Troponin-Tropomyosin: An Allosteric Switch or a Steric Blocker?

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ABSTRACT The interaction of myosin subfragment 1 (S1) with actin-tropomyosin-troponin (regulated actin) is highly nucleotide dependent. The binding of S1 or S1-ADP (but not S1-ATP nor *N*,*N*′-ρ-phenylenedimaleimide-modified S1-ATP) to regulated actin activates ATP hydrolysis even in the absence of Ca^{2+}. Investigations with S1 and S1-ADP have led to the idea that some actin sites are directly blocked toward the binding of S1 either by tropomyosin or troponin. The blocked state is thought to occur only at ionic strengths greater than 50 mM. The question is whether nonactivating S1 binding is blocked under the same conditions. We show that troponin inhibits binding of the nonactivating state, *N*,*N*′-ρ-phenylenedimaleimide-S1-ATP, to actin but only when tropomyosin is absent. A lag in the rate of binding of activating S1 to actin (an indicator of the blocked state) occurs only in the presence of tropomyosin. Thus, tropomyosin inhibits binding of rigor S1 but not S1-ATP-like states. No evidence for an ionic strength-dependent change in the mechanism of regulation was observed either from measurements of the rate of activating S1 binding or from the equilibrium binding of nonactivating S1 to actin. At all conditions examined, *N*,*N*′-ρ-phenylenedimaleimide-S1-ATP bound to regulated actin in the absence of Ca^{2+}. These results support the view of regulation in which tropomyosin movement is an allosteric switch that is modulated by activating myosin binding but that does not function solely by regulating myosin binding.

INTRODUCTION

The actin-binding complex of tropomyosin and troponin regulates skeletal and cardiac muscle contraction. Ca^{2+} binding to the troponin C (TnC^1) component of troponin causes rearrangements in the troponin complex (troponin I (TnI), troponin T, and TnC) and changes the orientation of tropomyosin on actin (Huxley, 1972; Haselgrove, 1972; Parry and Squire, 1973; Kress et al., 1986; Lehman et al., 1994, 2000; Vibert et al., 1997; Xu et al., 1999). In the absence of Ca^{2+}, the position of tropomyosin on actin is such that it overlaps the putative binding site of rigor S1 but not the putative binding site of S1-ATP (Vibert et al., 1997; Xu et al., 1999; Craig and Lehman, 2001). Ca^{2+} causes tropomyosin to move from the outer domain of actin toward the inner domain where there is less potential overlap of the rigor S1 binding site. High concentrations of rigor S1 stabilize tropomyosin into a third position (Vibert et al., 1997; Poole et al., 1995) that may be associated with the highest rate of ATP hydrolysis (Eisenberg and Kielley, 1970; Bremel et al., 1972). Fluorescent probes placed on troponin I (Trybus and Taylor, 1980; Greene, 1986) and tropomyosin (Ishii and Lehrer, 1990) support the idea that there are at least three conformational states of regulated actin. Probes on TnI I respond both to changes in Ca^{2+} and to binding of "activating" cross-bridges to actin, whereas probes on tropomyosin respond primarily to binding of activating cross-bridges.

An outstanding question is how the movement of tropomyosin on actin results in a large increase in ATPase activity. Fig. 1 compares three potential mechanisms. In the classic steric blocking hypothesis (Fig. 1 *A*) actin exists in two states, one blocked toward myosin binding and one open to myosin binding. The reduction in binding of S1 to actin by tropomyosin directly results in a decrease in ATPase activity.

Subsequent experimentation showed that binding of high affinity or activating states to regulated actin was not totally inhibited but exhibited positive cooperativity as the S1 concentration was increased (Greene and Eisenberg, 1980, 1988; Lehrer and Morris, 1982). Note that activating states such as S1-ADP and rigor S1 can stabilize tropomyosin into the active position, whereas nonactivating states such as S1-ATP cannot (Chalovich et al., 1983; Brenner et al., 1999). Furthermore, binding of nonactivating states such as S1-ATP (Chalovich et al., 1981; Brenner et al., 1982), *N*,*N*′-ρ-phenylenedimaleimide-S1-ATP (ρPDM-S1-ATP (Chalovich et al., 1983), and S1-ATPγS (Resetar and Chalovich, 1995) are not greatly affected by Ca^{2+}. Alternative models were proposed in which Ca^{2+} and rigor type S1 are allosteric effectors of tropomyosin (Chalovich et al., 1981; Hill et al., 1981; Tobacman and Butters, 2000). In the Hill model (Fig. 1 *B*), different states of bound tropomyosin correspond to different levels of activity of actin in facilitating hydrolysis of ATP by myosin. Actin exists in two major states. State 1 is less active, and state 2 is more active. The binding of activating S1 stabilizes state 2. States 1 and 2 of actin have three substates depending on whether zero, one, or two molecules of Ca^{2+} are bound per troponin complex. Although tropomyosin inhibits the binding of activating states of S1 (strong binding such as S1 and

Submitted September 11, 2001, and accepted for publication April 4, 2002.

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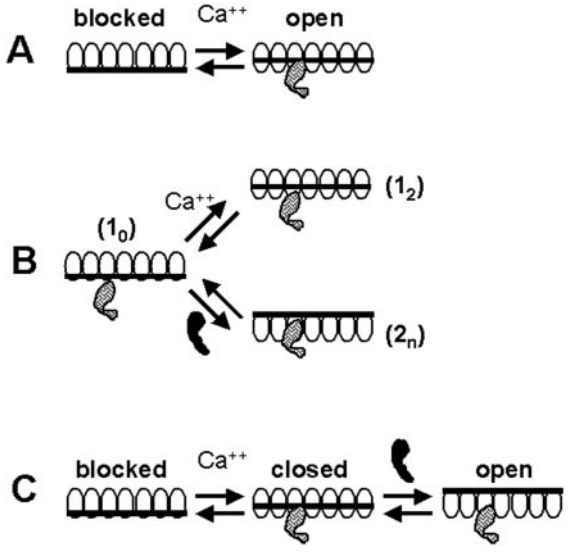

FIGURE 1 Models of binding of S1 to actin-tropomyosin-troponin. Actin is shown by ovals and tropomyosin by a solid bar. Unmodified S1-ATP is shown by the shaded figures, whereas S1 modified by *N*-ethylmaleimide is shown by the solid figures. (*A*) Classic steric blocking model. Ca^{2+} stabilizes the open state of the actin filament that is permissive for S1 binding. (*B*) Hill model of regulation. S1-ATP can bind to regulated actin in the absence of Ca^{2+} even though the position of tropomyosin overlaps the binding site of activating states of S1 such as S1-ADP and rigor S1. This is state $1_{(0)}$. Ca^{2+} changes the position of tropomyosin to stabilize actin in state $1_{(2)}$ where the subscript refers to the number of Ca^{2+} bound per troponin. The ATPase rate is much higher in the presence of saturating Ca^{2+} but is not maximal. Binding of rigor type S1 (solid S1 shape) to actin causes yet another change in tropomyosin position to form the state 2. For clarity, the rigor S1 is not shown bound to the actin filament. In state 2 the affinity of activating forms of S1 is higher, but there is little change in S1-ATP affinity. Actin is most active in accelerating ATP hydrolysis in state 2 regardless of the number of Ca^{2+} bound per troponin. (*C*) McKillop and Geeves model. Ca^{2+} stabilizes the closed state of the actin filament that is permissive for low affinity S1 binding. Occupancy of the actin filament with rigor type S1 stabilizes the open state. S1 bound in the open state can isomerize to a more stable complex. Force can be generated only when actin is in the open state.

S1-ADP) to actin there is little effect on the binding of nonactivating states (weak binding such as S1-ATP, and S1-adenosine 5′-(γ-thiotriphosphate) (S1-ATPγS)). Activation results from acceleration of the rate of transition from a bound S1 complex with low ATPase activity to a bound complex with high ATPase activity.

The Hill model had an apparent discrepancy between the predicted effects of Ca^{2+} on the equilibrium binding of S1 to regulated actin and the kinetics of binding (Trybus and Taylor; 1980; McKillop and Geeves, 1993). To overcome this discrepancy, the Hill model was revised with the inclusion of a blocked state to which S1 could not bind (McKillop and Geeves, 1993). The McKillop and Geeves model (Fig. 1 *C*) retains elements of the two earlier models. In the absence of Ca^{2+}, 80% of the sites of regulated actin are thought to be blocked or unavailable for binding to rigor S1 (McKillop and Geeves, 1993). Some researchers have suggested that blocking of binding may be due to troponin rather than tropomyosin. This blocked state was thought to be destabilized below 50 mM ionic strength (Head et al., 1995). The loss of the blocked state at low ionic strength and the assumption that only a fraction of actin sites were blocked was used to explain the earlier observations that S1-ATP-like state binding to regulated actin was unaffected by Ca^{2+}. Binding of Ca^{2+} to troponin shifts the equilibrium from the blocked state to a closed state that permits binding but not acceleration of ATPase activity. Binding of activating states of S1 causes formation of an open state that activates ATP hydrolysis and force production. Presumably substates exist in this model also, and the properties of the closed and open states change depending on the number Ca^{2+} bound to TnC. Activation results from unblocking the binding of myosin to actin and also by a change in the rate of transition between bound states.

The Hill model can predict the effects of Ca^{2+} on steady-state ATP hydrolysis both at the limits of saturating actin and saturating S1 (Hill et al., 1981). It is unclear if the McKillop and Geeves model can make similar predictions because that model has not been extended beyond binding to ATPase activities. Because of the success of the Hill model in simulating the regulation of ATPase activity we have reexamined some of the apparent discrepancies of that model. For example, we recently showed that the Hill model does correctly predict the binding kinetics of the S1-actin interaction (Chen et al., 2001). We have now taken another look at the possibility that the binding of nonactivating states of S1 may be blocked under some conditions. We examined the effect of tropomyosin-troponin, troponin alone, and TnI on the binding of ρPDM-S1-ATP, S1, and S1-ADP to actin. ρPDM-S1-ATP was used as a model of a nonactivating state because its low ATPase activity permitted binding to be measured at high concentrations of modified S1. The binding constant of ρPDM-S1-ATP to actin is only approximately twofold stronger than that of S1-ATP but 0.001 of that of the binding of S1-ADP to actin (Chalovich et al., 1983; Greene et al., 1986; Kirshenbaum et al., 1993). In the presence of ATP, ρPDM-S1 does not activate the regulated filament in the absence of Ca^{2+} (Greene et al., 1986).

Our results do not support the hypothesis that the binding of nonactivating states of S1 to regulated actin is blocked. We also have failed to find convincing evidence for a change in mechanism of regulation as the ionic strength is lowered below 50 mM. We suggest that the Hill model is a reasonable description of regulation of striated muscle contraction. The primary function of the change in tropomyosin binding to actin is to alter the ability of actin to act as a cofactor in ATP hydrolysis. In terms of regulation it may be more important that myosin affects tropomyosin binding than tropomyosin affects myosin binding.

MATERIALS AND METHODS

Materials

[$\gamma-^{32}$P] ATP was obtained from New England Nuclear (Wilmington, DE) and [1,4-^{14}C] maleic anhydride was from Amersham Pharmacia Biotech (Piscataway, NJ). *N*-((2-(iodoacetoxy)ethyl)-*N*-methyl)amino-7-nitrobenz-2-oxa-1,3-diazole (IANBD) was obtained from Molecular Probes (Eugene, OR), and ATP from Sigma (St. Louis, MO). All other chemicals were reagent grade. [^{14}C]- ρPDM, synthesized as described by Wells and Yount (1982), and ρPDM from Aldrich (Milwaukee, WI) were purified by sublimation.

Protein preparations

Skeletal muscle actin (Spudich and Watt, 1971) and myosin (Kielley and Harrington, 1960) were isolated from rabbit back and leg muscles. Myosin subfragment 1, S1, was prepared by digestion of myosin with chymotrypsin (Weeds and Taylor, 1975). S1 was modified with ρPDM as described elsewhere (Wells and Yount, 1982). Contaminating unmodified S1 was removed by sedimentation in the presence of actin (Chalovich et al., 1983). Troponin and tropomyosin were prepared using hydroxyapatite chromatography (Eisenberg and Kielley, 1974). Troponin was labeled with IANBD as described by Trybus and Taylor (1980). TnI was isolated from the pure troponin complex (Potter, 1982).

Protein concentrations were determined by ultraviolet absorption at 280 nm and the extinction coefficients used were 750 cm^2/g for S1, 1150 cm^2/g for F-actin, 290 cm^2/g for tropomyosin, 450 cm^2/g for troponin, 380 cm^2/g for the troponin-tropomyosin complex, and 397 for TnI. For determination of molar concentrations, the following molecular weights were used: S1, 120,000; actin, 42,000; tropomyosin, 68,000; troponin, 80,000; the troponin-tropomyosin complex, 150,000; and TnI, 21,000.

Equilibrium binding assays

Binding assays were done either with ρPDM-S1 or with ^{14}C-ρPDM-S1. Mixtures of ρPDM-S1, actin, and various combinations of tropomyosin, troponin, or troponin subunits were centrifuged at 135,000 $\times$ *g* for 25 min to separate free ρPDMS1 from actin-bound ρPDMS1. ATP was present in all binding assays to insure that the S1 was in the nonactivating or "weak binding" state (Greene et al., 1986). In studies with unlabeled ρPDMS1, pellets were suspended in sodium dodecyl sulfate-sample buffer and the proteins were separated by 10% polyacrylamide-sodium dodecyl sulfate electrophoresis. Gels were stained with Coomassie Blue, and protein bands were analyzed by densitometry with a Hewlett Packard Scanner Jet iicx/t (Palo Alto, CA) and IMAGE Quant software (Molecular Dynamics, Sunnyvale, CA). In the case of ^{14}C-labeled S1, aliquots of the supernatant were analyzed in a scintillation counter and compared with the total counts present before centrifugation to determine the fraction bound to actin. In all cases, the amount of ρPDMS1-ATP binding to actin was corrected for the sedimentation of ρPDMS1-ATP in the absence of any actin. This was typically less than 4% of the total ρPDMS1-ATP concentration.

Because ρPDM-S1 remains in a purely nonactivating state only in the presence of ATP (Greene et al., 1986), it was important to insure that ATP was not depleted during the binding measurement. The ATPase activity of every ρPDMS1 preparation was measured, and the concentrations of ρPDM-S1 and ATP present in the assays were adjusted to insure that sufficient ATP remained to maintain a nonactivating state. The ρPDMS1 preparations had ATPase activities $<$1% that of unmodified S1. In some experiments ^{32}P-ATP was included in the binding assay so that the amount of ATP remaining could be measured directly.

Binding data were analyzed using the MLAB modeling program (Civilized Software, Bethesda, MD) or Mathematica (Wolfram Research, Inc., Champaign, IL). To examine the possible existence of a regulated actin state that was blocked toward the binding of S1-ATP-like states, binding was simulated by the following model:

$$B \leftrightarrows C; \qquad K1 = [C]/[B]$$

$$C + M \leftrightarrows CM; \qquad K2 = [CM]/[C] \times [M]$$

in which B and C are the blocked and closed forms of actin, respectively, and M is myosin with equilibrium constants as shown. In the McKillop and Geeves model, the open state is not populated in the absence of Ca^{2+} so it was not included in this analysis. The total actin concentration, $A_T = B + C + CM$, and the total myosin concentration is represented by M_T. The parameter of interest is θ, which is the fraction of actin sites occupied by myosin or $[CM]/[A]$. θ can be written in terms of A_T and M_T as shown in Eq. 3.

$$\theta = 1/(2 \times K1 \times K2) \times (1 + K1 + A_T \times K1 \times K2 + K1 \times K2 \times M_T - \mathrm{Sqrt}[(-4 \times A_T \times K1\text{^}2 \times K2\text{^}2 \times M_T) + (-1 - K1 - A_T \times K1 \times K2 - K1 \times K2 \times M_T)\text{^}2])/A_T$$

To simulate the case where there was no blocked state, $K1$ was set to 100. In the case where 80% of the actin was assumed to be in the blocked state, $K1$ was set to 0.25. The value of $K2$ was allowed to float to obtain the best fit of Eq. 3 to the data sets.

Stopped flow kinetic studies

Measurements were made with an Applied Photophysics DX17.MV/2 sequential stopped flow spectrofluorometer (Leatherhead, UK). The binding of S1 to actin was done with light scattering using excitation at 460 nm and measuring emission through a 455-nm-long pass filter. Activation of thin filaments by S1 or S1-nucleotides in the absence of Ca^{2+} was monitored as a decrease in IANBD fluorescence with a filter having 0% transmittance at 510 nm and 80% transmission at 540 nm using excitation at 492 nm. Averages of at least three traces were analyzed with the software provided in the Applied Photophysics package. The averaging improved the signal to noise ratio but did not change the shape of the curves because the curves in a single experiment were very similar to each other. In some experiments a lag preceded the exponential phase of binding. The lag duration was first estimated by eye. An exponential function was fitted to that part of the curve between 1.5 times the estimated lag duration to the end of the reaction. The lag was then defined by the intersection of the fitted curve with the abscissa or time axis. The observed changes were far greater than the variations in estimating lag durations.

ATPase assays

Unmodified S1 was used to determine the effect of regulatory components on the actin-activated ATPase rate. Hydrolysis of ATP by was measured by the liberation of ^{32}Pi from [$\gamma-^{32}$P]-ATP as described previously (Chock and Eisenberg, 1979). In cases where the concentration of S1 was very high a slight modification to the ATPase assay was made to improve extraction of ^{32}Pi (Hemric et al., 1993). Aliquots were removed at four times during the reaction to determine the initial velocity of the reaction. In experiments where a protein component was varied the solution was maintained at constant ionic strength.

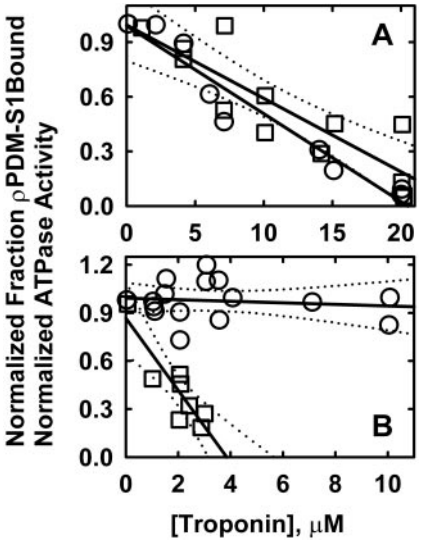

FIGURE 2 Effect of whole troponin and troponin-tropomyosin on the ATPase activity of S1 (*squares*) and on the binding of ρPDM S1 to actin (*circles*). Conditions: 25°C, 20 μM actin, 1.6 μM pPDM-S1-ATP, or 0.1 μM unmodified S1 in a buffer containing 1 mM ATP, 3 mM $MgCl_2$, 10 mM imidazole, 0.5 mM EGTA, and 1 mM dithiothreitol. (*A*) Absence of tropomyosin. The fraction of ρPDM-S1-ATP bound in the absence of troponin was ≈35%. The ATPase activity in the absence of troponin was ≈4 s^{-1}. All other data are expressed as the fraction of these values measured in the absence of troponin. The dotted lines are the 95% confidence limits of the binding. (*B*) Presence of tropomyosin; conditions same as in *A*. The ATPase rate in the absence of troponin and presence of tropomyosin was ≈2.3 s^{-1}. The 95% confidence intervals for both sets of data are shown by dotted curves.

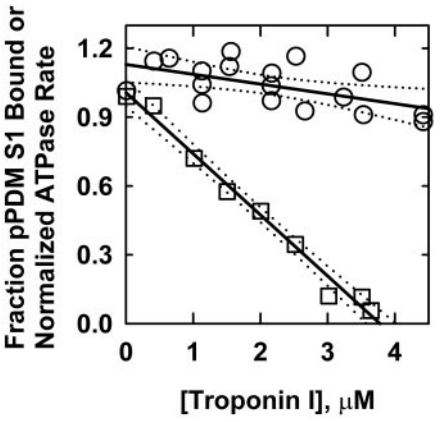

FIGURE 3 Effect of TnI on the ATPase activity of S1 (*squares*) and on the binding of ρPDM S1 to actin-tropomyosin (*circles*). Note that the affinities of ρPDM-S1 and unmodified S1 are similar in the presence of ATP (see Fig. 8). The conditions are the same as in Fig. 2. In all cases the concentrations of actin and tropomyosin were 20 and 4.3 μM, respectively. The 95% confidence limits for the data are shown by dotted lines.

RESULTS

We first wished to determine if individual components of the troponin complex could inhibit the binding of myosin-ATP-like states to actin. That is, could a modified steric blocking model exist in which the blocked state (Fig. 1, *A* and *C*) resulted from troponin itself inhibiting myosin binding? Fig. 2 *A* shows that troponin alone (no tropomyosin) inhibits the binding of ^{14}C-labeled ρPDM-S1-ATP (circles) to actin at low ionic strength. The actin-activated ATPase activity of S1 decreased in parallel with the inhibition of S1 binding.

Tropomyosin completely restored the binding of ρPDM-S1-ATP to actin even in the presence of excess troponin (Fig. 2 *B*) but did not restore the ATPase activity. In fact, less troponin was required to inhibit the ATPase activity in the presence of tropomyosin. This result is consistent with the idea that troponin acts by influencing the position of tropomyosin rather than by producing an inhibitory effect that is amplified by tropomyosin.

Fig. 3 shows the effect of troponin I on the binding of ρPDM-S1-ATP to actin-tropomyosin (circles) and on the ATPase activity (squares). Because pure troponin I is insoluble at low ionic strength the study was limited to the case where a saturating concentration of tropomyosin was present. As with whole troponin the inhibition of ATPase activity was maximal under conditions where there was little inhibition of binding of ρPDM-S1-ATP to actin. The pattern of Fig. 3 resembles that of Fig. 2 *B* in that tropomyosin permits inhibition of ATPase activity while diminishing the effect on S1 binding.

Another measure of a blocked state is the appearance of a lag in the rate of binding of rigor S1 to actin. Because whole troponin exhibited blocking activity in the absence of tropomyosin we determined the effect of troponin on the kinetics of rigor S1 binding to actin. Light scattering was measured to follow the rate of binding at 170 mM ionic strength in the absence of Ca^{2+}. Examples of time courses for the binding of an excess of S1 to pure actin, actin-tropomyosin-troponin, and actin-tropomyosin-TnI are shown in Fig. 4 *A*. The binding of S1 to pure actin occurred with a simple time course (curve a). We observed a lag in the presence of tropomyosin-TnI (b) but with a smaller duration than obtained with tropomyosin-troponin (curve c). The effect of tropomyosin-TnI on S1 binding was slightly less than reported by Geeves et al. (2000). However, we reached the same conclusion that troponin I acts to stabilize tropomyosin in an inhibited state. We also noted that a high concentration of troponin, in the absence of tropomyosin, reduced the rate of binding slightly but did not produce a lag (data not shown). Whereas inhibition of binding of ρPDM-S1-ATP occurred with troponin only in the absence of tropomyosin, inhibition of the rate of binding of rigor S1 occurred with troponin only in the presence of tropomyosin. Therefore, an observation of regulation of the rate of rigor S1 binding does not imply that the binding of S1-ATP-like states will be blocked.

Fig. 4 *B* shows the duration of the lag as a function of the concentration of TnI at saturating tropomyosin, whole tro-

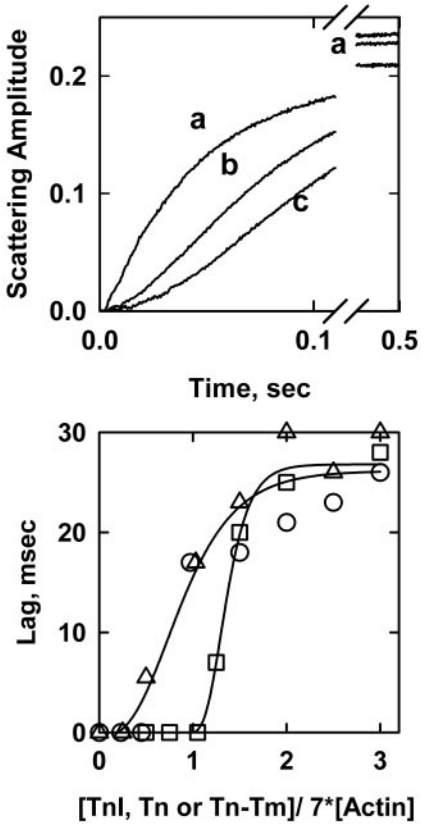

FIGURE 4 Time course of light scattering changes as S1 binds to actin-tropomyosin. (*A*) Binding of 8 μM S1 to 1 μM actin-tropomyosin (curve a), to 1 μM actin-tropomyosin-TnI (curve b), and to 1 μM actin-tropomyosin-troponin (curve c) at 25°C in a buffer containing 145 mM KCl, 20 mM imidazole, 3 mM $MgCl_2$, 2 mM EGTA, 1 mM dithiothreitol, pH 7.0. Curves b and c are sigmoidal having lags at the initial part of the time course. (*B*) Magnitude of the lag for binding of S1 to actin in the presence of varied TnI and 0.214 μM tropomyosin (*circles*), varied troponin, and 0.214 μM tropomyosin (*triangles*) or varied troponin-tropomyosin (*squares*).

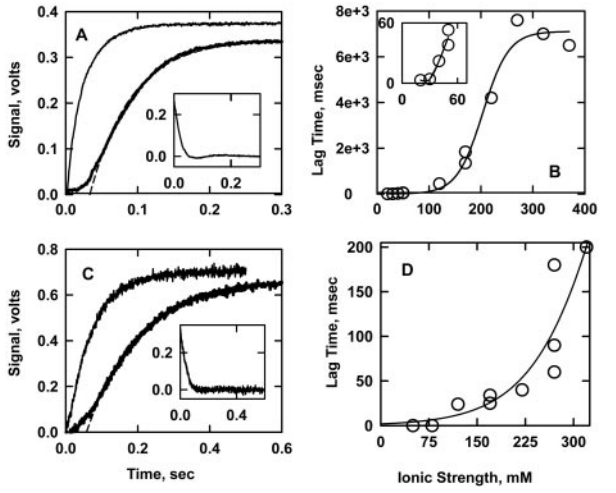

FIGURE 5 Effect of ionic strength on the rate of S1 binding to regulated actin. Binding was measured by light scattering in the presence and absence of Ca^{2+} at 25°C. Conditions: 10 mM imidazole, pH 7.0, 2 mM $MgCl_2$, 2 mM EGTA or 0.2 mM $CaCl_2$, 0 or 5 mM MgADP, 1 μM actin, 0.25 μM troponin-tropomyosin, and 8 μM S1. Ionic strength was varied from 30 to 320 mM with KCl. (*A*) S1-ADP binding at 50 mM ionic strength in the presence and absence (*bold line* with a lag) of Ca^{2+}. A single exponential function accurately describes the major part of the curve obtained in the absence of Ca^{2+} (*dashed line*). Intercept of the dashed line with the abscissa defines the lag. The inset shows the residuals (experimental curve, *fitted line*) for the data in the absence of Ca^{2+}. (*B*) Lag duration versus ionic strength for S1-ADP in the absence of Ca^{2+}. The curve through the data is arbitrary. (*C*) Binding of rigor S1 at 270 mM ionic strength in the presence or absence (*bold line*) of Ca^{2+}. The extrapolated dashed line from the exponential fit to the curve defines the lag. The inset shows the residuals for the data in the absence of Ca^{2+}. (*D*) Lag duration versus ionic strength for rigor S1 in the absence of Ca^{2+}. The solid curve is not a fit to the data.

ponin at saturating tropomyosin, and variable concentrations of the tropomyosin-troponin complex. Increasing the concentration of either TnI (triangles) or troponin (circles) at a fixed saturating tropomyosin concentration produced a hyperbolic increase in the lag duration. The effects of TnI-tropomyosin and troponin-tropomyosin were similar. In another experiment, the concentrations of both troponin and tropomyosin were varied together (squares). In this case, no lag was seen until more than 1 troponin-tropomyosin complex was added per 7 actin monomers. At that point there was a sharp increase in the duration of the lag. These results are consistent with models in which tropomyosin is a cooperative switch that impedes the binding of S1 and that troponin or TnI stabilize the tropomyosin in the inactive state. This is the more common view. The data shown here do not support models in which troponin blocks actin binding sites, whereas tropomyosin affects isomerization rates between attached states.

The results presented thus far show that regulation of the rate of rigor S1 binding does not act as a predictor of the effect on the equilibrium binding of S1-ATP-like states. We next explored the possibility that blocking of activating S1 binding occurs only above 50 mM ionic strength and that the binding of ρPDM-S1-ATP is blocked under the same conditions. We measured the lag in binding over a wide range of ionic strength conditions in both the presence of ADP and in rigor to help us identify the conditions where a blocked state is possible. Binding was assessed as an increase in light scattering after rapid mixing of S1 or S1-ADP and regulated actin in a stopped flow device (Fig. 5). In the presence of ADP and at 50 mM ionic strength a lag of 35 to 50 ms was evident in the absence of Ca^{2+}. No lag was observed in the presence of Ca^{2+} (Fig. 5 *A*). At ionic strengths below 50 mM, the lag became very small (Fig. 5 *B*). An expanded view of the ionic strength dependence of the lag duration is shown in the inset to Fig. 5 *B*. Below 30 mM ionic strength the lag was undetectable. Above 150 mM ionic strength, the duration of the lag increased greatly with increases in salt concentration. It was unclear if a real plateau was reached at 300 mM ionic strength.

The extended lag at high ionic strength could have resulted from a change in the affinity of S1 for ADP with increasing ionic strength. This artifact was ruled out by repeating the experiment in the absence of nucleotide (Fig. 5, *C* and *D*). The duration of the lag was much smaller in the case of rigor binding. No appreciable lag was observed below 120 mM ionic strength. As the ionic strength was increased above 120 mM, the duration of the lag increased as it had done in the presence of ADP (Fig. 5 *D*). These results do not support the idea that the mechanism of regulation changed at 50 mM ionic strength. Rather, there was a continuous change in lag over the entire range of conditions examined and the point at which a lag could first be observed depended on the nucleotide bound to S1. We do concur with others that there is a minimal ionic strength at which a lag is observed. A measurable the lag was observed above 30 mM ionic strength in the presence of ADP. If the binding of S1-ATP-like states is blocked under any conditions it is likely that they are blocked above 30 mM ionic strength.

The binding of ρPDM-S1-ATP to actin-tropomyosin-troponin was measured at 25, 60, and 100 mM ionic strength conditions. S1 modified with ρPDM was used to reduce the rate of ATP hydrolysis so that S1 would remain in an ATP bound state throughout the binding assay. At 25 mM ionic strength it was possible to obtain 80% saturation of the actin filament with ρPDM-S1-ATP (Fig. 6 *A*). The binding curve was hyperbolic with a fitted endpoint of 1 ρPDM-S1-ATP bound per actin monomer. At 60 mM ionic strength (Fig. 5 *B*), 60% saturation was reached in the absence of Ca^{2+} (open symbols) and the fitted endpoint was 1.0. Binding was also measured in the presence of saturating Ca^{2+} (solid symbols). There was no significant Ca^{2+}-dependence to the binding. Note that binding in the presence of Ca^{2+} was limited to lower ρPDM-S1 concentrations because ATP was depleted at higher concentrations due to residual ATPase that was Ca^{2+} sensitive.

The binding data obtained in the absence of Ca^{2+} could be fitted equally well with a simple binding isotherm and with a model that incorporates a rapid equilibrium between a blocked state (80%) and a closed state (Eq. 3). However, this model leads to the conclusion that Ca^{2+} weakens the binding of S1 to regulated actin (see Discussion).

Binding was difficult to measure at 100 mM ionic strength because of the low affinity under that condition (Fig. 6 *C*). ^{14}C-ρPDM-labeled S1 was used in this experiment to facilitate measurement of the low fraction of binding. The highest reliable measured fraction of saturation was 0.4, and the fitted endpoint was 1.0. If 80% of the actin sites were blocked toward the binding of ρPDM-S1-ATP and the blocked state were stable, one would expect to observe a maximal degree of saturation of 0.2 (dotted curve). This is not the case. If the blocked state were in rapid equilibrium with a nonblocked state (the closed state) then the degree of saturation would appear unchanged but the overall affinity would be reduced. We will show later that the measured affinity is equal to that for actin in the absence of regulatory proteins; that is the binding curve is consistent with the absence of a blocked state.

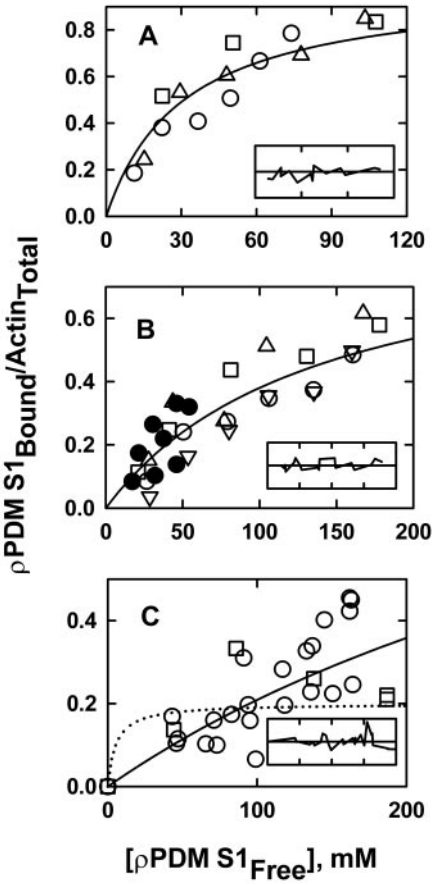

FIGURE 6 Binding of ρPDMS1-ATP to regulated actin. Reaction conditions: 10 mM imidazole, pH 7.0, 2 mM $MgCl_2$, 2 mM EGTA or 0.2 mM $CaCl_2$, and 1.6 mM MgATP (25 mM ionic strength studies) or 7.5 mM MgATP (60 and 100 mM ionic strength studies). Ionic strength was adjusted with potassium propionate because propionate salts are less disruptive of protein-protein interactions than chloride salts. Different symbols indicate different protein preparations. (*A*) 25 mM ionic strength. The binding constant is 21,000 M^{-1}. (*B*) 60 mM ionic strength. Binding in the absence (*open symbols*) or presence (*closed circles*) of calcium. The solid curve is a simulation using Eq. 3. If the blocked state was assumed to be virtually unpopulated $K_2 = 6700\ M^{-1}$ but if 80% of the actin was assumed to be in the blocked state K_2 increased to 33,600 M^{-1}. (*C*) Binding at 100 mM ionic strength was measured using both ^{14}C ρPDM (*circles*) and unlabeled ρPDM (*squares*). Fitting the data without a blocked state gave an affinity, $K_2 = 2300\ M^{-1}$. Assuming that 80% of the sites were blocked the value of K_2 became 11,000 M^{-1}. The dotted curve is the best fit to the data assuming that 80% of the sites are stably blocked (see text). The insets show the residuals for the respective binding data and a model with a single binding site with a stoichiometry of 1 S1 per actin monomer. The ordinates of the insets are ±0.4.

Conclusions drawn from Fig. 6 are valid only if the actin filament did not become activated as a result of binding of large amounts of ρPDM-S1. Several experiments were done to test for activation during ρPDM-S1 binding. One char-

TABLE 1 Rate of ATP hydrolysis by ρPDMS1 at μ = 60 mM ionic strength

[ρPDMS1], μM	ATPase Rate, s^{-1}	EGTA or Ca^{2+}
27	0.118	EGTA
82	0.087	EGTA
188	0.082	EGTA
82	0.857	Ca^{++}

acteristic of activation of regulated actin is that the rate of ATP hydrolysis per unit S1 increases with the concentration of S1 so that the rate approaches that observed in the presence of Ca^{2+}. Table 1 shows that from 27 to 188 μM ρPDM-S1-ATP, the ATPase rates were constant and roughly 10% of the rate in the presence of Ca^{2+}.

Activation of regulated actin can also be seen as an increase in both the affinity and rate of binding of S1 to actin as the occupancy of actin with S1 is increased. An example of the change in the rate of binding is a lag in binding as shown in Figs. 4 and 5. Fig. 7 *A* shows the time course of light scattering as S1 binds to actin in the absence of Ca^{2+}. In the case of unmodified S1 (curve a), there was a large and rapid increase in light scattering that reached equilibrium within 200 ms as the S1 bound to the regulated actin. This curve establishes the maximum expected extent of S1 binding. In the case of ρPDMS1 with no added ATP (curve b), there was slow increase in light scattering indicating that as the bound nucleotide was released from the ρPDM-S1 the binding became tighter and the actin filament became activated. In the presence of either 1 or 7.5 mM ATP there was no evidence for an increase in binding over the course of 1000 s.

Activation of the actin filament can also be shown by a decrease in the fluorescence of an NBD probe on troponin I (Trybus and Taylor, 1980; Greene, 1986). Fig. 7 *B* shows that the binding of unmodified S1 to regulated actin resulted in a very rapid decrease in fluorescence (curve a). ρPDMS1 binding resulted in a slow decrease in fluorescence (curve b). The slow rate may be due to the rate of release of nucleotide bound to the ρPDM-S1. In the presence of either 1 or 7.5 mM ATP (curves c and d) there was no evidence of a fluorescence change over the course of 1000 s. As a further test, ρPDMS1 was preincubated with 1 mM ATP for 1 h to promote ATP hydrolysis before being assayed in the stopped flow. Even with the longer exposure of ATP to ρPDM-S1, there was no evidence of activation of the actin filament (data not shown). These results confirm that under the conditions of our binding studies the actin filament was not activated.

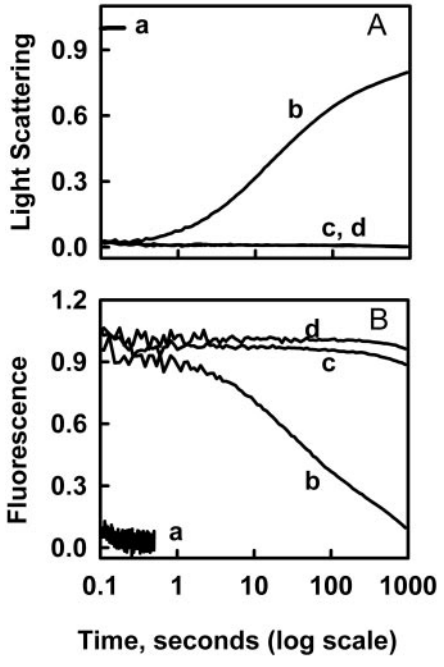

FIGURE 7 Time course of binding of S1, ρPDMS1, or ρPDMS1-ATP to regulated actin at 100 mM ionic strength, 20°C. Reaction conditions were 10 mM imidazole, 2 mM $MgCl_2$, 2 mM EGTA, and 10 μM actin, 2.5 μM troponin-tropomyosin, and 40 μM S1 or 100 μM ρPDMS1. MgATP concentrations ranged from 0 to 7.5 mM. Ionic strength was adjusted with KCl. (*A*) Light scattering traces for binding of S1 (*a*), ρPDMS1 (*b*), ρPDMS1 with 1 mM (*c*) or 7.5 mM MgATP (*d*). (*B*) Changes in IANBD-TnI fluorescence upon the binding of S1 (*a*), ρPDMS1 (*b*), and ρPDMS1 with 1 mM (*c*), or 7.5 mM (*d*) MgATP.

DISCUSSION

Fig. 1 *B* illustrates the model of regulation of contraction that we favor. In this model tropomyosin is an allosteric switch that responds to Ca^{2+} and rigor S1 to control the pathway of ATP hydrolysis (Hill et al., 1980, 1981; see Fig. 1 *B*). The Hill model correctly predicts the kinetics of ATP hydrolysis. It is unclear that other models can simulate the steady-state kinetics of ATP hydrolysis both with an excess of actin and with an excess of S1. The Hill model also incorporates the observed nucleotide-dependent difference in the binding of S1 to regulated actin. In particular, tropomyosin-troponin does not greatly inhibit the binding of S1-ATP and S1-ADP-Pi to actin even in the absence of Ca^{2+} (Chalovich et al., 1981; Chalovich and Eisenberg, 1982). In terms of the model, nonactivating myosin can bind to actin even when tropomyosin is in the fully inhibitory position with no bound Ca^{2+} (state $1_{(0)}$).

Another feature of the Hill model is that there is no assumption that the catalytic activity of pure actin is equal to that of the actin-tropomyosin-troponin complex. This provides the possibility of explaining how the ATPase rate in the presence of regulatory proteins can exceed that obtained with pure actin (Bremel et al., 1972). That is, an increase in Ca^{2+} favors state $1_{(2)}$ over state $1_{(0)}$ and increases the ATPase activity to a submaximal level. Note that in Fig. 1, *B* and *C*, all of possible substates of Ca^{2+} bound to troponin are not shown. The maximal rate of ATP hydrolysis occurs when rigor-type myosin (shown in black in

Fig. 1) binds to actin and stabilizes actin in state 2. The ATPase rate in state 2 may be higher than that observed in the absence of regulatory proteins because the regulatory proteins stabilize state 2 in these conditions. Activating S1 (S1-ADP and rigor S1) binds tighter to actin-tropomyosin-troponin that is in state 2, thus stabilizing state 2. In this model the change in affinity of strong binding or activating or rigor type S1 is important in altering the state of activity of actin. This gives rise to a different pathway where ATP hydrolysis is more rapid. That is, whereas the increase in activating myosin binding is part of the activating signal, it is the change in the pathway of hydrolysis that causes the large increase in ATPase rate.

Several shortcomings of the Hill model have been reported, and we have reexamined various aspects of the model. For example, although the Hill model explained the effect of Ca^{2+} on the equilibrium binding of S1 to regulated actin, there appeared to be a discrepancy with the Ca^{2+} effect on the rate of binding (Trybus and Taylor, 1980; McKillop and Geeves, 1993). We have recently shown that the Hill model does predict the correct kinetics of binding (Chen et al., 2001). The suggestion was also made that steric blocking occurs only above 50 mM ionic strength and that only a fraction of the potential myosin binding sites are blocked (Head et al., 1995). Furthermore, the observation that fluorescent probes on troponin are sensitive to changes in Ca^{2+} and activating S1 binding whereas probes on tropomyosin are sensitive only to S1 binding (Ishii and Lehrer, 1990) caused some to speculate that troponin might be responsible for covering the myosin binding site on actin. These later suggestions caused us to reevaluate the possible blocking of nonactivating S1 states at higher ionic strengths and under conditions where blocking of 80% of the sites could be detected. We also examined the possibility that troponin blocked the binding of S1 to actin-tropomyosin.

We examined the possibility that whole troponin or the TnI component of troponin might inhibit the binding of S1 to actin. Isolated troponin I inhibits actin activation of ATPase activity but only when bound to actin in a 1:1 ratio (Wilkinson et al., 1972; Perry et al., 1972). Our present data indicate that whole troponin does inhibit the binding of ρPDM-S1-ATP to actin in the absence of tropomyosin. The inhibition of ATPase activity is roughly correlated with the inhibition of S1 binding. However, in the presence of saturating tropomyosin, troponin and TnI have very little effect on the binding of ρPDM-S1-ATP to actin although the ATPase activity is markedly inhibited.

Troponin binds less tightly to pure actin than to actin-tropomyosin at moderate and high ionic strength (Potter and Gergely, 1974; Hitchcock, 1975). However, at low ionic strength, such as that used in Fig. 2, tropomyosin has less effect on the affinity of troponin to actin (Hitchcock, 1975). It is possible that at the highest concentrations of troponin used in Figs. 1 and 2 that the amount of troponin and TnI bound to actin may exceed the normal 1:7 ratio (Hitchcock, 1975). In the presence of tropomyosin, an amount of troponin or TnI sufficient to reduce the ATPase activity to less than 15% of the initial value has virtually no effect on the binding of ρPDM-S1-ATP to actin. A concentration of whole troponin that is 6.5 times the concentration required to give 50% inhibition of ATPase activity reduced the fraction of ρPDM-S1-ATP bound to actin by only 2%. Regardless of possible effects of having an excess troponin bound to the actin-tropomyosin the binding of S1-ATP-like states to actin was not inhibited, but the ATPase activity was inhibited normally. This can only mean that as long as tropomyosin is present (as in the normal physiological case) neither troponin nor tropomyosin inhibit nonactivating S1 binding to actin. This result is consistent with earlier studies reviewed elsewhere (Chalovich, 1992).

The commonly held view is that troponin acts indirectly by modulating the binding of tropomyosin to actin (e.g., Geeves et al., 2000); the data of Fig. 3 *B* support that view. When actin was titrated with tropomyosin-troponin no lag occurred until a nearly saturating amount of the tropomyosin-troponin complex was added. If the amount of tropomyosin added to actin was constant a lag was seen even with subsaturating amounts of troponin or TnI. Troponin either induced the binding of tropomyosin to actin or it stabilized tropomyosin in an inhibitory state. In either case the tropomyosin component of the regulatory complex is responsible for the inhibition of binding of activating S1 to actin.

The other concern that we addressed is whether the binding of nonactivating type S1 states to actin is blocked under conditions that a blocked state is supposed to exist, that is at ionic strengths greater than 50 mM. One criterion for a blocked state is a lag in binding of S1 to regulated actin in the absence of Ca^{2+} when S1 is in excess over actin (Trybus and Taylor, 1980). This lag in binding has been interpreted as a delay caused by the transition from the "blocked" state to the "closed" state of actin (McKillop and Geeves, 1993). Binding studies used to argue for a blocked state were done with S1 or S1-ADP, but the assumption was made that the blocked state applied to the binding of S1-ATP-like states also. This was contrary to earlier studies that showed binding of S1-ATP-like states to regulated actin at ionic strengths $\geq$50 mM both in solution (Chalovich and Eisenberg, 1982; El-Saleh and Potter, 1985) and in single muscle fibers (Kraft et al., 1995). Furthermore the high level of regulation of ATPase activity at low ionic strength (Chalovich and Eisenberg, 1982) is inconsistent with the loss of a major contributing factor to regulation under those conditions. More recently there has been recognition that S1-ATP-like states may be different from the low affinity intermediate observed in experiments done with rigor S1 (Holmes, 1995; Geeves and Conibear, 1995). However, this has not been tested systematically.

To establish the conditions necessary to produce a blocked state we examined the rate of binding of rigor S1

and S1-ADP to regulated actin over a range of ionic strength conditions. We did not observe a discrete change in the lag as might be expected if a blocked state occurred only above 50 mM ionic strength. Rather, the lag duration increased continuously as the ionic strength was increased. The first evidence of what may be called a lag appeared by 30 mM ionic strength in the presence of ADP. The lag duration increased above 30 mM but the greatest change in the lag occurred when the ionic strength was increased above the physiological value (≈150 mM).

The change in lag duration was most likely the result of a change in the regulated actin filament. This change could be in the number of bound molecules of S1 required to stabilize the active state, the rate of transition from the inactive to the active state, or the equilibrium constant between the two states. Preliminary simulations suggest that changes in the affinity or rate of association of S1 to actin have little effect on the lag. Any explanation of the reason for the ionic strength dependence of the lag will be model dependent. Chen et al. (2001) have shown that the Hill model (Hill et al., 1980, 1981) is able to simulate the lag in binding and the effect of Ca^{2+} on the rate of S1 binding without incorporating a blocked state. A detailed analysis of the lag by different models will be presented elsewhere. At present it is sufficient to note that what may be called a blocked state exists at ionic strengths greater than 30 mM.

To determine the existence of a blocked state that is refractory to the binding of the nonactivating S1 to regulated actin we measured the binding at several conditions. Binding was measured at increasing concentrations of ρPDM-S1-ATP so that high levels of saturation could be achieved allowing the examination of blocking of a fraction of the total binding sties. The data were well described by a simple hyperbolic binding mechanism at 25, 60, and 100 mM ionic strength. In no case could the data be fitted with a model in which 80% of the S1 binding sites of actin were stably blocked in the absence of Ca^{2+}. This can be seen even in the case of the highly scattered binding at 100 mM ionic strength (Fig. 6 *C*, dotted curve).

If, on the other hand, there were a rapid equilibrium between blocked and nonblocked states then the binding constants would change in a discontinuous way to compensate for the loss of available sites on actin. Association constants calculated using a simple binding model with no blocked state are shown as solid circles in Fig. 8. The ionic strength dependence of these constants is the same as for the binding to actin in the absence of regulatory proteins where no blocked state is possible. This too shows that the binding of nonactivating S1 to regulated actin is not blocked. Another point to consider is that at 60 mM ionic strength, where blocking is supposed to occur, the affinity of binding of ρPDM-S1-ATP to actin is the same in the presence and absence of Ca^{2+} (Fig. 6) even though the population of the blocked state is supposed to be very small in the presence of Ca^{2+}. Note that we have restricted ourselves to a discussion

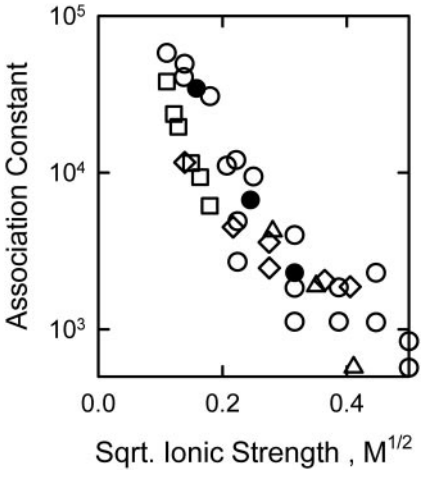

FIGURE 8 Ionic strength dependence of nonactivating S1 binding to actin. The constants for the binding of high concentrations of ρPDMS1 to regulated actin (*solid circles*) were calculated from Eq. 3 assuming that virtually none of the actin was in a blocked state (from Fig. 6). These data are compared with results of single point measurements of ρPDMS1-ATP binding to actin with actin in large excess over pPDM-S1-ATP so that very little of the actin contains bound ρPDM-S1-ATP (*open circles*). The diamonds represent the binding of pPDM-S1-ATP to actin in the absence of troponin-tropomyosin (Kirshenbaum et al., 1993). Also shown is the binding of S1-ATP to actin with actin in large excess over S1 (*squares*) (taken from Chalovich et al., 1983; Highsmith and Murphy, 1992; Frisbie et al., 1998). The triangles are for the binding of S1 to pure actin in the presence of saturating AMP-PNP where S1 forms a nonactivating state (Frisbie et al., 1998).

of myosin S1 because this fragment has been used for the generation of both the Hill model and the McKillop and Geeves model.

It was possible to simulate individual binding curves with a model in which blocked and nonblocked states were in rapid equilibrium. That is, theoretical curves for Fig. 6 were similar for rapid equilibrium models whether we assumed that either 0% or 80% of the actin sites were blocked (Eq. 3). However, the results of a rapid equilibrium blocked model were unreasonable. The calculated binding constants for the nonblocked states have to increase by a factor of 5 to compensate for the effective lowering of the actin concentration caused by blocking. That is, in the absence of Ca^{2+}, the affinity of the nonblocked states for S1 is fivefold greater than in the presence of Ca^{2+}. Such behavior is inconsistent with all current models of regulation.

Our data do not support the concept that regulation by tropomyosin-troponin occurs primarily by blocking the binding of all myosin states to actin. Nonactivating states of S1 bind to regulated actin under conditions where the binding of activating states is inhibited or "blocked". The transition from nonactivating "weak binding" states to activating "strong binding" states appears to be regulated. We suggested earlier that this could occur either by inhibition of the rate of phosphate release (Chalovich and Eisenberg, 1982) or by inhibiting the transition of actin from an inhib-

ited state to an active state (Hill et al., 1981). This topic was reviewed in some detail earlier (Chalovich, 1992). Others have suggested that the position of tropomyosin in relaxed muscle might allow binding of an electrostatic "collision complex" of myosin to actin but partially block the stronger binding that involves electrostatic and hydrophobic interactions (Holmes, 1995; Geeves and Conibear, 1995). The complex that we have described here and elsewhere does not appear to be a collision complex (for details on the S1-ATP state, see Chalovich, 1992). In the Hill model S1-ATP and S1-ADP-Pi are discrete states. However, in that model it is possible to incorporate a two-step binding of activating-S1 to actin (electrostatic followed by electrostatic and hydrophobic) to accommodate the details of both types of interaction (Chen et al., 2001). Thus, for this reason and others stated earlier, the Hill model remains a reasonable framework for understanding regulation of striated muscle contraction.

The role of tropomyosin in this striated muscle regulatory system appears to be different from its role in conjunction with smooth muscle caldesmon. In the case of actin-tropomyosin-caldesmon, it is the caldesmon that is responsible for inhibition of the rate of binding of S1 to actin (Sen et al., 2001). Furthermore, tropomyosin-troponin has a much greater effect on the equilibrium binding of activating states (i.e., S1-ADP and S1) than on nonactivating states, whereas caldesmon has its greatest effect on the equilibrium binding of nonactivating states (Chalovich et al., 1987). Tropomyosin may actually enhance the ability of caldesmon to compete with activating S1 binding to actin (Chen and Chalovich, 1992). These different functions of tropomyosin may result from the different positions that tropomyosin occupy on actin in the presence of caldesmon and in the presence of troponin (Hodgkinson et al., 1997).

CONCLUSIONS

The data shown here are consistent with the Hill model shown in simplified form in Fig. 7. The Hill model can also simulate the cooperative equilibrium binding of S1 to actin (Hill et al., 1980), the steady-state ATPase activity at both high actin and high S1 conditions (Hill et al., 1981), the relationship between the rate of force redevelopment, the extent of activation of single muscle fibers (Brenner and Chalovich, 1999), and also the lag in the rate of binding in the absence of Ca^{2+} (Chen et al., 2001). In the Hill model the primary function of the movement of tropomyosin is to alter the ability of the actin filament to accelerate ATP hydrolysis. That is, the real importance of the overlap between the inhibitory positions of tropomyosin on actin and the strong binding or activating states of S1 may be that this arrangement permits S1 to act as a switch, in addition to Ca^{2+} to alter the activity of the actin filament.

We wish to thank Michael Vy-Freedman and Anmei Cai for their excellent assistance. The National Institutes of Health grant AR40540 (to J.M.C.) supported this research.

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