Mechanism of Action of Troponin-Tropomyosin

INHIBITION OF ACTOMYOSIN ATPase ACTIVITY WITHOUT INHIBITION OF MYOSIN BINDING TO ACTIN

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The regulation of vertebrate skeletal muscle contraction by the troponin-tropomyosin complex is generally thought to be the result of tropomyosin physically blocking the myosin binding site of actin in the absence of Ca++. This mechanism was tested during steady state ATP hydrolysis by comparing the degree of association of myosin subfragment 1 (S-1) with the actin-tropomyosin-tropomyosin complex in the absence and presence of Ca++. Binding in the presence of ATP was determined by stopped flow absorbance measurements at 25°C. Although the steady state ATPase rate was reduced 96% in the absence of Ca++, the association constant of S-1 with regulated actin was virtually the same in the absence of Ca++. The association constant of Ca++ with actin was determined (1.3 x 10^14 M^-1) as in the presence of Ca++. The association constant of S-1 to regulated actin in the presence of Ca++ was similar to the association constant of S-1 to unregulated actin. These results suggest that the troponin-tropomyosin complex does not inhibit the actin-activated ATPase activity by preventing the binding of S-1.ATP or S-1.ADP-P, to actin; rather, it may act by blocking the release of P from the acto-S-1.ADP-P complex.

Contraction of vertebrate-striated muscle occurs when the thick myosin filaments slide past the thin actin filaments, a process driven by the cyclic interaction of the myosin cross-bridges with actin as ATP is hydrolyzed (2-4). Relaxation of vertebrate-striated muscle occurs when the sarcoplasmic reticulum sequesters myofibrillar Ca++ so that the concentration of free Ca++ falls below 10^-7 M. At this low Ca++ concentration, the myosin cross-bridges no longer cyclically interact with the actin filaments and the actin-activated myosin ATPase activity drops to a very low level. The effect of Ca++ on the actin-myosin interaction is mediated by the troponin-tropomyosin complex, a complex of proteins which binds to F-actin; in the absence of troponin-tropomyosin, Ca++ has no effect on the interaction of actin and myosin (5, 6).

Structural studies have shown that 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 (7). There is also strong evidence that the binding of Ca++ to troponin determines the position of the tropomyosin molecule on the F-actin filament; when the Ca++ concentration is lowered, the tropomyosin moves away from the central groove of the F-actin filament toward a position where it might interfere with the binding of the myosin cross-bridge to actin (8, 9). On this basis, Haselgrove and Huxley (8, 9) proposed the steric blocking model of tropomyosin action. This model suggests that relaxation occurs when tropomyosin in the "relaxed" position physically blocks the binding of the myosin cross-bridge to actin.

As yet, there has been no direct in vitro evidence for the steric blocking model. Recently, however, Greene and Eisenberg found that the troponin-tropomyosin complex has a marked effect on the binding of S-1.GDP to F-actin (10). At low levels of occupancy of the F-actin-troponin-tropomyosin complex (regulated F-actin) with S-1.ADP, the S-1.ADP binds over 100-fold more weakly to the regulated actin than it does to unregulated actin. On the other hand, at high levels of saturation of the regulated F-actin with S-1.ADP, the S-1.ADP binds even more strongly to regulated actin than to unregulated actin. On the basis of these data, Greene and Eisenberg suggested that the regulated actin filament could exist in two forms, a "weak" binding form and a "strong" binding form, with Ca++ and S-1.ADP acting as allosteric effectors shifting the equilibrium between the two forms.

Since the troponin-tropomyosin complex has the ability to greatly weaken the binding of S-1.ADP to actin, it seemed possible that troponin-tropomyosin might cause relaxation by also blocking the binding of myosin with bound ATP or ADP + P, to actin as suggested by the steric blocking model. Stein et al. previously measured the binding of S-1.ATP and S-1.ADP-P to unregulated actin using stopped flow absorbance measurements (11). Therefore, in the present study, we used this method to investigate the binding of S-1.ATP and S-1.ADP-P to regulated actin both in the presence and absence of Ca++. Surprisingly, our results show that at 25°C, μ = 0.018 M in the absence of Ca++, as well as in the presence of Ca++, the troponin-tropomyosin complex has very little effect on the binding of S-1.ATP or S-1.ADP-P, to actin although the actin-activated ATPase activity is 96% inhibited in the absence of Ca++. These results suggest that the troponin-tropomyosin complex does not inhibit the actin-activated ATPase activity by preventing the binding of S-1.ATP or S-1.ADP-P to actin; rather, it may act by blocking the release of P from the acto-S-1.ADP-P complex.

MATERIALS AND METHODS

Rabbit skeletal myosin, S-1, and actin were prepared as described by Stein et al. (11). Native tropomyosin was prepared according to Eisenberg and Kielley (12). The molecular weights used for actin, S-1, and native tropomyosin were 42,000, 120,000, and 150,000, respectively. Protein concentrations were determined 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 native tropomyosin. Regulated actin was prepared by mixing actin with a 50% excess (1.5 mol/7 mol of actin) of native tropomyosin and dialyzing 12 h at 4°C against 2 mM ATP, 4 mM MgCl₂, 10 mM imidazole, pH 7.0, 0.25 mM diethiothreitol, and 1 mM EGTA (or 0.5 mM CaCl₂).

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

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Stopped flow absorbance measurements were made at 25°C with incident light of 340 nm using the apparatus previously described (13, 14). Absorbance was calculated by comparing the measured transmitted light intensity of the buffer and the sample (11). In a binding experiment, one syringe contained S-1 in 2 mM MgCl₂, 10 mM imidazole, pH 7.0, 0.25 mM dithiothreitol, and 1 mM EGTA (or 0.5 mM CaCl₂). The other syringe contained actin or regulated actin in a similar buffer containing 4 mM MgCl₂ and 2 mM ATP. In this way, the free Mg²⁺ was always maintained at 2 mM to ensure that the F-actin was fully saturated with troponin·tropomyosin. The fraction of S-1 bound at a given actin concentration was determined as described by Stein et al. (11). Association constants were determined from double reciprocal plots which were computer-fitted using the Marquardt compromise (15).

RESULTS

In this study, the binding of S-1 to regulated actin in the presence of ATP was studied using the stopped flow absorbance method of Stein et al. (11). Since we found that 2 mM free Mg²⁺ is required to keep the troponin·tropomyosin complex strongly bound to actin, it was necessary to work at a higher ionic strength (18 mM) than was employed by Stein et al. Therefore, to obtain the strongest possible binding, we performed our experiments at 25°C rather than at 15°C.

In stopped flow absorbance measurements carried out with actin in excess over S-1, the initial absorbance provides a measure of the fraction of S-1 bound to actin in the presence of ATP while, after all of the ATP is hydrolyzed, the absorbance rises to the value obtained when all of the added S-1 is complexed with actin (11). Fig. 1 shows that, both in the presence and absence of troponin·tropomyosin, the absorbance of the acto-S-1 complex obtained after all of the ATP is hydrolyzed is proportional to the amount of S-1 bound to actin. Although the regulated actin, by itself, has a higher absorbance than unregulated actin, the binding of a given amount of S-1 causes the same increase in absorbance with regulated actin as with unregulated actin. This strongly suggests that troponin·tropomyosin does not interfere with the use of absorbance as a measure of S-1 binding to actin. Comparison of the open circles and solid circles in Fig. 1 also shows that the stopped flow apparatus yields the same absorbance values as in a standard spectrophotometer.

Fig. 2A shows double reciprocal plots of the fraction of S-1 bound to actin versus free actin concentration, in the presence of ATP, as a function of free actin concentration. The conditions are the same as in Fig. 1 with 20 μM S-1, except where noted. A, binding in the presence of calcium. EGTA was replaced with 0.5 mM CaCl₂. Binding to regulated (closed symbols) and unregulated actin (open symbols) are shown. B, binding in the absence of calcium. S-1 concentration was varied from 10 μM (A) to 40 μM (Δ). In both A and B, different symbols represent different protein preparations. The solid lines are least squares fits to the data (see "Materials and Methods").

Note that, as found previously by Stein et al. (11), the ordinate intercept of the double reciprocal plot in Fig. 2A has a value close to 1. This strongly suggests that the absorbance of the acto-S-1 complex is similar in the presence and absence of ATP; if the absorbance of acto-S-1 were less in the presence of ATP than in the absence of ATP, the ordinate intercept of Fig. 2A would be greater than 1. Even if this were the case, however, it would not invalidate the use of absorbance as a measure of the binding of S-1 to actin providing that the increase in absorbance is proportional to the fraction of S-1 bound to actin in the presence of ATP. In this case, the association constant obtained from the double reciprocal plot in Fig. 2A would still be a true measure of binding.

We next measured the binding of S-1 to regulated actin in the presence of ATP and EGTA. Based on the steric blocking model of troponin·tropomyosin action, we expected that the binding would decrease markedly. Unexpectedly, however, we found that S-1 binds to regulated actin almost as strongly in the presence of EGTA as in the presence of Ca²⁺ (Fig. 2B). The double reciprocal plot of the fraction of S-1 bound to actin versus free actin concentration was linear and yielded an association constant of 1.3 × 10⁴ M⁻¹, only slightly weaker than the value obtained in the presence of Ca²⁺. As shown by the open and closed triangles in Fig. 2B, about the same binding occurred at 10 μM and 40 μM S-1 suggesting that there
is no cooperativity in the binding of S-1 to regulated actin in the presence of ATP and EGTA. The relatively strong binding of S-1-ATP to actin shown in Fig. 2B occurred despite the fact that under these conditions the ATPase activity was 96% inhibited by EGTA. Therefore, there seems to be no correlation between the fraction of S-1 bound to actin and the inhibition of the acto-S-1 ATPase by troponin-tropomyosin in the presence of EGTA.

This point is shown even more clearly in Fig. 3 where we plot the fraction of S-1 bound as a function of time both with unregulated actin and with regulated actin in the presence and absence of Ca2+. In all three cases, we adjusted the actin concentration so that about 70% of the S-1 was bound to actin in the presence of ATP. Despite this identical binding, it took only 5 s for all of the ATP to be hydrolyzed with regulated actin in the presence of Ca2+ (ATPase rate = 25 s⁻¹) while in the absence of Ca2+ it took more than 70 s (ATPase rate = 1.4 s⁻¹). Clearly, the steady state ATPase activity shows no correlation with the fraction of S-1 bound to actin in the presence of ATP.

Stein et al. (11) showed that, with unregulated actin, the steady state absorbance level was reached almost instantaneously after S-1, ATP, and actin were mixed. On this basis, they concluded that S-1-ATP and S-1-ADP-Pi bind to actin with about the same equilibrium constant. Fig. 4 shows the time course of the light intensity change which occurred immediately after 40 μM S-1 was mixed with 120 μM regulated actin + ATP in the presence of EGTA; under this condition, 35% of the S-1 is bound to regulated actin. There is a slight increase in light intensity during the first 20 ms after the S-1, actin, and ATP are mixed (upper trace), but an almost identical increase is observed when the regulated actin is mixed with buffer in the absence of S-1 (lower trace). This small change in light intensity is probably an artifact caused by rapid dilution of concentrated regulated actin solutions. Therefore, it appears that S-1-ATP and S-1-ADP-Pi bind to regulated actin with about the same equilibrium constant although it will be necessary to have more data on the relative levels of S-1-ATP and S-1-ADP-Pi in the presence of regulated actin to substantiate this conclusion.

**DISCUSSION**

The most widely accepted mechanism of troponin-tropomyosin action is the steric blocking model (8, 9). This model suggests that troponin-tropomyosin in the relaxing position blocks the binding of the myosin cross-bridge to actin. Based on this model, it might be expected that when troponin-tropomyosin inhibited the acto-S-1 ATPase activity in the absence of Ca2+ it would also block the binding of S-1 to actin. However, we find that under our conditions, troponin-tropomyosin strongly inhibits the acto-S-1 ATPase activity without having a significant effect on the binding of S-1-ATP to actin. Therefore, our data suggest that the troponin-tropomyosin complex inhibits the acto-S-1 ATPase activity by blocking some other step in the kinetic cycle.

Stein et al. (11) have proposed the following kinetic model for the acto-S-1 ATPase (M, myosin; A, actin):

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\begin{align*}
M &\rightarrow M \cdot ATP \\
M \cdot ATP &\rightarrow M \cdot ADP \cdot P_i \\
M \cdot ADP \cdot P_i &\rightarrow M \cdot ADP \cdot P_{ii} \\
M \cdot ADP \cdot P_{ii} &\rightarrow M \cdot ADP \\
M \cdot ADP &\rightarrow M \\
M &\rightarrow M \cdot ATP \\
A \cdot M \cdot ATP &\rightarrow A \cdot M \cdot ADP \cdot P_i \\
A \cdot M \cdot ADP \cdot P_i &\rightarrow A \cdot M \cdot ADP \cdot P_{ii} \\
A \cdot M \cdot ADP \cdot P_{ii} &\rightarrow A \cdot M \cdot ADP \\
A \cdot M \cdot ADP &\rightarrow A \\
A &\rightarrow A \cdot M \
\end{align*}
\]

Both the ATP hydrolysis step (M • ATP → M • ADP • P_i) and the transition from the refractory to the nonrefractory state (M • ADP • P_{ii} → M • ADP), which is the rate-limiting step in the presence of actin, occur at about the same rate whether or not S-1 is bound to actin (11, 16). On the other hand, the step from M • ADP • P_{ii} to M • ADP is very slow in the absence of actin. Therefore, it seems likely that troponin-tropomyosin inhibits the ATPase activity in the presence of actin by markedly decreasing the rate of P_i release from A • M • ADP • P_{ii}. It is possible that with regulated actin in the absence of Ca2⁺, the rate of P_i release is almost as slow when S-1 is bound to actin as when it is dissociated from actin.

In contrast to the negligible effect of troponin-tropomyosin on the binding of S-1-ATP and S-1-ADP-P_i to actin, Greene and Eisenberg found that troponin-tropomyosin strongly inhibits the binding of S-1 and S-1-ADP to regulated actin (10). Interestingly, S-1-ADP and S-1-ADP-P_i bind several orders of magnitude more strongly to actin than do S-1-ATP and S-1-ADP-P_i (11). On the basis of this latter finding, Eisenberg and Greene have proposed a cross-bridge model in which the myosin states are grouped into two major categories. The states which retain a bound phosphate at the active site (M • ATP and M • ADP • P_i) are postulated to bind to actin weakly at a preferred angle of about 90° while the
states without a bound phosphate (M-ADP and M) are postulated to bind to actin strongly at a preferred angle of about 45° (17). The key assumption here is that the preferred angle of attachment of a cross-bridge to actin is correlated with its binding affinity to actin. The marked difference in the effect of troponin-tropomyosin on states with and without bound phosphate supports the assumption that these two groups of states not only bind to actin with different affinities but also with different orientations. On this basis, our data might be interpreted as suggesting that troponin-tropomyosin has very little effect on the binding of myosin states which attach to actin at a preferred angle of 90° but can greatly weaken the binding of states which attach to actin at a preferred angle of 45°. Furthermore, troponin-tropomyosin might not only destabilize the binding of the 45° state to actin but, in a related effect, might also decrease the rate of the transition from the 90° state to the 45° state, i.e. decrease the rate at which P is released from A-M-ADP-P.

Although it is tempting to speculate that troponin-tropomyosin inhibits the acto-S-1 ATPase activity simply by blocking the transition from the 90° state to the 45° state, the complete mechanism of relaxation might well be more complex. In particular, Greene and Eisenberg have found that troponin-tropomyosin can greatly weaken the binding of S-1-ADP to actin in the presence of Ca^{2+}, as well as in its absence (10). Yet, in the presence of Ca^{2+}, the acto-S-1 ATPase activity is quite high. Before we can conclude that troponin-tropomyosin acts simply by destabilizing the 45° state and thus decreasing the rate of the transition from the 90° state to the 45° state, the relationship between ATPase activity and the strength of binding S-1-ADP to regulated actin must be clarified.

The fact that, under the conditions of our experiments, troponin-tropomyosin inhibits the acto-S-1 ATPase activity without blocking the binding of S-1-ATP to actin raises the possibility that a certain fraction of the myosin cross-bridges remain attached to actin in relaxed muscle. Although it might, at first, appear that attached cross-bridges would make the relaxed muscle stiff, weakly attached cross-bridges in rapid equilibrium with unattached bridges might only be detected as a slight viscosity effect (18, 19) because they attach and detach from actin so rapidly. Furthermore, if attachment of myosin cross-bridges with bound ATP and ADP-P, to actin is energetically favorable in active muscle, then energy must be supplied to keep these cross-bridges detached from actin in relaxed muscle, and the source of this energy is not obvious at the present time. Of course, extrapolation of our results to the situation in vivo is tentative: we do not know how ionic strength affects the action of troponin-tropomyosin, how strongly the myosin cross-bridge with bound ATP binds to actin in vivo, nor whether the interaction between myosin molecules in the myosin filament could affect the binding of the myosin cross-bridge to actin. Therefore, the results presented in this paper only raise the possibility that, to some extent, myosin cross-bridges are attached to actin in relaxed muscle. However, these results do strongly suggest that troponin-tropomyosin affects the kinetic step involving P release in addition to any other effect it might have on cross-bridge binding to actin in vivo.

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