Inhibition of Actomyosin ATPase Activity by Troponin-Tropomyosin without Blocking the Binding of Myosin to Actin

Joseph M. Chalovich and Evan Eisenberg
From the Laboratory of Cell Biology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20205

Abstract

Relaxation of vertebrate skeletal muscle is thought to occur in the absence of Ca\(^{2+}\) as a result of tropomyosin physically blocking the binding of myosin to actin. This steric blocking model of muscle relaxation predicts that myosin subfragment 1 (S-1) will not bind to actin under conditions where the acto-S-1 ATPase rate is inhibited. Using stopped flow absorbance as a measure of binding, we have previously shown that when the rate of ATP hydrolysis is only 4% of the rate in the presence of Ca\(^{2+}\), S-1·ATP and S-1·ADP·Pi bind to actin-troponin-tropomyosin (regulated actin) with almost the same affinity as in the presence of Ca\(^{2+}\). This result has now been confirmed using sedimentation in an air-driven ultracentrifuge to directly measure the binding at pH 7.0, 25 °C, and \(\mu = 18\) m\(\text{M}\). In the presence of Ca\(^{2+}\), the rate of ATP hydrolysis is more than 20 times greater than in the absence of Ca\(^{2+}\). In contrast, the association constant of S-1·ATP and S-1·ADP·Pi with regulated actin is virtually the same in the absence of Ca\(^{2+}\) (1.4 \times 10^4 \text{M}^{-1}) as in the presence of Ca\(^{2+}\) (1.5 \times 10^4 \text{M}^{-1}). Similarly, at 50 m\(\text{M}\) ionic strength, the ATPase rate is inhibited about 98% in the absence of Ca\(^{2+}\) although the association constant is not significantly changed compared to that in the presence of Ca\(^{2+}\). Finally, it has been shown that, at 18 m\(\text{M}\) ionic strength, the inhibition of the actin-activated ATPase rate in the absence of Ca\(^{2+}\) is due to a large decrease in the maximum ATPase rate (to 4% of the Ca\(^{2+}\) value) with only a small change in the apparent binding constant of S-1 to actin. These data do not support a simple steric blocking model of muscle relaxation. Rather they suggest that, in the absence of Ca\(^{2+}\), troponin-tropomyosin inhibits a kinetic step, perhaps Pi release, in the cycle of ATP hydrolysis.

Vertebrate skeletal muscle contraction is the result of a cyclic interaction of thick myosin filaments with the thin filaments, composed primarily of actin, troponin, and tropomyosin, causing these two sets of filaments to slide past each other (2,3). This cycling is driven by the hydrolysis of ATP by myosin in a reaction which is activated by actin. When the sarcoplasmic reticulum lowers the free Ca\(^{2+}\) concentration from \(10^{-5}\) to \(<10^{-7}\) \text{M}\, muscle contraction ceases and the associated actin-activated myosin ATPase activity is inhibited. The proteins troponin and tropomyosin are responsible for this effect of Ca\(^{2+}\) on the interaction between myosin and actin (4,5).

The tropomyosin molecules lie end to end along the two grooves of the F-actin filament with each tropomyosin molecule binding to seven actin monomers (6). Troponin consists of three subunits, and one troponin molecule is bound to each tropomyosin molecule. The binding of Ca\(^{2+}\) to troponin determines the position of tropomyosin on the F-actin filament. At levels of Ca\(^{2+}\) low enough to cause relaxation, tropomyosin is positioned away from the central groove of the F-actin filament where it appears that it might interfere with the binding of the myosin cross-bridge (7–9). This structural work formed the basis of the steric blocking hypothesis which suggests that relaxation occurs when tropomyosin, in the “relaxed” position, physically

---

*A preliminary report of this work has been presented at the Biophysical Society Meeting, 1981 (February 23, 1981, Denver) (1).*
blocks the binding of the myosin cross-bridge to actin. Three-dimensional reconstructions from electron micrographs have suggested that the myosin cross-bridge and the tropomyosin molecule may be in close contact with each other on the actin filament, a requirement for a steric blocking type model (10,11).

The steric blocking model predicts that in the absence of Ca\(^{2+}\) the degree of association of S-1\(^1\) should be much weaker with regulated actin than with unregulated actin. In fact, Greene and Eisenberg (12) have recently demonstrated that, in the absence of Ca\(^{2+}\), the binding of S-1·ADP to regulated actin is strongly inhibited in a cooperative manner. At low levels of saturation of the actin filament with S-1·ADP, the binding of S-1·ADP to the regulated actin filament is about 10\(^3\) weaker than at high levels of saturation. However, the fact that S-1·ADP binds weakly to regulated actin does not prove the steric blocking model since, in relaxed muscle, the cross-bridges normally exist with bound ATP (or ADP·P\(_i\)) and not bound ADP (13,14). Therefore, the steric blocking model predicts that troponin-tropomyosin should inhibit the binding of S-1·ADP·P\(_i\) as well as S-1·ADP to regulated actin in the absence of Ca\(^{2+}\).

Using stopped flow turbidity measurements, we have previously measured the effect of Ca\(^{2+}\) on the association of S-1 · ATP and S-1 · ADP · P\(_i\) with regulated actin (15). Surprisingly, in the absence of Ca\(^{2+}\), the binding constant of S-1 · ATP or S-1 · ADP · P\(_i\) to regulated actin was only decreased to 56% of the value in the presence of Ca\(^{2+}\) although the rate of ATP hydrolysis under the same conditions was decreased to 6% of the rate with Ca\(^{2+}\) present. These data suggest, in disagreement with the steric blocking model, that inhibition of the rate of ATP hydrolysis, in the absence of Ca\(^{2+}\), is not the result of inhibition of the binding of S-1 to regulated actin.

In the present study, we have reinvestigated this problem using a different and more direct measurement of binding. Free S-1 was separated from actin-bound S-1 in the presence of ATP by rapid centrifugation in an air-driven ultracentrifuge and the free S-1 concentration was then determined by an ATPase assay. As in our earlier turbidity studies, we find very little effect of troponin-tropomyosin on the binding of S-1 to regulated actin in the presence of ATP. Similar confirmation of our turbidity results has already been reported by Wagner and Giniger (16). In the present study, we have also measured the binding at a higher ionic strength (50 mM) and here too have found no correlation between the inhibition of ATPase rate and the binding of S-1·ATP or S-1·ADP·P\(_i\) to regulated actin. Finally, we demonstrate that the removal of Ca\(^{2+}\) affects the rate of regulated actin-activated S-1-ATPase activity primarily by lowering the maximum ATPase rate (\(V_{\text{max}}\)) rather than the apparent binding constant of S-1 to actin (\(K_{\text{ATPase}}\)). These data imply that, in the absence of Ca\(^{2+}\), troponin-tropomyosin inhibits the ATPase activity by inhibiting a kinetic step in the cycle of ATP hydrolysis, perhaps P\(_i\) release.

MATERIALS AND METHODS

Proteins

Rabbit skeletal myosin, S-1, and actin were prepared as described by Stein et al. (17). Troponin-tropomyosin was prepared according to Eisenberg and Kielley (18). All protein solutions containing S-1 or troponin-tropomyosin also contained 1 mM dithiothreitol. The molecular weights used for actin, S-1, and native tropomyosin were 42,000, 120,000, and 150,000, respectively. Protein concentrations were determined by the Lowry method (19) or spectrophotometrically using the following absorption coefficients: 1150 cm\(^2\)/g at 280 nm for F-actin, 750 cm\(^2\)/g at 280 nm for S-1, and 380 cm\(^2\)/g at 278 nm for troponin-tropomyosin.

\(^1\)The abbreviations used are: S-1, myosin subfragment 1; EGTA, ethylene glycol bis(\(\beta\)-aminoethyl ether) N,N',N'-tetraacetic acid.

\(J\) Biol Chem. Author manuscript; available in PMC 2005 October 26.
Regulated actin was prepared by mixing actin with a 50% excess (1.5 mol/7 mol of actin) of native tropomyosin and dialyzing overnight at 4 °C against 3 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM EGTA, 10 mM imidazole, pH 7.0.

**Actin-activated ATPase Assays**

Actin-activated S-1 ATPase activities were measured at 25 °C both by measuring the rate of liberation of $[^{32}\text{P}]P_i$ from $[^{\gamma-32}\text{P}]\text{ATP}$ (20) and by the pH-stat method (21). Assays based on $[^{32}\text{P}]P_i$ were carried out in 1.5 ml of solution containing 1 mM ATP, 3 mM MgCl₂, 1 mM EGTA (or 0.5 mM CaCl₂), 10 mM imidazole, pH 7.0, with or without 32 mM KCl, giving 18 or 50 mM ionic strength, respectively. A single assay usually consisted of four or five time points. Measurements with the pH-stat were performed in a 3.5-ml solution of similar composition except that 10 mM imidazole was replaced by 2 mM imidazole and 4 mM KCl.

**Binding Assay**

Measurement of binding of S-1 to actin in the presence of ATP is made difficult by the rapid hydrolysis of ATP by S-1, especially in the presence of Ca²⁺ or in the absence of regulatory proteins. In an earlier paper, we had circumvented this problem by using stopped flow absorbance measurements which were completed prior to appreciable ATP hydrolysis (15). In the present study, we have directly measured the association of S-1 with actin by rapidly sedimenting the actin-bound S-1 and measuring the concentration of free S-1 in the supernatant by the NH₄⁺-EDTA ATPase assay.

Binding of S-1 to actin or regulated actin at 18 mM ionic strength was measured at 25 °C in 1 mM ATP, 3 mM MgCl₂, 1 mM EGTA or 0.5 mM CaCl₂, 10 mM imidazole, pH 7.0, in a total volume of 1.5 ml. At 50 mM ionic strength, ATP was increased to 2 mM, MgCl₂ was increased to 4 mM and 27 mM KCl was included. The binding mixture, excluding S-1, was equilibrated with stirring in a thermostatted water jacket. S-1 was added in a volume of 50–100 μl and stirred briefly. Fractions of 0.2 ml were centrifuged in a Beckman Airfuge at 178,000 × g for 20 min. The final sample temperature was 25 ± 1 °C. The supernatant was assayed for free S-1 concentration by the NH₄⁺-EDTA ATPase assay. The total time from the addition of S-1 to the binding mixture until the start of centrifugation was less than 3 min.

The NH₄⁺-EDTA ATPase activity of S-1 was measured at 25 °C by the release of $[^{32}\text{P}]P_i$ from $[^{\gamma-32}\text{P}]\text{ATP}$ in a solution containing 5 mM ATP, 0.4 mM NH₄Cl, 35 mM EDTA (Tris salt), 25 mM Tris, pH 8.0. This condition is similar to that used by Seidel (22) except for a large increase in the EDTA concentration for chelation of 1 mM Mg²⁺ which was present in the supernatant from the binding studies. This assay was linear for at least the first 25% of the reaction and was directly proportional to the S-1 concentration over the entire range investigated from 0.004 to 0.23 μM S-1 (Fig. 1). Note that for clarity several points in the range 0.004 to 0.02 μM S-1 have been omitted from Fig. 1. Addition of 8 μM actin to the assay had no effect on the rates. This is roughly five times the maximum amount of actin expected to be carried over from the binding studies.

The sedimentation time of actin was estimated by measuring the concentration of actin remaining in the supernatant, by the Lowry protein determination, after various times of centrifugation. Under our conditions, 75% of the actin was sedimented at 10 min, 95% at 15 min, and 98% at 20 min. We have observed no detectable sedimentation of 1 μM S-1 in the absence of actin after a 20-min centrifugation, as determined by NH₄⁺-ATPase activity, although at 0.06 μM S-1 up to 10% of the ATPase activity was lost, probably due to slight denaturation. To avoid this problem, control experiments measuring sedimentation of S-1 in the absence of actin were carried out in the presence of either troponin-tropomyosin or 2 mg/ml bovine serum albumin.

*J Biol Chem.* Author manuscript; available in PMC 2005 October 26.
The S-1 concentrations used for the binding assays were 1 \( \mu M \) in the presence of EGTA and 0.06 \( \mu M \) in the presence of Ca\(^{2+}\). The concentration of S-1 was limited in each case by the amount of ATP hydrolysis which occurred during the centrifugation. As shown in the first three lines of Table I, these concentrations of S-1 were sufficiently low that a large fraction of the ATP remained intact at the end of the binding assay. It was particularly important in this study to avoid overestimation of the binding in the presence of EGTA. This was further tested by measuring the binding at S-1 concentrations different from the 1 \( \mu M \) typically used (Table 1). Decreasing the S-1 concentration 4-fold from 1.1 to 0.23 \( \mu M \) had virtually no effect on the fraction bound. Increasing the concentration 3-fold significantly increased the fraction of S-1 bound as a result of excessive ATP utilization. This can be compared to the case in the absence of ATP where all of the S-1 is bound to actin. Therefore, it is likely that the binding that we are measuring at 1 \( \mu M \) S-1 concentration, in the presence of ATP, represents the binding of the S-1 substrate states S-1·ATP and S-1·ADP·P\(_i\) to actin.

RESULTS

We first examined the relationship between the regulated actin-activated ATPase rate and the fraction of S-1 bound to regulated actin at 18 m\( M \) ionic strength, the same condition used in our earlier work. Fig. 2A is a direct plot of the actin-activated S-1 ATPase activity, in the presence of excess native tropomyosin, as a function of the concentration of regulated actin. All of the rates have been corrected for the rate of ATP hydrolysis by S-1 alone (0.09 s\(^{-1}\)). Both in the presence and absence of Ca\(^{2+}\), the ATPase rates increase with regulated actin concentration although the magnitudes of the rates are much greater in the presence of Ca\(^{2+}\) than in the absence of Ca\(^{2+}\). The ATPase rates for regulated actin estimated from the time to maximum turbidity rise on the stopped flow (15) are roughly twice the values reported here. This is probably because the stopped flow values are averaged over the entire reaction time although the rate of ATP hydrolysis increases with time as the ATP concentration becomes low (23). In addition, the stopped flow studies used a much higher concentration of S-1 (20–40 \( \mu M \)) which may have partially “turned on” the actin filament (24).

Fig. 2B shows the fraction of S-1 bound to regulated actin, under the same conditions used for the steady state ATPase measurement, as a function of the free regulated actin concentration. In contrast to the ATPase activity which was greatly affected by Ca\(^{2+}\), the degree of association of S-1 with regulated actin is virtually the same in the presence and absence of Ca\(^{2+}\). Therefore, with ATP present in the absence of Ca\(^{2+}\), the troponin-tropomyosin complex inhibits actin activation of the S-1 ATPase rate without blocking the binding of S-1 to actin.

Binding constants for the association of S-1 to unregulated or regulated actin were determined from double reciprocal plots of the fraction of S-bound versus free actin concentration (Fig. 3). The best fits to the binding data were determined using the Marquardt compromise (25). Fig. 3A shows that the binding constant of S-1 to pure actin, in the presence of ATP, is 2.1 \( \times \) 10\(^4\) M\(^{-1}\). This agrees well with the value of 2.3 \( \times \) 10\(^4\) M\(^{-1}\) determined earlier by stopped flow turbidity (15). Similarly, in Fig. 3B, the binding constant of S-1 to regulated actin in the presence of Ca\(^{2+}\) is 1.5 \( \times \) 10\(^4\) M\(^{-1}\) which agrees well with the earlier value of 2.3 \( \times \) 10\(^4\) M\(^{-1}\). The binding constant of S-1 to regulated actin in the absence of Ca\(^{2+}\) is 1.4 \( \times \) 10\(^4\) M\(^{-1}\) which agrees well with the earlier value of 2.3 \( \times \) 10\(^4\) M\(^{-1}\). The binding constant of S-1 to regulated actin in the presence of Ca\(^{2+}\) is 1.4 \( \times \) 10\(^4\) M\(^{-1}\) compared to the stopped flow turbidity value of 1.3 \( \times \) 10\(^4\) M\(^{-1}\). In short, both methods agree that, in the presence of ATP, S-1 binds to actin with about the same affinity whether or not native tropomyosin is present and whether or not Ca\(^{2+}\) is present. Both methods also show that at 18 m\( M \) ionic strength in the absence of Ca\(^{2+}\) the inhibition of the actin-activated S-1 ATPase rate by native tropomyosin is not the result of inhibition of binding of S-1 to actin.

We next repeated these experiments at 50 m\( M \) ionic strength to determine if there was a similar lack of correlation of ATPase rate with the fraction of S-1 bound at a higher ionic strength.
4 shows that, at 50 mM ionic strength, native tropomyosin remains an effective inhibitor of the actin-activated ATPase rate in the absence of Ca\textsuperscript{2+}. The rate in the presence of EGTA is generally 2% of the rate in the presence of Ca\textsuperscript{2+} over our working range. All rates were corrected for the rate of ATP hydrolysis by S-1 alone (0.1 s\textsuperscript{-1}).

The association of S-1 with regulated actin in the presence of ATP at 50 mM ionic strength is shown in Fig. 5. Binding at 50 mM ionic strength was measured with 2 mM Mg-ATP rather than 1 mM Mg-ATP to compensate for a higher rate of ATP hydrolysis in the presence of Ca\textsuperscript{2+} at 50 mM ionic strength than at 18 mM ionic strength (compare Figs. 2 and 4). The association constant in the presence of Ca\textsuperscript{2+} is roughly \(3 \times 10^3 \text{ M}^{-1}\) which is similar to the value of \(2 \times 10^3 \text{ M}^{-1}\) obtained in the absence of Ca\textsuperscript{2+}. In the absence of native tropomyosin, the binding constant is also about \(3 \times 10^3 \text{ M}^{-1}\) (data not shown). These values of the binding constants could be slightly in error because it was not possible to work at a higher actin concentration where more binding occurs. Nevertheless, it is clear from Fig. 5 that the situation at 50 mM ionic strength is analogous to that at 18 mM ionic strength; although native tropomyosin greatly inhibits the actin-activated ATPase rate in the absence of Ca\textsuperscript{2+}, it has little effect on the degree of association of S-1 to actin in the presence of ATP.

Our binding studies suggest that, in the absence of Ca\textsuperscript{2+}, native tropomyosin inhibits the actin-activated S-1 ATPase rate by affecting a rate process rather than the binding of S-1 to actin at a saturating ATP concentration. On this basis, it might be expected that the effect of troponin-tropomyosin on the double reciprocal plot of ATPase versus actin would be to change \(V_{\text{max}}\) rather than \(K_{\text{ATPase}}\). In contrast to this prediction, several previous studies (one from our laboratory) suggested that the effect of troponin-tropomyosin was on \(K_{\text{ATPase}}\) rather than \(V_{\text{max}}\) (26,27). However, these studies were carried out at relatively low actin concentrations and if, as is often the case, \(K_{\text{ATPase}}\) were stronger than \(K_{\text{binding}}\), the actin-activated ATPase rate might be quite high, although very little binding of S-1 to actin was occurring during steady-state ATP hydrolysis. Therefore, we reinvestigated the effect of troponin-tropomyosin on the double reciprocal plot under conditions where there is considerable binding of S-1 to regulated actin in the presence of ATP.

The open symbols in Fig. 6 show that, as the ratio of troponin-tropomyosin to actin is increased, there is marked inhibition of the ATPase activity in the presence of EGTA. When the molar ratio of troponin-tropomyosin to actin is increased from 0 to 1.5/7, \(K_{\text{ATPase}}\) increases 3-fold (from 20 to 60 \(\mu\text{M}\)) but the major change is in \(V_{\text{max}}\) which decreases to 4% of the initial value (from 22 to 0.8 s\textsuperscript{-1}). Increasing the ratio of troponin-tropomyosin to actin from 1.5/7 to 4.7 resulted in no further inhibition. The addition of Ca\textsuperscript{2+} to the actin fully saturated with troponin-tropomyosin (solid symbols) yields a double reciprocal plot very similar to the plot obtained in the absence of troponin-tropomyosin. Compared to the plot obtained in the absence of Ca\textsuperscript{2+}, \(V_{\text{max}}\) increases 22-fold (from 0.8 to 18 s\textsuperscript{-1}) and \(K_{\text{ATPase}}\) becomes about 1.5-fold stronger (60 to 40 \(\mu\text{M}\)). Therefore, the inhibition of the actin-activated ATPase activity caused by troponin-tropomyosin in the absence of Ca\textsuperscript{2+} is mainly due to an effect on \(V_{\text{max}}\) with a much smaller effect on \(K_{\text{ATPase}}\).

**DISCUSSION**

The results of this study suggest that inhibition of the actin-activated S-1 ATP hydrolysis by native tropomyosin, in the absence of Ca\textsuperscript{2+}, is not due to a steric blocking of the association of S-1 to actin. We have found, in agreement with our earlier work (15), that, in the presence of ATP, the affinity of S-1 for regulated actin is relatively Ca\textsuperscript{2+} insensitive. In contrast, the ATPase rate in the absence of Ca\textsuperscript{2+} is decreased to about 4% of the rate in the presence of Ca\textsuperscript{2+}. The lack of correlation between binding in the presence of ATP and the ATPase rate occurs both at low (18 mM) and moderate (50 mM) ionic strengths. Since the regulatory proteins...
troponin and tropomyosin have little effect on the binding of S-1 to actin in the presence of ATP, it is likely that these regulatory proteins affect a rate process in the pathway of ATP hydrolysis. This conclusion is supported by our steady state kinetic measurements. Fig. 6 shows that, for the most part, native tropomyosin acts as a noncompetitive inhibitor of ATP hydrolysis in the absence of Ca$^{2+}$. These kinetic results strongly indicate that native tropomyosin and S-1-ATP do not compete for the same site on actin. Inhibition of the ATPase rate is primarily the result of a large decrease in the maximum velocity which suggests that a kinetic step is being affected. In considering which kinetic step is affected by troponin-tropomyosin, we will refer to the model of Stein et al. (17) since it includes all of the proposed kinetic steps (Fig. 7).

The work of Greene et al. (12), confirmed by Trybus and Taylor (28) and Murray et al. (29), has shown that troponin-tropomyosin has a major effect on the binding of M and MD (for abbreviations, see kinetic model in Fig. 7) to actin. Troponin-tropomyosin greatly inhibits this binding at low ratios of M or MD to actin while at high ratios the binding becomes even stronger than in the absence of troponin-tropomyosin. Hill et al. (30) have modeled this cooperative response in terms of two states of the actin filament: a weak binding state and a strong binding state. Ca$^{2+}$ affects the binding of S-1 and S-1·ADP by changing the equilibrium constant between these two states.

Although the cooperative binding of S-1 induced by troponin-tropomyosin is of great interest, it cannot explain the ability of troponin-tropomyosin to inhibit the acto-S-1 ATPase activity; how strongly M or MD bind to actin will not directly affect the rate of ATP hydrolysis. Of course, if troponin-tropomyosin were to affect the binding constant of $M \cdot D \cdot P_i$ to actin ($K_{14}$), it would affect the actin-activated ATPase activity since $K_{ATPase} = K_{14}k_{-7}/(k_{-8} + k_{10})$ (17). However, this effect would manifest itself as an effect on $-K_{ATPase}$ not on $V_{max}$, i.e., increasing the actin concentration would overcome the inhibitory effect of the troponin-tropomyosin. In fact, Fig. 6 shows that the major effect of troponin-tropomyosin is on $V_{max}$. Therefore, it seems very unlikely that tropomyosin acts by weakening $K_{14}$. Our data clearly show that the binding constants of $MT$ ($K_{i}$) and $MDP_i$ ($K_{13}$) to actin are essentially unaffected by troponin-tropomyosin. Therefore, troponin-tropomyosin must be affecting a step other than the binding of one of the S-1 states to actin; it must be affecting a kinetic step which occurs when S-1 is attached to actin.

Troponin-tropomyosin cannot inhibit the acto-S-1 ATPase activity by affecting the rate of the ATP hydrolysis step or the rate of transition from the refractory to the nonrefractory state when the S-1 is bound to actin because these steps occur at almost the same rate when the S-1 is detached from actin (17,31). Since S-1-ATP and S-1-ADP-P$_i$ are in rapid equilibrium with acto-S-1. ATP and acto-S-1·ADP·P$_i$, respectively, inhibition of these steps would not inhibit the acto-S-1 ATPase activity at moderate actin concentrations. Release of ADP cannot be the rate-limiting step because then the major species present in the inhibited system would be MD which binds tightly to actin; we find that S-1 binds weakly to actin in the inhibited system. Therefore, it seems most likely that the step which is inhibited by troponin-tropomyosin is the conformational change associated with P$_i$ release from $A\cdot M\cdot D\cdot P_i$. P$_i$ release is, of course, very slow in the absence of actin. Strong inhibition of P$_i$ release, in the presence of actin, would greatly decrease the value of $V_{max}$ obtained from the double reciprocal plot of ATPase activity versus actin. At the same time, decreasing the rate of P$_i$ release ($k_{10}$) would make $K_{ATPase} = K_{14}k_{-7}/(k_{-8} + k_{10}) = K_{13}k_{-9}/(k_{-8} + k_{10})$ (17). This would explain why troponin-tropomyosin has a small effect on $K_{ATPase}$ as well as a large effect on $V_{max}$.

It is of interest to consider how the inhibitory effect of troponin-tropomyosin on P$_i$ release is related to its effect on the binding of S-1-ADP to actin. One of the most striking features of troponin-tropomyosin action is the marked difference between its effect on the binding of S-1·ATP (or S-1·ADP-P$_i$) to actin compared with its effect on S-1 and S-1·ADP binding.

*J Biol Chem.* Author manuscript; available in PMC 2005 October 26.
Eisenberg and Greene have suggested that the S-1 states with bound P_i (M·T and M·O·P_i) bind to actin at a ~90° angle while states without bound P_i (M·D and M) bind at a ~45° angle (13). This hypothesis provides a natural explanation for the differential effect of troponin-tropomyosin on the two groups of states. Troponin-tropomyosin would cooperatively inhibit the binding of states which attach at a 45° angle but would have no effect on states which bind at a 90° angle.

This hypothesis also provides a natural explanation for the inhibitory effect of troponin-tropomyosin on the rate of P_i release; the troponin-tropomyosin would interfere with the rotation of the S-1 from the 90° state to the 45° state and thus inhibit the release of P_i from the acto-S-1. Of course, if there were sufficient AMD complexes occurring along the regulated actin filament to push the troponin-tropomyosin complex over to the strong form, troponin-tropomyosin would no longer interfere with the rotation of the 90° state to the 45° state and thus P_i release would not be inhibited.

This hypothesis suggests that, although troponin-tropomyosin is not acting by sterically blocking the binding of S-1·ADP·P_i to actin, it may act, in a sense, by “sterically blocking” the rotation of S-1 and the associated P_i release. Of course, this interference of rotation of the S-1 would not be an all or none effect. Rather the activation energy associated with S-1 rotation would be increased because movement of the troponin-tropomyosin as well as S-1 would be required during P_i release. Therefore, the rate constant for P_i release would be decreased. On this basis, the role of Ca^{2+} might be either to partially shift the weak form of actin to the strong form or to shift the position of the tropomyosin on the actin so that rotation of the S-1 is not associated with as high an activation energy and thus the rate of P_i release is increased. We therefore conclude from our data that troponin-tropomyosin does not sterically block the binding of S-1·ATP and S-1·ADP·P_i to actin. However, it may interfere with the rotation of the S-1 on the actin, thus increasing the activation energy for this step and decreasing the rate of P_i release. This interference may be a direct steric effect or an indirect conformational change in the actin.

Our finding that Ca^{2+} has little effect on the binding of S-1·ADP or S-1·ADP·P_i to regulated actin in vitro opens up the possibility that some fraction of the myosin cross-bridges is attached to actin in relaxed muscle. In fact, three possibilities arise. First, cross-bridges in state M·T or M·D·P_i may not be attached to actin in either active or relaxed muscle. Whether they are attached depends on the rate of attachment of the cross-bridges to actin in vitro, i.e. on the “effective actin concentration.” An experimental value for the rate of attachment of the cross-bridge is not yet available, but calculations based on the rate of diffusion of the cross-bridge suggest that M·T and M·D·P_i might well be attached to actin in vivo (32). A second possibility is that although M·T and M·D·P_i bind to actin in active muscle, something other than troponin-tropomyosin prevents their binding in relaxed muscle. For example, the structure of the myosin filament in relaxed muscle may prevent cross-bridge attachment. But when Ca^{2+} binds to troponin, it may allow force-producing (45° state) bridges to form which in turn may cooperatively change the structure of the myosin filament so that more cross-bridges can attach.

The third possibility, of course, is that cross-bridges are attached to actin in relaxed muscle. Relaxed muscle may be easily extensible because the attached cross-bridges are in rapid equilibrium with detached cross-bridges. Slipping of the attached cross-bridges along actin may result in only a small viscosity effect.\(^2\) Data from recent x-ray diffraction (33) and ESR studies (34) seem to argue against attached cross-bridges in relaxed muscle. However, x-ray studies may not detect the attached cross-bridges because it is possible that less than half of the myosin molecules are attached by one head so that the mass of attached cross-bridges in

\(^2\)M. Schoenberg, personal communication.
active muscle is ¼ of the mass of attached cross-bridges in rigor. This would lead to an x-ray pattern in active muscle which is less than 1/16 as strong as in rigor muscle, making it rather difficult to detect. As for physical methods like ESR, it is possible that cross-bridges attached at a 90° angle are somewhat flexible both at their point of attachment to actin and in the neck region of the S-1 molecule near S-2. This could mean that cross-bridges attached to actin in relaxed muscle are somewhat more flexible than cross-bridges in rigor muscle. This is one of the possibilities suggested by Thomas et al. (35).

Further work will be required to determine which of the three possibilities discussed above is correct. We can conclude from our work that, whether or not cross-bridges are attached to actin in relaxed muscle, troponin-tropomyosin certainly does not act by simply blocking the binding of cross-bridges to actin. Troponin-tropomyosin inhibits a kinetic step in the ATPase cycle.

References
1. Chalovich JM, Eisenberg E. Biophys J 1981;33:232a.
2. Huxley AF, Niedergerke R. Nature (Lond) 1954;173:971–973. [PubMed: 13165697]
3. Huxley HE, Hanson J. Nature (Lond) 1954;173:973–976. [PubMed: 13165698]
4. Perry SV. Biochem Soc Trans 1979;7:593–617. [PubMed: 157897]
5. Weber A, Murray JM. Physiol Rev 1973;53:612–673. [PubMed: 4577547]
6. Ebashi S. Proc R Soc Lond B Biol Sci 1980;207:259–286. [PubMed: 6102396]
7. Haselgrove JC. Cold Spring Harbor Symp Quant Biol 1972;37:341–352.
8. Huxley HE. Cold Spring Harbor Symp Quant Biol 1972;37:361–376.
9. Parry DAD, Squire JM. J Mol Biol 1973;75:33–55. [PubMed: 4713300]
10. Seymour J, O’Brien EJ. J Muscle Res Cell Motil 1980;1:499.
11. Taylor KA, Amos LA. J Mol Biol 1981;147:297–324. [PubMed: 7288881]
12. Greene LE, Eisenberg E. Proc Natl Acad Sci U S A 1980;77:2616–2620. [PubMed: 6930656]
13. Eisenberg E, Greene LE. Annu Rev Physiol 1980;42:293–309. [PubMed: 6996582]
14. Taylor EW. CRC Crit Rev Biochem 1979;6:103–164. [PubMed: 156624]
15. Chalovich JM, Chock PB, Eisenberg E. J Biol Chem 1981;256:575–578. [PubMed: 6450206]
16. Wagner PD, Giniger E. Biophys J 1981;33:232a.
17. Stein LA, Schwarz RP Jr, Chock PB, Eisenberg E. Biochemistry 1979;18:3895–3909. [PubMed: 158378]
18. Eisenberg E, Kiellay RW. J Biol Chem 1974;249:4742–4748. [PubMed: 4276966]
19. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. J Biol Chem 1951;193:265–275. [PubMed: 14907713]
20. Chock SP, Eisenberg E. J Biol Chem 1979;254:3229–3235. [PubMed: 155064]
21. Eisenberg E, Moos C. J Biol Chem 1967;242:2945–2951. [PubMed: 4226122]
22. Seidel JC. Biochim Biophys Acta 1969;189:162–170. [PubMed: 4242604]
23. Murray JM, Knox MK, Trueblood CE, Weber A. FEBS Lett 1980;114:169–173. [PubMed: 6892903]
24. Pemrick S, Weber A. Biochemistry 1976;15:5193–5198. [PubMed: 136272]
25. Bevington, P. R. (1969) in Data Reduction and Error Analysis for the Physical Sciences, pp. 235–240, McGraw-Hill, New York.
26. Eisenberg E, Kiellay WW. Biochem Biophys Res Commun 1970;40:50–56. [PubMed: 4248005]
27. Parker L, Pyun HY, Hartshorne DJ. Biochim Biophys Acta 1970;223:453–456. [PubMed: 4251402]
28. Trybus KM, Taylor EW. Proc Natl Acad Sci U S A 1980;77:7209–7213. [PubMed: 6938966]
29. Murray JM, Weber A, Knox MG. Biochemistry 1981;20:641–649. [PubMed: 6452158]
30. Hill TL, Eisenberg E, Greene L. Proc Natl Acad Sci U S A 1980;77:3186–3190. [PubMed: 10627230]
31. Stein LA, Chock PB, Eisenberg E. Proc Natl Acad Sci U S A 1981;78:1346–1350. [PubMed: 6453345]
32. Hill TL, Eisenberg E. Biophys Chem 1980;11:271–281. [PubMed: 6989413]
33. Huxley HE, Farqui AR, Bordas J, Koch MHJ, Milch JR. Nature 1980;284:140–143. [PubMed: 7189013]
34. Thomas DD, Cooke R. Biophys J 1980;32:891–906. [PubMed: 6266539]
35. Thomas DD, Ishiwata S, Seidel JC, Gergely J. Biophys J 1980;32:873–890. [PubMed: 6266538]
Fig. 1. Standard curve of the NH₄⁺ EDTA ATPase assay used for determining the concentration of free S-1 in binding studies
Conditions: 5 mM ATP, 0.4 mM NH₄Cl, 35 mM EDTA, 25 mM Tris, pH 8.0, 25 °C.
Fig. 2. Rate of ATP hydrolysis (A) and fraction of S-1 bound to regulated actin in the presence of ATP (B) as a function of the concentration of regulated actin at 18 mM ionic strength. Measurements were made in the presence (•) and absence (○) of Ca\(^{2+}\). In A, all rates were corrected for the rate of hydrolysis by S-1 (0.09 s\(^{-1}\)) and the S-1 concentration was 0.1 or 1.5 μM in the presence of Ca\(^{2+}\) and 0.27 or 2.7 μM in the absence of Ca\(^{2+}\). Conditions: 1 mM ATP, 3 mM MgCl\(_2\), 1 mM EGTA (or 0.5 mM CaCl\(_2\)), 10 mM imidazole, pH 7.0, 25 °C.
Fig. 3. Double reciprocal plots of the fraction of S-1 bound, in the presence of ATP at 18 mM ionic strength, as a function of free actin concentration

Binding was measured using unregulated actin in the presence of Ca\(^{2+}\) (□), regulated actin in the presence of Ca\(^{2+}\) (•), and regulated actin in the absence of Ca\(^{2+}\) (○).
Fig. 4. Rate of ATP hydrolysis by S-1 as a function of the concentration of regulated actin at 50 mM ionic strength in the presence (•) and absence (○) of Ca\(^{2+}\) 
All rates are corrected for the rate of hydrolysis by S-1 alone (0.1 s\(^{-1}\)). The S-1 concentration was 0.2 μM in the presence of Ca\(^{2+}\) and 2.0 μM in the absence of Ca\(^{2+}\). Conditions: 1 mM ATP, 3 mM MgCl\(_2\), 1 mM EGTA (or 0.5 mM CaCl\(_2\)), 10 mM imidazole, 32 mM KCl, pH 7.0, 25 °C.
Fig. 5. Double reciprocal plots of the fraction of S-1 bound, in the presence of ATP at 50 mM ionic strength, as a function of free actin concentration.

Binding was measured with regulated actin in the presence (•) or absence (○) of Ca$^{2+}$.

Conditions: 2 mM ATP, 4 mM MgCl$_2$, 1 mM EGTA (or 0.5 mM CaCl$_2$), 10 mM imidazole, 27 mM KCl, pH 7.0, 25°C.
Fig. 6. Double reciprocal plots of S-1 ATPase against actin concentration at various ratios of native tropomyosin to actin at 18 mM ionic strength

*Open symbols* are in the presence of EGTA at molar ratios of native tropomyosin to actin of 0/7 (□), 1/7 (♦), 1.2/7 (▲), 1.5/7 (○), and 2/7 (+). For clarity, no line is drawn through the 0/7 ratio points. In the presence of Ca$^{2+}$ (●), the ratio of tropomyosin to actin is 1.5/7. Conditions: 1 mM ATP, 3 mM MgCl$_2$, 1 mM EGTA (or 0.5 mM CaCl$_2$), 10 mM imidazole, pH 7.0, 25 °C.
Fig. 7. Kinetic model of ATP hydrolysis by myosin
In this model, $M$ is myosin or S-1, $A$ is actin, $T$ is ATP, $D$ is ADP, $TP\cdot TM$ is troponintropomyosin, and the subscripts $R$ and $N$ are the refractory and nonrefractory states, respectively. Equilibrium constants involving formation of actin complexes are dissociation constants (e.g. $K_3 = [A][M\cdot T]/[A\cdot M\cdot T]$). For all other equilibrium constants, products are considered to be to the right of the reactants (e.g. $K_{10} = [A\cdot M\cdot D]\cdot P_i/[A\cdot M\cdot D\cdot P_{iN}]$).
| [S-I] | ATP hydrolyzed<sup>a</sup> | Fraction of S-I bound<sup>b</sup> |
|------|----------------|-----------------|
|      | ×10<sup>6</sup> μ | %               |
| Unregulated actin | 0.06 | 60 |
| Regulated actin + Ca<sup>2+</sup> | 0.06 | 44 |
| Regulated actin + EGTA | 1.0 | 37 |
| Regulated actin + EGTA | 0.23 | 0.42 |
| Regulated actin + EGTA | 1.1 | 0.46 |
| Regulated actin + EGTA | 3.0 | 0.62 |
| Regulated actin + EGTA, no ATP | 2.4 | 0.99 |

<sup>a</sup> [γ-<sup>32</sup>P]ATP replaced ATP in the binding mixture and [<sup>32</sup>P]P<sub>i</sub> was measured in the supernatant after centrifugation; the total actin concentration was 100 μM.

<sup>b</sup> Fifty-four μM actin total.