those myosins. Consistent with this is the lack of movement upon ADP dissociation seen in the cryo-electron microscopy maps of Dictyostelium myosin II (Fig. 7) and the previously published EPR data comparing skeletal and smooth muscle myosin (11). Thus, it would appear that that the large scale structural change inferred by the temperature dependence of the isomerization between the two strong smooth actomyosin:ADP states is the structural change observed in movement of the light chain domain upon ADP release from smooth muscle myosin. It is interesting that the ADP/rigor position of the Dictyostelium lever arm is similar to the rigor position of the smooth lever arm. On the other hand, previously published fast skeletal EM maps and EPR data (10, 11) reveal that the ADP/rigor lever arm position of skeletal myosin is similar to the ADP position of smooth. This could reflect either differences in the size of the power stroke among these myosins or differences in the connection between the light chain helix and the converter domain of the myosin motor. In particular the position of the essential light chain density of Dictyostelium myosin II appears somewhat different with respect to the motor domain than that of smooth muscle myosin II (Fig. 7).

It is ironic that neither the myosin for which there is the most functional data (fast skeletal myosin II) nor the myosin for which there is the most structural data (Dictyostelium myosin II) demonstrate an additional transition associated with ADP release from actomyosin. It is likely that the coupling that produces the additional movement has been lost to allow for greater velocities of filament sliding. However, this appears to be in conflict with data from skeletal muscle fibers undergoing isometric contraction, conditions under which ADP release is greatly slowed, yet the myosin remains attached strongly enough to support force transduction. The resolution of this paradox likely results from a strain-induced uncoupling of strong actin and ADP binding. We propose that there is sufficient compliance within the myosin molecule to allow a strong actin interface to form without completion of the lever arm swing. Indeed, in contracting muscle there is evidence for lever arm positions that are in between the unstrained positions revealed by the crystal structures (17, 36, 37). If ADP release cannot occur rapidly until the lever arm approaches the unstrained position, then this would provide a means to have both strong actin binding and strong ADP binding during isometric contraction. However, in the absence of such a load, as occurs in solution, the lever arm would reach its unstrained position coincident with strong actin binding, and this would give rise to the reciprocal relationship between actin and ADP binding that we observe for fast skeletal and Dictyostelium myosin II.

By contrast, smooth muscle myosin II, brush border myosin I, and myosin VI all display large scale movements associated with ADP release (10, 13, 34). This movement indicates that there is loose coupling between actin and ADP binding and implies the existence of a state that binds both actin and ADP strongly in the absence of imposed strain. The critical difference between these myosins on the one hand and fast skeletal and Dictyostelium myosin II on the other is that the former will have a long lived ADP state in the absence of load. This feature may be critical for a number of unconventional myosins in order to generate a long duty cycle in the absence of load. For smooth muscle myosin, this structural transition could generate long lived, force-producing cross-bridges that would allow the sustained force generation seen in this tissue. It is reasonable to assume that other members of the myosin family, such as myosin V, will also display such movements. Indeed, in the case of myosin V, DeLaCruz et al. (2) have argued that the kinetics of nucleotide binding by this myosin could be best explained by invoking the presence of an additional, strongly bound actomyosin ADP state. In all cases, the structural coupling between an ADP-occupied nucleotide pocket and the myosin lever arm will generate strain-dependent ADP release that could be exploited for a range of physiologic purposes.
REFERENCES

1. Mehta, A. D., Rock, R. S., Rief, M., Spudich, J. A., Mooseker, M. S., and Cheney, R. E. (1999) Nature 400, 590–593
2. DeLaCruz, E. M., Wells, A. L., Safer, D., Rosenfeld, S. S., Ostap, E. M., and Sweeney, H. L. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 13726–13731
3. Ruppel, K. M., and Spudich, J. A. (1996) Annu. Rev. Cell Dev. Biol. 12, 543–573
4. Arner, A., and Malmqvist, U. (1998) Acta Physiol. Scand. 164, 363–372
5. Ritchie, M. D., Geeves, M. A., Woodward, S. R., and Manstein, D. J. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 8619–8623
6. Guilford, W. H., Dupuis, D. E., Kennedy, G., Wu, J., Patlak, J. B., and Warshaw, D. M. (1997) Biophys. J. 72, 1066–1021
7. VanBuren, P., Guilford, W. H., Kennedy, G., Wu, J., and Warshaw, D. M. (1995) Biophys. J. 68, 256–258
8. Rosenfeld, S. S., and Taylor, E. W. (1984) J. Biol. Chem. 259, 11908–11919
9. Spudich, J. A., and Taylor, E. W. (1987) J. Biol. Chem. 262, 9994–9999
10. van Buren, P., Guilford, W. H., Kennedy, G., and Warshaw, D. M. (1995) Biophys. J. 68, 256–258
11. Gollub, J., Cremo, C. R., and Cooke, R. (1996) Nat. Struct. Biol. 3, 796–802
12. Poole, K. I. V., Lorenz, M., Ellison, P., Evans, G., Rosenbaum, G., Boesecke, P., Holmes, K. C., and Cremo, C. R. (1997) J. Muscle Res. Cell Motil. 18, 179–268
13. Jontes, J. D., Wilson-Kubalek, E. M., and Milligan, R. A. (1995) Nature 378, 751–753
14. Jontes, J. D., Milligan, R. A., Pollard, T. D., and Ostap, E. M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 14332–14337
15. Cremo, C. R., and Geeves, M. A. (1998) Biochemistry 37, 1969–1978
16. Dantzig, J. A., Barsotti, R. J., Manz, S., Sweeney, H. L., and Goldman, Y. E. (1999) Biophys. J. 77, 386–387
17. Gollub, J. V., and Jontes, J. D. (1996) J. Muscle Res. Cell Motil. 18, 179–268
18. Fisher, A. J., Smith, C. A., and Rayment, I. (1995) Biochemistry 34, 8960–8972
19. Hirtz, S. T. (1983) Biochim. Biophys. Acta 742, 496–508
20. Persechini, A., and Hartshorne, D. J. (1983) Biochemistry 22, 470–476
21. Rosenfeld, S. S., and Taylor, E. W. (1987) J. Biol. Chem. 262, 9994–9999
22. Whittaker, M., Wilson-Kubalek, E., Safer, L., Milligan, R. A., and Sweeney, H. L. (1995) Nature 378, 748–751
23. Gollub, J., Cremo, C. R., and Cooke, R. (1996) Nat. Struct. Biol. 3, 796–802
24. VanBuren, P., Guilford, W. H., Kennedy, G., Wu, J., and Warshaw, D. M. (1995) Biophys. J. 68, 256–258
25. Rosenfeld, S. S., and Taylor, E. W. (1993) Biochemistry 22, 4866–4871
26. Liepnieks, J. J., and Light, A. (1971) J. Biol. Chem. 246, 4866–4871
27. Summers, M. D., and Smith, G. E. (1990) J. Biol. Chem. 265, 1677–1683
28. Cooper, J. A., Walker, S. B., and Pollard, T. D. (1983) J. Muscle Res. Cell Motil. 4, 253–258
29. Rosenfeld, S. S., Correia, J. J., Xing, J., Rener, B., Gong, W., and Cheung, H. C. (1996) J. Biol. Chem. 271, 30212–30222
30. Sosa, H., Dias, D. P., Heonger, A., Whittaker, M., Wilson-Kubalek, E., Sablin, E., Fletterick, R. J., Vale, R. D., and Milligan, R. A. (1997) Cell 90, 217–224
31. Sweeney, H. L., Rosenfeld, S. S., Brown, F., Faust, L., Smith, J., Xing, J., Stein, L., and Sellers, J. (1996) J. Biol. Chem. 273, 6282–6270
32. Sweeney, H. L., and Warshaw, D. M. (1991) Biochemistry 30, 4317–4322
33. Sleep, J. A., and Hutton, R. L. (1980) Biochemistry 19, 1276–1283
34. Onew, A. L., Lin, A. W., and Hutton, R. L. (1980) Biochemistry 19, 1276–1283
35. Hopp, T. P., Prickett, K. S., Prince, V., Libby, R. T., March, C. J., Cerretti, P., and Hutton, R. L. (1988) Bio/Technology 6, 1205–1210
36. Corrie, J. E., Brandmeier, B. D., Ferguson, R. E., Frentham, R. D., Kendrick-Jones, J., Hopkins, S. C., van der Heide, U. A., Goldman, Y. E., Sabido-David, C., and Irving, M. (1999) Nature 400, 425–430
37. Dominguez, R., Freyzon, Y., and Cohen, C. (1998) Cell 94, 559–571