Cooperative binding of myosin subfragment-1 to the actin–troponin–tropomyosin complex

(muscle relaxation/cooperativity/regulated actin)

LOIS E. GREENE AND EVAN EISENBERG

Laboratory of Cell Biology, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, Maryland 20205

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ABSTRACT The binding of myosin subfragment-1 (S-1) to the F-actin–troponin–tropomyosin complex (regulated F-actin) was examined in the presence of ADP (ionic strength, 0.23 M; 22°C) by using the ultracentrifuge and S-1 blocked at SH2 with iodo[¹⁴C]acetamide. S-1-ADP binds with positive cooperativity to regulated F-actin, both in the presence and absence of calcium; it binds independently to unregulated actin. With and without Ca²⁺ at very low levels of occupancy of the regulated actin by S-1-ADP, S-1-ADP binds to the regulated actin with <1% of the strength that it binds to unregulated actin, whereas at high levels of occupancy of the regulated actin by S-1-ADP, S-1-ADP binds 3-fold more strongly to the regulated actin than it does to unregulated actin. The major difference between the results obtained in the presence and absence of Ca²⁺ with regulated F-actin is that, in the absence of Ca²⁺, the binding of S-1-ADP remains weak until a higher free S-1-ADP concentration is reached and the transition to strong binding is much more cooperative. These results are consistent with a model that is basically similar to the cooperative binding model of Hill [Hill, T. L. (1952) J. Chem. Phys. 20, 1259–1273] and of Monod et al. (Monod, J., Wyman, J., & Changeux, J. (1965) J. Mol. Biol. 12, 35–46). The regulated actin filament can exist in two forms, a weak-binding and a strong-binding form; and Ca²⁺ and Ca²⁺-S-1-ADP, acting as allosteric effectors, shift the equilibrium between the two forms.

It is now generally accepted that muscle contraction is caused by myosin and actin filaments sliding past each other as ATP is hydrolyzed. In skeletal muscle the regulation of muscle contraction appears to be controlled by the troponin–tropomyosin complex which binds to the actin filament to form regulated F-actin. The most widely accepted mechanism of troponin–tropomyosin action is the steric blocking model (1–3) which suggests that the position of the tropomyosin molecule on the actin filament controls the actin–myosin interaction. This model proposes that, in the absence of Ca²⁺, tropomyosin takes a position where it blocks the binding of myosin to the thin filament whereas, when Ca²⁺ binds to tropomyosin, the tropomyosin is thought to move toward the thin filament of actin, enabling the myosin to interact with actin. Because the tropomyosin molecule spans seven F-actin monomers (4), the position of the tropomyosin is thought to be effective over the entire actin unit and, therefore, this model suggests that cooperativity is an inherent part of regulation.

On a biochemical level, the removal of Ca²⁺ from the troponin–tropomyosin complex generally causes marked inhibition of the actomyosin ATPase activity. However, this is not always the case, as was observed by Bremel and Weber (5). At low ATP concentration and high ratios of subfragment 1 of myosin (S-1) to actin, they found that the ATPase activity of regulated actin–S-1 complex (acto-S-1) is no longer sensitive to Ca²⁺. They suggested that this cooperative response was due to the binding of a few S-1 molecules, free of ATP, to the actin filament and pushing the tropomyosin away from its inhibitory position, thus preventing inhibition of the ATPase activity even in the absence of Ca²⁺. Cooperative responses have also been observed in the presence of Ca²⁺. Weber and coworkers (6) found that at high S-1 concentrations the ATPase activity of regulated acto-S-1 can be potentiated so that it is higher than the ATPase activity of acto-S-1 in the absence of tropomyosin.

The cooperative responses observed with regulated actin are fundamental to our understanding of the biochemical basis of regulation. Up to the present time, there have not been any studies on the binding of S-1 to regulated actin in the absence of ATP. Equilibrium binding studies are generally easier to interpret than steady-state ATPase studies. Therefore, in the present study, using a method previously used in our studies on the binding of S-1-nucleotide complexes to unregulated F-actin (7), we investigated the binding of S-1-ADP to regulated F-actin both in the presence and in the absence of Ca²⁺. At ionic strength = 0.23 M at 22°C, although S-1-ADP binds independently to unregulated actin, it binds with positive cooperativity to regulated F-actin, both in the presence and absence of Ca²⁺. With and without Ca²⁺ at very low levels of occupancy of the regulated actin by S-1-ADP, S-1-ADP binds to the regulated actin with <1% of the strength that it binds to unregulated actin, whereas at high levels of occupancy of the regulated actin by S-1-ADP, S-1-ADP binds 3-fold more strongly to the regulated actin than it does to unregulated actin. Our results are consistent with a typical cooperative binding model (8–10) in which the regulated actin filament can exist in two forms, a weak-binding and a strong-binding form. Ca²⁺ and S-1-ADP, acting as allosteric effectors, shift the equilibrium toward the strong form.

MATERIALS AND METHODS

Rabbit skeletal myosin, S-1, and actin were prepared as described by Stein et al. (11). The troponin–tropomyosin complex was prepared according to Eisenberg and Kielley (12). The molecular weights used for S-1, actin, and troponin–tropomyosin complex were 120,000, 42,000, and 150,000, respectively. Protein concentrations were determined by UV absorption and the following extinction coefficients: 750 cm⁻²/g at 280 nm for S-1, 1150 cm⁻²/g at 280 nm for F-actin, and 380 cm⁻²/g at 278 nm for the troponin–tropomyosin complex. The rabbit myosin was labeled with iodo[¹⁴C]acetamide, as described (13), resulting in 1 ± 0.1 mol of iodoacetamide incorporated per mol of S-1.

Binding studies were conducted in the preparative centrifuge (Beckman L-2-65B). The SH₂-blocked S-1 and regulated actin in a total volume of 4 ml were stirred for several minutes and

Abbreviations: S-1, subfragment 1 of myosin; acto-S-1, complex of actin with S-1; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N',N' tetraacetic acid.
then allowed to remain for 30 min at 22°C before centrifugation. All binding studies were conducted at ionic strength = 0.23 M and 22°C in the presence of 2.5 mM ADP to saturate both the S-1 and acto-S-1 with ADP. Diadenosine pentaphosphate (5 mM) was added to inhibit myokinase activity. The order of addition of the proteins typically was actin, S-1, and then the troponin–tropomyosin complex. Aliquots (3-ml) were then centrifuged for 1 hr at 80,000 × g. An 0.5-ml aliquot removed prior to centrifugation and the supernatant after centrifugation were assayed for radioactivity in a Beckman LS-250 liquid scintillation counter to determine the total and free S-1 concentrations, respectively. In the absence of actin, >97% of the S-1 remained in the supernatant after centrifugation. Centrifugation of the actin alone showed that >96% sedimented, as determined by absorbance. The binding data were plotted by using the Scatchard equation (14) and the value of the slope for linear regions of the Scatchard plots was determined by linear regression analysis.

ADP and diadenosine pentaphosphate were from PL Biochemicals and iodo[14C]acetamidamide was from Amersham.

**RESULTS**

We first compared the binding of S-1-ADP to regulated and unregulated actin in the absence of Ca2+. using SH2-blocked S-1 modified with iodo[14C]acetamidamide. These studies were done at an ionic strength of 0.23 M to obtain detectable amounts of dissociation of acto-S-1 by ADP. The binding of S-1-ADP to unregulated actin gave a linear Scatchard plot (Fig. 1), confirming that there is no cooperativity in the binding of S-1-ADP to unregulated F-actin. In contrast, with regulated actin, the Scatchard plot shows a marked convex curvature, indicating that the binding of S-1-ADP is highly cooperative in the absence of Ca2+.

This cooperativity is shown more clearly in Fig. 1B where the fraction of actin saturated with S-1 is plotted as a function of free S-1 concentration. In contrast to the situation with unregulated actin, at a free S-1 concentration less than 1 μM there was almost no binding of S-1-ADP to regulated actin. Then, over a narrow range of S-1 concentration (1 to 1.5 μM), the fraction of regulated actin saturated with S-1 increased from about 0.05 to 0.5 which is about twice the level of saturation observed with unregulated actin at 1.5 μM S-1. Finally, above a free S-1 concentration of 1.5 μM, the binding of S-1 to regulated actin no longer was cooperative but was about 3 times stronger than the binding of S-1 to unregulated actin. This can best be seen by comparing the slopes of the Scatchard plots for regulated and unregulated actin at θ > 0.5 in Fig. 1A.

In general, for cooperative binding systems, noncooperative regions of binding occur at both very low and very high levels of saturation and, therefore, binding constants can be obtained for these regions. The binding constant of S-1 to regulated actin at high levels of saturation can be obtained from the slope of the Scatchard plot at θ > 0.5. This binding constant has a value of 7 × 10⁵ M⁻¹, about 3 times that obtained with unregulated actin, 2 × 10⁵ M⁻¹.

To obtain an accurate binding constant at very low levels of saturation, binding experiments were conducted at a higher actin concentration (56 μM). Much less S-1 bound to regulated actin than to unregulated actin at low free S-1 concentration (Table 1). From the concentrations of unbound S-1, bound S-1, and regulated actin, a value of 10⁹ M⁻¹ was calculated for the binding constant of S-1-ADP to regulated actin at very low levels of saturation in the absence of Ca²⁺. However, because only a small amount of S-1 bound to the regulated actin, this value is probably an upper limit; the binding constant could be even weaker than 10⁹ M⁻¹.

To make certain that the cooperative binding of S-1 to regulated actin is a real effect, several control experiments were carried out (Table 2). First, the amount of dissociated S-1 was not significantly different at 1.2 and 2.5 mM ADP. Therefore, at 2.5 mM ADP both the S-1 and acto-S-1 are saturated with ADP. Second, our results were unaffected by a 5-fold increase in the concentration of the troponin–tropomyosin complex, which shows that the actin was saturated with the troponin–tropomyosin complex. Third, to test whether the system was in equilibrium, the order of addition of S-1, troponin–tropomyosin, and EGTA was varied. The 55% of the added S-1

![Fig. 1. Binding of S-1-ADP to regulated (♦) and unregulated (△) F-actin in the absence of Ca²⁺. Varying amounts of SH2-blocked S-1 were added to a fixed concentration of either unregulated or regulated actin. Conditions were 0.2 M KCl, 12 mM imidazole, 5 mM MgCl₂, 5 mM KPi, 2.5 mM ADP, 0.5 mM dithiothreitol, 3 μM diadenosine pentaphosphate, and 1 mM ethylene glycol bis(β-aminoethyl ether)-N,N',N″,N‴-tetraacetic acid (EGTA) at pH 7.0 and 22°C. In the binding study using regulated actin, 0.4–12.2 μM SH2-blocked S-1 was added to 5.5 μM F-actin and 1.6 μM troponin–tropomyosin complex. In the binding study using unregulated actin, 1.0–28.5 μM SH2-blocked S-1 was added to 5.5 μM F-actin. (A) Data are plotted according to the Scatchard equation. (B) Same data are plotted as fraction of actin saturated with S-1 (θ) vs. free S-1 for values of θ obtained at free S-1 ≤ 5 μM. θ is mol of S-1 bound per mol of F-actin monomer.](image)

<table>
<thead>
<tr>
<th>Total S-1, μM</th>
<th>Bound S-1, μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regulated actin</td>
<td>0.29</td>
</tr>
<tr>
<td>+ 1 mM EGTA</td>
<td>0.65</td>
</tr>
<tr>
<td>+ 0.5 mM CaCl₂</td>
<td>0.91</td>
</tr>
<tr>
<td>Unregulated actin</td>
<td>0.29</td>
</tr>
<tr>
<td>+ 0.5 mM KPi</td>
<td>0.62</td>
</tr>
<tr>
<td>+ 3 mM ADP, and 3 μM diadenosine pen- taphosphate</td>
<td>0.94</td>
</tr>
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</table>

| Conditions were: 0.2 M KCl, 12 mM imidazole, 5 mM MgCl₂, 5 mM KPi, 0.5 mM dithiothreitol, 3 mM ADP, and 3 μM diadenosine pentaphosphate at 22°C and pH 7.0. The actin concentration was 56 μM. In the experiments using regulated actin, the troponin–tropomyosin complex was at 16 μM. |
Table 2. Factors affecting the binding of S-1-ADP to regulated actin

<table>
<thead>
<tr>
<th>Condition</th>
<th>% S-1 bound</th>
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<tbody>
<tr>
<td>ADP at 1.2 mM</td>
<td>10</td>
</tr>
<tr>
<td>ADP at 2.5 mM</td>
<td>8</td>
</tr>
<tr>
<td>Troponin–tropomyosin complex at 2.2 μM</td>
<td>4</td>
</tr>
<tr>
<td>Troponin–tropomyosin complex at 11 μM</td>
<td>4</td>
</tr>
<tr>
<td>Actin + S-1</td>
<td>55</td>
</tr>
<tr>
<td>Actin + troponin–tropomyosin, then S-1 added</td>
<td>8</td>
</tr>
<tr>
<td>Actin + S-1, then troponin–tropomyosin added</td>
<td>8</td>
</tr>
<tr>
<td>CaCl2 at 0.125 mM, no added EGTA</td>
<td>55</td>
</tr>
<tr>
<td>CaCl2 at 0.125 mM, then 5 mM EGTA added*</td>
<td>5</td>
</tr>
</tbody>
</table>

Conditions were: 0.2 M KCl, 12 mM imidazole, 5 mM KPO4, 5 mM MgCl2, 0.5 mM diithiothreitol, and 3 μM diadenosine pentaphosphate at 22°C and pH 7.0. Free S-1 < 1.0 μM; actin = 6–8 μM; and troponin–tropomyosin complex ≈ 2 μM (except line 4). ADP was at 2.5 mM (except line 1) and EGTA was at 1 mM (except lines 8 and 9). *EGTA was added 10 min after the addition of CaCl2 (solution at 22°C); the mixture was centrifuged 30 min later.

that bound to unregulated F-actin was almost completely dissociated upon addition of the troponin–tropomyosin complex. Thus, the amount of S-1 bound decreased to the same low level (≈5%) as occurred when S-1 was added directly to the troponin–tropomyosin–actin complex. Similarly, the 55% of the added S-1 that bound to F-actin in the presence of Ca2+ (see below) was almost completely dissociated when EGTA was added. Therefore, the binding of the S-1 to regulated actin is reversible and the system appears to be in equilibrium. Finally, although SH2-blocked S-1 was used in these binding studies, the same results were obtained in preliminary experiments using unmodified S-1.

We next examined the binding of S-1-ADP to regulated actin in the presence of Ca2+. Because the regulated actomyosin ATPase activity is at a high level in the presence of Ca2+ both in vivo and in vitro (15), it was expected that the binding of S-1-ADP to regulated actin would not show cooperativity. However, contrary to expectations, the results obtained in the presence of Ca2+ were similar to the results obtained in the presence of EGTA; the convex Scatchard plot in Fig. 2A shows that the binding of S-1-ADP to regulated F-actin is quite cooperative. However, there are important differences between the binding in Ca2+ and EGTA. Comparison of Fig. 2B with Fig. 1B shows that the transition from weak to strong S-1 binding occurred at a lower free S-1 concentration with Ca2+ than with EGTA. In addition, the cooperative transition was not as steep as that obtained in the presence of EGTA. Nevertheless, the binding in the presence of Ca2+ clearly was cooperative.

As in the presence of EGTA, binding constants can be obtained for the noncooperative regions at very low and very high levels of saturation of actin with S-1-ADP. At high levels of occupancy of the regulated actin by S-1, the value of the binding constant obtained from the slope of the Scatchard plot is 6 × 105 M−1. This is almost identical to the value obtained in the presence of EGTA at high levels of occupancy.

Because the cooperative transition occurs at such a low free S-1 concentration in the presence of Ca2+, only an approximate value could be obtained for the upper limit of the binding constant of S-1-ADP to actin at very low levels of occupancy. As with EGTA, this binding constant was estimated at a regulated actin concentration of 56 μM. With 0.29 μM S-1 added, the value of the binding constant is about 109 M−1 (Table 1).

At higher added S-1 concentration, much more S-1 is bound, probably because there is cooperativity at these S-1 concentrations. Therefore, both in the presence and absence of Ca2+, S-1-ADP binds to regulated actin with 1% of the strength that it binds to unregulated actin at very low levels of occupancy of the actin sites by S-1 and about 3-fold more strongly at high levels of occupancy.

**DISCUSSION**

The results presented in this paper show that the binding of the S-1-ADP complex to regulated F-actin is a highly cooperative phenomenon. Because cooperative binding occurs both in the presence and absence of Ca2+, it seems reasonable to use as a framework for analyzing our data one of the classic allosteric models for cooperative binding (8–10). Monod et al. (10) have applied such a cooperative model to the regulatory properties of allosteric enzymes. We use their simple formulation, which is a special case of Hill’s treatment (8, 9), to present the basic elements of a cooperative model that accounts for our data.

Both in the presence and absence of Ca2+, each cooperative unit along the regulated actin filament is assumed to occur in two forms, a “weak-binding” and a “strong-binding” form. The equilibrium constant L between the two forms of the cooperative unit is defined as $L = [\text{weak form}] / [\text{strong form}]$. When the cooperative unit is in the weak form, each of the actin monomers in the cooperative unit is assumed to bind S-1 with an association constant $K_w$, while the cooperative unit is in the strong form, each of the monomers is assumed to bind S-1 with an association constant $K_s$. In agreement with the model of Monod et al., the values of $K_w$ and $K_s$ are assumed to be independent of the amount of S-1 bound to the F-actin. On this basis, we have the following model:

\begin{align*}
A_n & \leftrightarrow \overset{\text{A}_{n}^{w}}{\text{A}_{n}^{w}} \cdot \text{M}_1 \leftrightarrow \overset{\text{A}_{n}^{w}}{\text{A}_{n}^{w}} \cdot \text{M}_2 \leftrightarrow \cdots \leftrightarrow \overset{\text{A}_{n}^{w}}{\text{A}_{n}^{w}} \cdot \text{M}_{n-1} \leftrightarrow \overset{\text{A}_{n}^{w}}{\text{A}_{n}^{w}} \cdot \text{M}_n \\
\text{L} & \leftrightarrow \overset{\text{A}_{n}^{s}}{\text{A}_{n}^{s}} \cdot \text{M}_1 \leftrightarrow \overset{\text{A}_{n}^{s}}{\text{A}_{n}^{s}} \cdot \text{M}_2 \leftrightarrow \cdots \leftrightarrow \overset{\text{A}_{n}^{s}}{\text{A}_{n}^{s}} \cdot \text{M}_{n-1} \leftrightarrow \overset{\text{A}_{n}^{s}}{\text{A}_{n}^{s}} \cdot \text{M}_n
\end{align*}
in which $A_n^w$ and $A_n^s$ are cooperative units in the weak and strong forms, respectively, $M$ is $S-1$, and $n$ is the number of actin monomers in a cooperative unit.

In applying this model to our data, $n$ was set equal to 7 because one tropomyosin molecule binds to seven actin monomers. $K_w$ was taken to be $10^9$ M$^{-1}$ and $K_s$ $7 \times 10^9$ M$^{-1}$ in both the presence and absence of Ca$^{2+}$ (Figs. 1 and 2, Table 1). The data were then fitted to the above model by varying the value of $L$. As shown in Fig. 3 (a replot of the data in Figs. 1B and 2B), the data obtained in the presence of Ca$^{2+}$ can be fitted reasonably well to a theoretical plot with $L = 7$ (the solid line). However, we could not duplicate the high degree of cooperativity observed in the absence of Ca$^{2+}$ with any value of $L$. The best fit we could obtain is shown by the dashed line in Fig. 3, which is the theoretical plot with $L = 60$. Although it was assumed in our modeling that $K_w = 10^9$ M$^{-1}$, a decrease in this association constant would not significantly affect the theoretical plots. A change in $K_s$ (e.g., 10%) would affect the shape of the theoretical plots, but it would still not be possible to fit the data obtained in the absence of Ca$^{2+}$. It is possible to fit these highly cooperative data if we use a value of $n$ much greater than 7. However, there is no physical basis for using a larger value of $n$ because an indefinite array of tropomyosin molecules along the actin filament could be cooperatively linked.

A more realistic (and somewhat more complex) cooperative model in which the interaction between adjacent tropomyosin molecules is taken into consideration has recently been developed by Hill et al. (16). It is essentially identical with the simple cooperative model presented above except that a function has been added which allows for variable interaction between adjacent tropomyosin molecules. By making this interaction stronger in the absence than in the presence of Ca$^{2+}$, it is possible to obtain an excellent fit to our data, in particular the high level of cooperativity observed in the absence of Ca$^{2+}$.

Therefore, the basic concept that, both in the presence and absence of Ca$^{2+}$, the regulated actin filament can occur in either a weak form or a strong form appears to be a reasonable way to account for the cooperative data presented in this paper.

It is of interest to consider the implications of our data for the mechanism of skeletal muscle relaxation. Our data show that the troponin–tropomyosin complex blocks the binding of S-1-ADP to regulated actin in the weak form. It might then be expected, on the basis of the steric blocking model (1–3), that the myosin cross-bridge with bound ATP or ADP + P$_i$ would also be prevented from binding to regulated actin in the weak form but would bind quite well to regulated actin in the strong form. However, by using stopped-flow turbidity measurements (11) to determine the binding of S-1 to regulated actin in the presence of ATP, Chalovich et al. (17) found that troponin–tropomyosin has very little effect on the binding of S-1-ATP or S-1-ADP-P$_i$ to actin, in either the presence or absence of Ca$^{2+}$. This suggests that the troponin–tropomyosin complex may cause relaxation not by blocking the binding of S-1-ATP or S-1-ADP-P$_i$ to actin but rather by inhibiting the release of P$_i$ from the acto-S-1-ADP-P$_i$ complex.

The inhibition of P$_i$ release and the weakening of S-1-ADP binding to actin by the troponin–tropomyosin complex may have a common origin: an increase in the free energy (decrease in stability) of the acto-S-1-ADP complex when the actin filament is in the weak form. An increase in the free energy of acto-S-1-ADP relative to acto-S-1-ADP-P$_i$ would occur if the rate constant for P$_i$ release decreased without a corresponding decrease in the rate constant for P$_i$ binding. At the same time, an increase in the free energy of acto-S-1-ADP relative to S-1-ADP would decrease the association constant of S-1-ADP to actin.

On this basis, weak binding of S-1-ADP to regulated actin in the weak form would always be linked to an inability of the weak form to activate the S-1-ATPase.

The major advantage of this model is that the properties of the weak form are the same in the presence and absence of Ca$^{2+}$; Ca$^{2+}$ acts only as an allosteric effector, shifting the equilibrium between the weak and strong forms. The weak and strong forms are synonymous in this model with the "relaxed" and "active" states observed in vitro. Unfortunately, there are data that may not be compatible with this simple model. It may be necessary to assume that Ca$^{2+}$ actually affects the properties of the weak form so that, in the presence of Ca$^{2+}$, regulated actin in the weak form is able to activate the S-1-ATPase activity. The major finding that suggests a need for this more complex approach is that considerable ATPase activity is observed in the presence of Ca$^{2+}$, even at low ratios of S-1 to actin where our data suggest that most of the actin remains in the weak form. In addition, the x-ray diffraction studies by Haselgrove (2) suggest that, when the actin and myosin filaments are stretched out of overlap, the binding of Ca$^{2+}$ to troponin shifts the position of the tropomyosin on the actin filament from the "relaxed" to the "active" position, although again our data suggest that under these conditions much of the actin remains in the weak form. Of course, it is possible that the binding of Ca$^{2+}$ shifts enough of the regulated actin into the strong form to account for these x-ray diffraction results and for the increased ATPase activity that occurs in the presence of Ca$^{2+}$. However, if this turns out not to be the case, the most likely explanation for these data is that Ca$^{2+}$ can induce a conformational change in the weak form which causes a change in the x-ray diffraction pattern and allows the actin, still in the weak form, to activate the S-1-ATPase activity.

Whether or not Ca$^{2+}$ affects the kinetic properties of the weak form, it does appear that regulated actin must be in the weak form in order for relaxation to occur. At low ATP concentration, when S-1 or S-1-ADP binds to actin, removal of Ca$^{2+}$ does not cause inhibition in vitro (5) or relaxation in vitro (18, 19). Based on our results, it seems likely that this is because at low ATP concentration, even in the absence of Ca$^{2+}$, most of the actin occurs in the strong form, shifted from the weak form by the binding of the allosteric effector S-1-ADP. It therefore
appears that the weak form of regulated actin is necessary for relaxation to occur.

We thank Mr. Louis Dobkin for technical assistance.