Regulation of the Interaction between Actin and Myosin Subfragment 1: Evidence for Three States of the Thin Filament

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ABSTRACT Equilibrium titrations and kinetic experiments were used to define the cooperative binding of myosin subfragment 1 (S1) to actin-troponin-tropomyosin. Both types of experiment require an equilibrium between two states of the thin filament in which one state (the off state) binds S1 less readily than the other. Equilibrium titrations are compatible with >95% of the actin$_7$·Tn·Tm units being in the off state in the absence of calcium and 80% in the off state in the presence of calcium. Kinetic binding data suggest that the presence of calcium switches the thin filament from 70% in the off state to <5%. The two experiments, therefore, define quite different populations of the off states. We propose a three-state model of the thin filament. A "blocked state" which is unable to bind S1, a "closed state" which can only bind S1 relatively weakly and an "open state" in which the S1 can both bind and undergo an isomerization to a more strongly bound rigor-like conformation. The equilibrium between the three states is calcium-dependent; K_B = [closed]/[blocked] = 0.3 and ≥ 16 and K_T = [open]/[closed] = 0.09 and 0.25 in the absence and presence of calcium, respectively. This model can account for both types of experimental data.

INTRODUCTION

The interaction between actin and myosin subfragment 1 (S1) in vertebrate striated muscle is regulated by the thin filament proteins tropomyosin (Tm) and troponin (Tn) (1). The steric blocking model is the most commonly quoted molecular interpretation of this regulation mechanism (2, 3) in which tropomyosin in the absence of calcium, physically blocks the myosin binding site on actin. Calcium binding to troponin causes a conformational change in the thin filament which results in tropomyosin moving over the surface of actin to a nonblocking position. In recent years the strongest evidence in favor of this interpretation has come from time-resolved x-ray scattering studies of intact muscle (4). These studies have been interpreted in terms of a change in structure of the thin filament which occurs before any significant attachment of myosin heads (crossbridges) to the thin filament and this significantly precedes force development.

The above model however appears to conflict with several studies of the behavior of the purified proteins in solution (5–7). Of particular significance is the observation that at low ionic strengths calcium binding to troponin regulates the acto·S1 ATPase but does not affect the degree of complex formation between S1·ATP and actin·Tm·Tn (8). This result led to the proposal that Tm/Tn regulates the ATPase not by controlling the association between actin and S1 but by controlling the rate-limiting P_i release step of the ATPase cycle.

In solution the binding of S1 to actin·Tm·Tn is cooperative both in the presence and absence of calcium (9). This has been interpreted in terms of a two state model of the thin filament by Hill et al. (10) in which the cooperative unit is the seven actin monomers bridged by a single Tm. In this model the actin is predominantly in the off state (weak S1 binding) in both the presence and absence of calcium. Calcium binding to Tn changes the equilibrium constant between on and off states of the thin filament from around 0.02 to 0.2 (9, 11). Thus calcium binding alone is not sufficient to turn on the thin filament. S1 binding is required in order to switch the thin filament completely into the on state. This observation is incompatible with a simple steric blocking model where calcium binding removes the block before S1 binds.

We have suggested a different interpretation of the cooperative binding of S1 and S1-nucleotide complexes to regulated actin (12, 13). The binding of S1 and S1-nucleotides to pure actin filaments has been shown to occur in at least two steps (14)

$$A + M \underset{K_1}{\rightleftharpoons} A\text{–}M \underset{K_2}{\rightleftharpoons} AM \qquad (1)$$

where step 1 results in formation of relatively weakly bound actin and strongly bound nucleotide (the A state). This complex isomerizes in step 2 to give strongly bound actin and weakly bound nucleotide (the R state). The formation of the R state is required both to accelerate the S1 ATPase rate and to generate mechanical force in muscle (for review see Ref. 15).

We proposed a model in which Tm·Tn regulates S1 binding to actin by controlling the isomerization step (Fig. 1*A*). Only the open conformation of the thin filament allows formation of the R state and so only the open conformation will accelerate the S1 ATPase rate and generate mechanical force. This is compatible with the work of Rosenfeld and Taylor (16) who proposed that the primary effect of regulation is on a conformational change which determines the rate of dissociation of ligands from the myosin active site.

This model is a special condition of the earlier Hill et al. model, identical in mathematical form but ascribing a specific role to the A to R isomerization of the acto·S1 complex.

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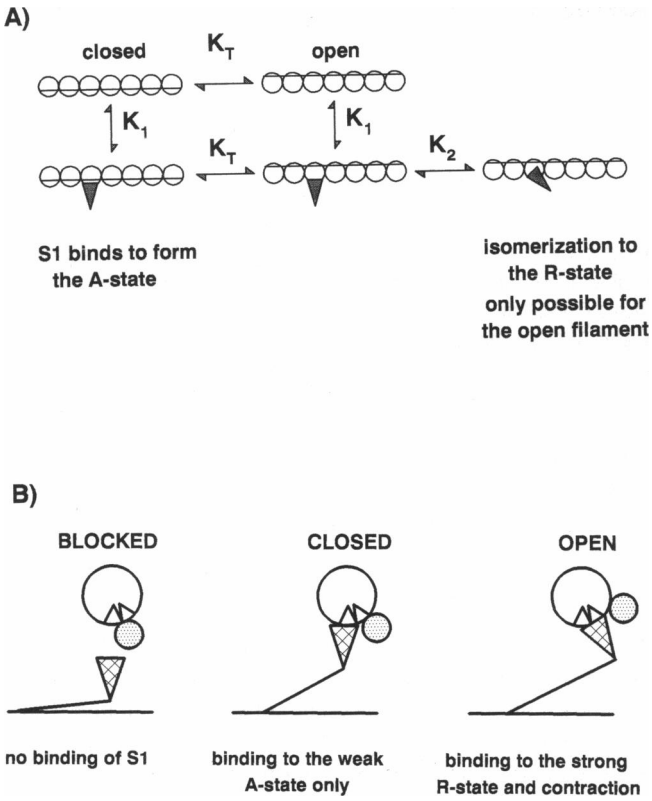

FIGURE 1 The two- and three-state models of the thin filament. (*A*) The two-state model of the thin filament where the A_7·Tm·Tn structural unit is shown as seven open circles connected by a linear tropomyosin. This unit exists in a dynamic equilibrium between the open and closed states shown as two positions of the line representing Tm. S1 (▼) can bind to both states with equal affinity to form the acto·S1 A state (shown as binding at 90° to filament axis, equilibrium constant K_1) but only the open filament allows the isomerization to the R state (drawn as 45° binding, equilibrium constant K_2). The ratio of open to closed structural units is defined by K_T in the absence of any bound S1. With a single S1 bound per A_7·Tm·Tn unit the ratio is $K_T(1 + K_2)$, for two S1 bound $K_T(1 + K_2 + K_2^2)$, for 3 S1 bound $K_T(1 + K_2 + K_2^2 + K_2^3)$, etc. (*B*) A diagrammatic representation of a direct steric blocking version of the three-state model. Formally this is identical to the two-state model with the addition of the blocked state which does not bind S1. As shown here in the blocked state, Tm prevents all binding of S1 to actin. In the closed state, Tn moves to uncover a binding site which allows S1 to form the relatively weakly bound A state. In the open state the full interaction of S1 with actin is possible and the R state is occupied. No attempt has been made to represent accurately the three-dimensional relationship between the three proteins and the evidence is just as compatible with an indirect effect of Tm on the two binding states of the crossbridge.

In addition, it did not require direct regulation of the rate of phosphate release (as proposed by Chalovich et al. (8), but only a binary switch between a state which could or could not form the R state with myosin. A second difference to the Hill et al. model was that we found no compelling evidence for interactions between tropomyosins and therefore analyzed the experimental data without including this extra cooperativity (12, 13).

As a test of this model the equilibrium constants (K_1, K_2) for the binding of various S1-nucleotide complexes to A_7Tm·Tn were measured in a manner which was independent of the model. The cooperative binding isotherms could then be defined by the model by fitting K_T, the equilibrium constant between the two forms of the A_7Tm·Tn units. This was shown to be independent of the nucleotide bound to S1 for a range of values of K_1 and K_2 which varied by a factor of 100 (13).

However, as noted by McKillop and Geeves (13), although this model could describe all of the equilibrium binding data, there remained the conflict with the time-resolved x-ray data on intact muscle (4). In addition solution studies with purified proteins showed that the kinetics of binding S1 to regulated thin filaments could not be accommodated. Trybus and Taylor had shown that the rate of S1 binding to actin·Tm·Tn in the presence of calcium was identical to that of S1 binding to pure actin filaments (17). In the absence of calcium the binding transients showed a marked lag phase which could be abolished by preincubating with 1–2 S1 molecules per A_7Tm·Tn unit. The simplest interpretation of the data is that a substantial fraction (≈70%) of the actin is unavailable for S1 to bind to in the absence of calcium until an S1 binds and traps the A_7Tm·Tn cooperative unit in the "on" state.

In the presence of calcium almost all of the sites (>90%) are available. Thus the kinetic data suggest that binding calcium switches the thin filament from 30 to >90% in the "on" state. This conflicts with the equilibrium data which suggests that in the presence of calcium only 20% of the cooperative units are in the "on" (strong S1 binding) state and <5% are in the "on" state in the absence of calcium.

We present here a model and experimental evidence supporting this model which can accommodate all of the above experimental findings from solution experiments. In essence the model is similar to the earlier Geeves and Halsall model (12) with the addition that the closed state of the thin filament can be subdivided into two states giving three states of the thin filament overall (Fig. 1).

THE MODEL

The thin filament is assumed to exist as a dynamic equilibrium between three states as shown in Fig. 1*B*. A *blocked state* which does not bind S1 significantly, a *closed state* which can only bind S1 to form the A state, and an *open state* which can bind S1 in the A state and isomerize to the R state. The equilibrium between the three states is defined by

$$K_B = [A_7\text{Tm}\cdot\text{Tn}]_{\text{closed}}/[A_7\text{Tm}\cdot\text{Tn}]_{\text{blocked}} \quad (2)$$

and

$$K_T = [A_7\text{Tm}\cdot\text{Tn}]_{\text{open}}/[A_7\text{Tm}\cdot\text{Tn}]_{\text{closed}}. \quad (3)$$

There is no direct equilibrium between the blocked and open states. Calcium binding to Tn can influence either equilibrium constant.

The other assumptions of the Geeves and Halsall (12) model are retained, i.e., 1) Cooperative unit is the seven actins bridged by a single Tm; 2) Each actin monomer acts independently of its neighbor in binding S1; 3) S1 binds to

actin in two steps; the formation of the A state is identical for closed and open A_7Tm·Tn units.

The equilibrium binding of S1 to regulated actin

The equations to define the equilibrium binding of S1 to actin in a titration were described by McKillop and Geeves (12) for the two-state model and for the three-state model are:

$$\text{Fraction of total actin sites occupied} = \frac{K_1[M](K_T(1 + K_2)P^6 + Q^6)}{K_T P^7 + Q^7 + 1/K_B} \quad (4)$$

where $[M]$ is the concentration of free myosin heads and $P = 1 + K_1[M](1 + K_2)$ and $Q = 1 + K_1[M]$. Fraction of actin sites with tightly bound S1, i.e.,

$$\text{R states} = \frac{K_T K_1 K_2 [M] P^6}{K_T P^7 + Q^7 + 1/K_B}$$

and this is related to the fractional change in pyrene fluorescence,

$$a = \frac{F_0 - F}{F_0 - F_\infty} = \frac{[\text{R states}]}{[\text{R states}]_{max}} = \frac{K_1[M]P^6(K_T(1 + K_2)^7 + 1)}{(K_T P^7 + Q^7 + 1/K_B)(1 + K_2)^6} \quad (5)$$

If $K_B \gg 1$ (i.e., no significant occupancy of the blocked state) then these equations are identical to those for the two-step model. (*Nota bene*: in the McKillop and Geeves paper the last term in the numerator of Eq. 6 was misquoted as K_2 in place of 1).

In principle the two- and three-state models can be distinguished by comparing light scattering titrations (which measure total occupied sites) and pyrene fluorescence titrations which monitor the number of R states formed (14, 18).

Kinetics of S1 binding to regulated actin

The kinetics of S1 binding to actin can be defined if the rates of the interconversion between the three states of the thin filament and the rate of step 2 are rapid compared to the rate of S1 binding in step 1, i.e. $(k_{+B} + k_{-B})$, $(k_{+T} + k_{-T})$ and $(k_{+2} + k_{-2}) \gg ([S1]k_{+1} + k_{-1})$.If the blocked state is not significantly occupied (assumed to be the case in the presence of calcium) then the rate of binding is defined by

$$-dA/dt = [A][S1]k_{+1} - k_{-1}[A\text{–}M] \quad (6)$$

where $[A - M]$ is the concentration of actin sites with weakly bound S1, i.e., A states. These are negligible when K_2 is large as it is in the absence of nucleotide. When $[S1] \gg [A]$ then an exponential process will be observed with $k_{obs} = [S1]k_{+1}$ identical to that seen in the absence of Tm·Tn. Both light scattering and pyrene fluorescence signals will give the same result.

In the absence of calcium the blocked state is occupied and the initial rate of binding will be slowed due to the lower concentration of available actin sites

$$-\frac{dA}{dt} = [S1][A]\left\{1 - \frac{1}{1 + K_B(1 + K_T)}\right\}k_{+1} - k_{-1}[A\text{–}M]. \quad (7)$$

Thus the initial rate of binding will be slower by a factor of $1 - 1/(1 + K_B(1 + K_T))$ and then accelerate (to that seen in the presence of calcium) as the binding of S1 to form R states shifts the equilibrium between the A_7·Tm·Tn units in favor of the open states.

The slow initial binding can be defined more readily by monitoring the reaction under conditions where $[A] \gg [S1]$. In the presence of calcium a single exponential will be observed as for the excess [S1] experiment $k_{obs} = [A]k_{+1}$. However, in the absence of calcium if only one S1 binds per A_7·Tm·Tn unit no acceleration will occur. A single exponential will be observed with $k_{obs} = [A]k_{+1}\{1 - 1/(1 + K_B(1 + K_T))\}$. If K_2 is sufficiently large to result in the majority of bound S1s being in the R state, then the reverse reaction is negligible. Thus the ratio of observed rate constants in the absence and presence of calcium defines $1 - 1/(1 + K_B(1 + K_T))$.

MATERIALS AND METHODS

Proteins

Myosin subfragment 1 was prepared by chymotryptic digestion of rabbit myosin (19). F-actin was prepared by the method Lehrer and Kewar (20) and labeled with pyrene iodoacetamide as described previously (21). Troponin and tropomyosin were prepared and copurified as described by Murray (22).

Spectroscopic measurements

Fluorescence titrations were carried out on a Perkin-Elmer LS-5B fluorimeter with the optics as described by McKillop and Geeves (13). Light scattering measurements were made in the same apparatus with both monochromators set to 405 nm. Initial titrations were performed with the constant speed motor-driven titrator described previously (13). Later titrations collected both light scattering and fluorescence data simultaneously during a titration using software running on an Epsom PC to control both data collection, monochromators and a Harvard Apparatus stepper motor titrator. The software allows a controlled volume of S1 to be added, and the fluorescence signal was monitored until it was judged to be stable. The monochromators are then changed to the wavelengths appropriate for light scattering and the stable reading recorded. The change in signal on the addition of S1 is used to estimate the volume of actin to be added in the next aliquot. This allows precise definition of both hyperbolic and sigmoid titration curves. Stopped flow measurements were made as described by McKillop and Geeves (13).

Curve fitting routines

Kinetic data were fitted to exponentials using a nonlinear least-squares fitting routine (23).

The general approach to fitting titration data was described in detail by McKillop and Geeves (13) for the two-step model, and an identical procedure was followed here using Eqs. 4 and 6 for the three-step model.

RESULTS

A key feature of both the two- and three-state models is that the equilibrium between the states of the thin filament is independent of the presence of nucleotide in the S1-active site. The equilibrium between the A and R states (defined by K_2) is however dependent on nucleotide. Thus titration experiments can be used to define K_T and K_B in the absence of nucleotide, and the same values should be obtained if the experiment is repeated in the presence of nucleotides or nucleotide analogues. This formed the basis of the test of the two-state model as presented previously (13). Here we wish to distinguish between the two- and three-state models described above. Under conditions where $K_2 \gg 1$ then titrations of S1 binding to A_7·Tm·Tn show sigmoid isotherms in the presence and absence of calcium, and both models predict identical sigmoid binding curves for both pyrene fluorescence and light scattering signals. This is illustrated in Fig. 2 which shows fluorescence titrations of pyrene actin with S1 and S1·ADP in the presence and absence of calcium. This is the same titration which was fitted to the two-state model in our previous paper (Ref. 13; Figs. 1 and 6). Data from light scattering titrations were superimposable on the fluorescence signals. The best fit to the three-state model is superimposed on the fluorescence signals. The titration curves do not contain sufficient information to define uniquely all four equilibrium constants. For these fits, K_1 in the absence of ADP and K_2 in the presence of ADP are well defined by independent solution experiments (13). K_B was defined as 0.3 in the absence of calcium (see Discussion) and 100 (an arbitrary value greater than 16; see Discussion) in the presence of calcium. The best fit was then obtained for the remaining two constants. This illustrates that, although a unique solution to the values cannot be defined, under these conditions where K_2 is estimated to be 200 and 18 in the absence and presence of ADP, respectively, both models can adequately describe the binding isotherms. Thus these titrations cannot distinguish between a thin filament in the blocked and closed states.

Under conditions where $K_2 \ll 1$ and then the R state is not substantially occupied, only weak binding of S1 to the A state is possible and both models predict hyperbolic binding isotherms in the presence of calcium. In the absence of calcium the two-step model would predict hyperbolic binding curves but sigmoid binding is possible in the three-state model. In theory this weak binding can be monitored in light scattering titrations but in practice the protein concentrations required at the ionic strength used here make the experiment impractical.

The two models can be distinguished when K_2 is close to 1. Under these conditions if there is no blocked state (i.e., the two-state model) then in a titration the light scattering signal should approximate to a hyperbola and lead the pyrene fluorescence signal which will be sigmoid. If a blocked state is present (only possible in the three-state model), then the lead of light scattering over fluorescence will be reduced or eliminated and the light scattering signal would be more obviously sigmoid. To test the model we need to define conditions where K_2 has a value close to one.

Butanedione monoxime (BDM) has been shown to inhibit the myosin and actomyosin ATPase (30), and this inhibition is compatible with the formation of a M·ADP·P_i·BDM quaternary complex (Ref. 31 and McKillop and Geeves, submitted for publication). We have examined the binding of actin to this complex and shown that a stable A·M·ADP·P_i·BDM complex can be formed with an apparent dissociation constant for actin of 11 μM and a K_2 of 0.7 in the absence of Tm·Tn. The presence of Tm·Tn and calcium increases K_2 to 3. These values of K_2 are obtained from kinetic and equilibrium experiments which are independent of the cooperative model assumed (13).

Fig. 3 shows fluorescence and light scattering of the titrations in the presence of BDM, ADP, and phosphate. Both are sigmoid, and there is only a small lead of light scattering over fluorescence. As discussed above this similarity in the light scattering and fluorescence titrations cannot be accommodated by the two-state model but is consistent with the

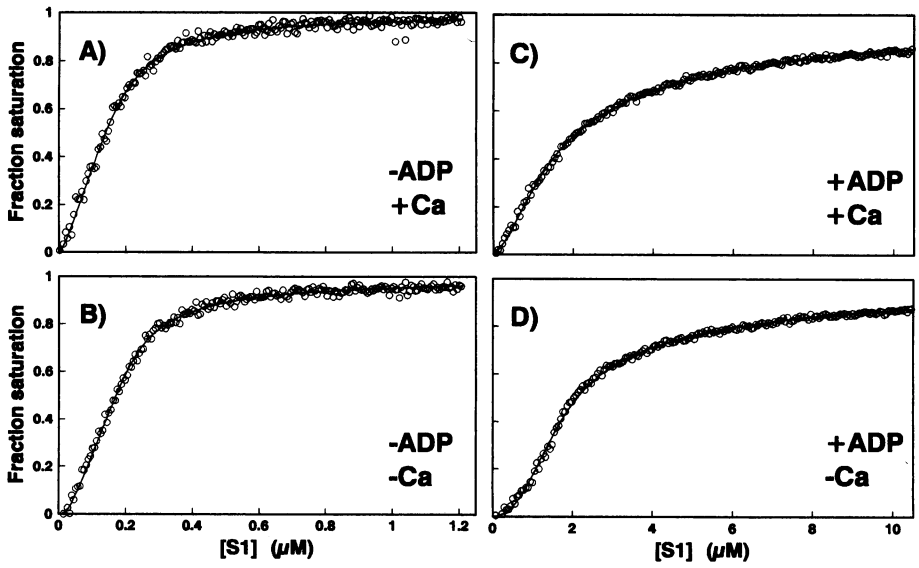

FIGURE 2 Fluorescence titrations of pyr·actin · Tm·Tn with S1. S1 was added continuously to a stirred cuvette containing pyr·actin Tm and Tn in the absence (*A* and *B*) and presence of ADP (*C* and *D*) and in the presence (*A* and *C*) and absence (*B* and *D*) of calcium. A fit to the three-state model is superimposed. Details of the fits are described in Table 1. Titration conditions: (*A* and *B*) 0.2 μM pyrene actin, 0.11 μM Tm·Tn; (*C* and *D*) 0.5 μM pyr·actin, 0.27 μM Tm·Tn, 2 mM ADP and 50 μM Ap_5A, 2 mM glucose, and 1 μM hexokinase to eliminate contaminant ATP. Buffer: 20 mM 4-morpholinepropanesulfonic acid, 140 mM KCl, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, pH 7.0, 20°C. In addition, the buffer contained either 2 mM $CaCl_2$ or 2 mM EGTA.

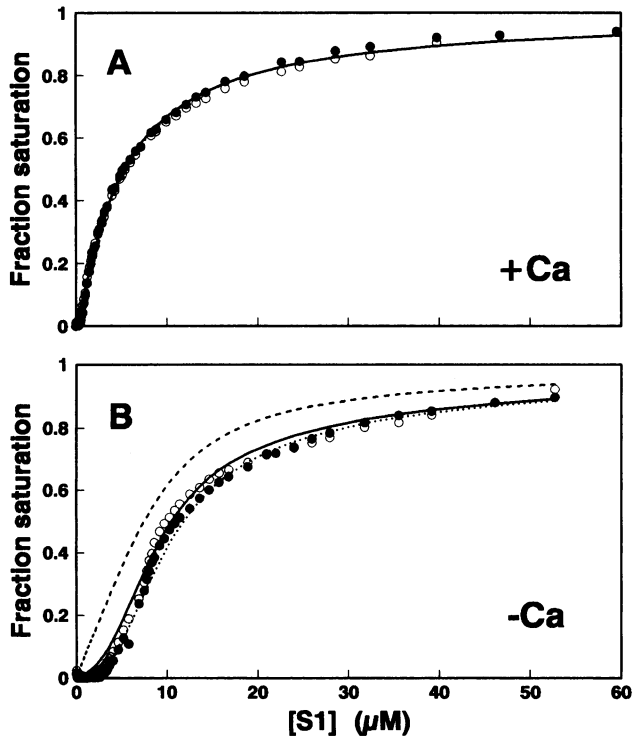

FIGURE 3 Titrations of pyr·actin·Tm·Tn with S1 in the presence of ADP, phosphate, and BDM. Titrations were performed with simultaneous measurement of light scattering (○) and fluorescence (●), and the scattering signal has been corrected for the scattering of S1 alone. The best fits to the three-state model are superimposed on *A* and *B* (*solid line*, light scattering; *dotted line*, fluorescence). In *B* the dashed line represents the fit to the light scattering for the two-state model with the fluorescence identical to the fit for the three-state model. Fitted parameters: (*A*) $K_1 = 5.5 \times 10^4$ M^{-1}, $K_2 = 3$, $K_T = 0.6$ and $K_B = 100$; (*B*) $K_1 = 4.0 \times 10^4$ M^{-1}, $K_2 = 3$, $K_T = 0.09$ and $K_B = 0.1$. Titration conditions: 0.5 μM pyr·actin, 0.27 μM Tm·Tn, 2 mM ADP, 90 mM P_i, 20 mM BDM, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, pH 7.0, 20°C. Hexakinase, glucose, Ap_5A, 2 mM $CaCl_2$, or EGTA were added to *A* and *B*, respectively.

three-state model. Fitting the data from both signals using the three-state model (as for Fig. 2) suggest a value of $K_B = 0.1$ and $K_T = 0.09$ in the absence of calcium and >10 and 0.6, respectively, in the presence of calcium. The form of the light scattering titration predicted from the two-state model is shown in Fig. 3 *b*, parameters used to calculate the curve were chosen so that the fluorescence data would superimpose on the curve predicted from the three-state model. The values of the constants obtained for the fit to the three-state model are compared with those obtained by refitting the data of McKillop and Geeves in Table 1 and are discussed below.

Thus the titration data in the presence of ADP, P_i, and BDM requires a three-state model. A more rigorous test of the model comes from analysis of the kinetic data which was previously known to be incompatible with the two-state model as defined by equilibrium titration experiments.

Kinetics

The observed reaction when an excess of S1 is mixed with actin·Tm·Tn both in the presence and absence of calcium is shown in Fig. 4 *a*. In the absence of Tm·Tn or in the presence of calcium single exponential processes are observed with similar observed rate constants as already reported (light scattering (17); pyrene fluorescence (24)). In the absence of calcium, a pronounced lag is seen in both the scattering and fluorescence signals. It is difficult to define the initial rate of S1 binding in these curves with any precision, but clearly the

TABLE 1 The calcium and nucleotide dependence of the equilibrium constants for the three-state model

	Calcium	K_1	K_2	K_T	K_B
		M^{-1}			
No nucleotide	+	1.3×10^5	**241**	**0.03**	100
No nucleotide	–	1.3×10^5	**188**	**0.013**	0.3
Phosphate	+	2.4×10^4	**79**	**0.57**	100
Phosphate	–	2.4×10^4	**64**	**0.18**	0.3
Sulfate	+	2.1×10^4	**286**	**0.09**	100
Sulfate	–	2.1×10^4	**237**	**0.07**	0.3
ADP	+	**3.1×10^4**	18	**0.24**	100
ADP	–	**3.6×10^4**	18	**0.12**	0.3
and BDM and Phosphate	+	**5.5×10^4**	3	**0.60**	100
and BDM and Phosphate	–	**4.0×10^4**	3	**0.09**	0.3

Numbers in *bold* were allowed to vary during the fitting procedure.

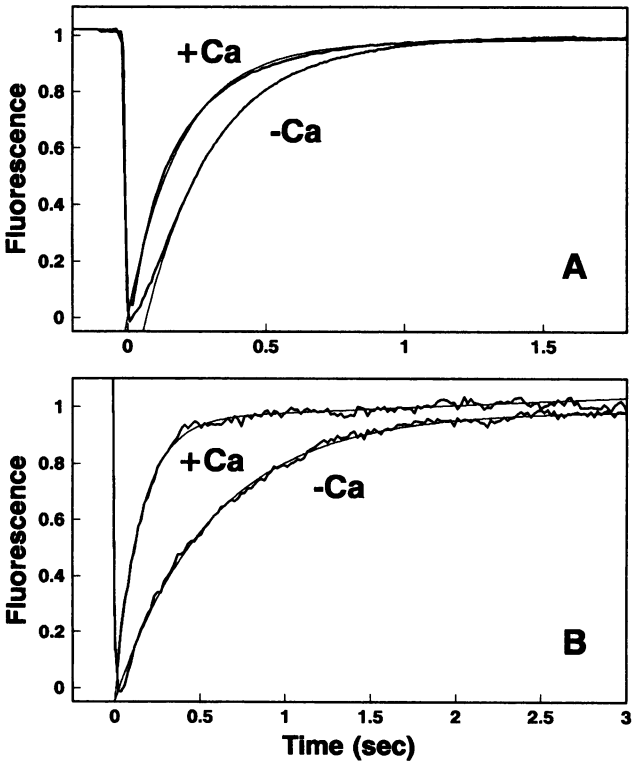

FIGURE 4 The observed fluorescence changes on S1 binding to actin·Tm·Tn in a stopped flow fluorimeter. (*A*) Mixing 5 μM S1 with 1 μM pyr·actin and 0.17 μM Tm·Tn in the presence and absence of calcium. A single exponential is fitted to both curves + Ca $k_{obs} = 5.5$ s^{-1} fitted to the whole curve. –Ca $k_{obs} = 3.6$ s^{-1} fitted to the last 30% of the amplitude and extrapolated to zero time. (*B*) Mixing 0.5 μM S1 with 5 μM actin and 0.75 μM Tm·Tn in the presence and absence of calcium. A single exponential is fitted to the whole reaction in both cases. Ca $k_{obs} = 6.5$ s^{-1}, –Ca $k_{obs} = 1.7$ s^{-1}. Other conditions as for Fig. 2.

data are compatible with a substantial fraction of the actin sites being initially unavailable for S1 to bind. It is easier to analyze the results in an experiment where actin is in excess of S1.

Such an experiment is shown in Fig. 4 *b*, where S1 is mixed with a 5-fold excess of actin. Under these conditions, results in the absence of Tm·Tn, and in the presence of calcium and Tm·Tn, are again similar and compatible with the second order rate constant determined with excess S1. In the absence of calcium a single exponential process is observed with a rate constant of 1.5 compared to 5.5 s^{-1} in the presence of calcium. The observed rate constant was independent of the ratio of actin to S1 over the range 5:1 to 20:1. The three-state model is compatible with this result if $1 - 1/[1 + K_B(1 + K_T)]$ is $1.5/5.5 = 0.27$ as defined above.

It can be seen from the kinetic equation derived above that the model predicts that the difference in observed rates in the presence and absence of calcium is independent of the value of $[A]k_{+1} + k_{-1}$. It is difficult to change the actin concentration over a wide range to test this feature of the model. However, the association rate constant can be changed by adding sulfate to the reaction buffer (13). Sulfate has been shown to bind to the myosin active site and to reduce the association rate constant k_{+1} significantly (25, 26). Under otherwise identical conditions to the experiments of Fig. 4, *a* and *b*, the observed reaction showed a clear lag phase for the excess S1 measurement in the absence of calcium (Fig. 5 *a*). In the excess actin experiment, single exponentials were observed in the presence and absence of calcium with observed rate constants of 0.87 and 0.21 s^{-1}, respectively (Fig. 5 *b*). Thus the presence of sulfate reduced the observed rates more than 7-fold, but the ratios of the observed rates ± calcium were 0.27 and 0.24 in the absence and presence of sulfate, respectively.

Thus both in the presence and absence of sulfate the three-state model would predict that approximately 74% of the actin in the thin filament is in the blocked state in the absence of calcium. In the presence of calcium this is reduced to <5%.

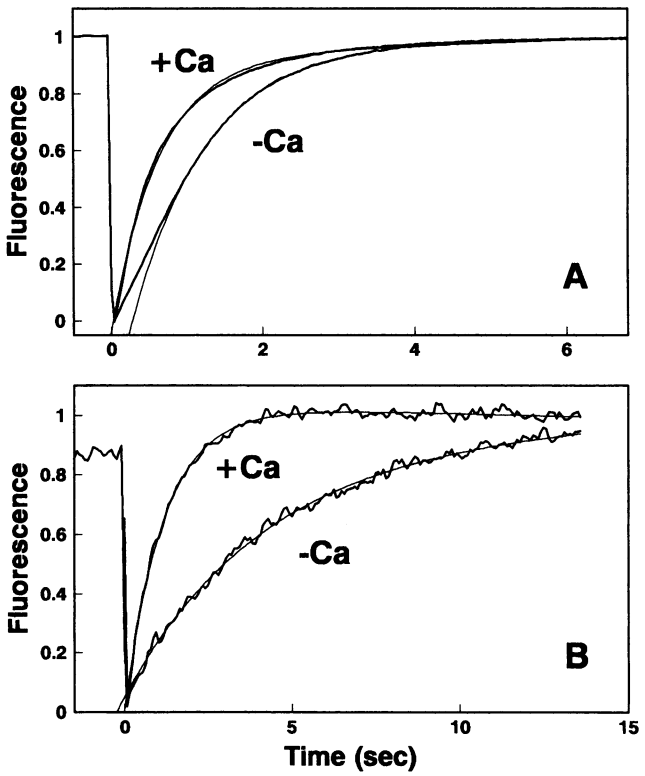

FIGURE 5 The observed fluorescence changes on binding S1 to actin·Tm·Tn in the presence of sulfate. (*A*) Mixing 5 μM S1 with 1 μM pyr·actin and 0.15 μM Tm·Tn in the presence and absence of calcium. Exponentials were fitted as for Fig. 4 *A* and were 1.56 and 0.86 s^{-1}, respectively. (*B*) Mixing 1 μM S1 with 5 μM pyr·actin and 0.75 μM Tm·Tn. Single exponentials fits to the + and – calcium curves gave k_{obs} of 0.87 and 0.21 s^{-1}, respectively.

DISCUSSION

The results we have presented, both the equilibrium titrations in the presence of BDM, ADP, and phosphate and the kinetics of the association between actin and S1, are not compatible with the two-state model of thin filament regulation presented previously (12, 13). The data are however compatible with the three-state model presented here.

The main objection to any two-state model is that the equilibrium and kinetic experiments define two quite separate populations of "off" states. Whether these two states are defined as the open and closed states, or the blocked and open states, no combination is compatible with the experimental data. In the presence of calcium the equilibrium measurements define 80% of the actin sites as being "off," whereas kinetically all those same sites appear to indistinguishable from "on" sites. We believe that subdividing the off states of the filament between the blocked and closed states is the simplest model which can account for all of the experimental observations. We will discuss the precision with which we can define K_T and K_B from our experimental data and the implications of the model for muscle regulation before going on to discuss alternative interpretation of our results.

The determination of the fraction of actin sites or A_7·Tm·Tn units in the blocked state is defined by the ratio of observed rate constants in the presence and absence of calcium for the excess actin kinetic binding experiments. In the presence of calcium the observed rate is similar to that seen in the absence of Tm·Tn. The simplest interpretation of this observation is that no measurable blocked state exists in the presence of calcium. However, the possibility that the presence of Tm·Tn increases k_{+1} but k_{obs} is unaltered because of the compensating presence of some blocked states cannot be completely eliminated. However if any significant blocked states were present, then a lag phase in the association reaction would have been apparent.

Assuming that no significant blocked states are present in the presence of calcium, then the results in the absence of calcium show that 76% of the actin is in the blocked state under our conditions. These kinetic experiments give no in-

formation on the proportion of the thin filament in the open and closed states. Analysis of the titration data is necessary to define the open and closed states.

All of the titration data previously presented cannot distinguish between the closed and blocked states. Analysis using the two-state model led to the conclusion that 20% of the actin was in the open conformation in the presence of calcium and this was reduced to ≤ 2% in the absence of calcium. The three-state model fits the same data equally well but is not sensitive to the partitioning of the actin between the closed and blocked states. Without any detailed refitting of the data presented in 1991 to the three-state model we can use the fits to equilibrium data to define the fraction of actin in the open state and the kinetic data presented here to define the fraction in the blocked state. These results are compatible with the percent occupancy of the three thin filament states (blocked/closed/open) being ≤ 5, ≈80, and ≈20% in the presence of calcium and ≈76, ≈22, and ≤2% in the absence of calcium. This gives values of $K_B \geq 16$ and $K_T \approx 0.25$ in the presence of calcium and ≈0.29 and ≤ 0.09, respectively, in the absence of calcium. This suggests that the main sensitivity of the thin filament to calcium may be in the equilibrium between the blocked and closed states with K_B changing by a factor of at least 55 and K_T by a factor of at least 3 on adding calcium. This interpretation leaves intact the conclusion of McKillop and Geeves (13) that the equilibrium between thin filament conformations is independent of the occupancy of the myosin nucleotide site.

A more rigorous direct fitting of the titrations presented here and our previous titration data (13) to the three-state model is compatible with these numbers. Assuming no significant blocked state in the presence of calcium and assuming a value of K_B of 0.3 in the absence of calcium gives the values shown in Table 1. In this table values shown in standard type were fixed and those in bold type were allowed to vary. A measure of the reliability of the numbers comes from the agreement of the values of K_2 (or K_1 where that was allowed to float) defined by the fit and those measured independently (13). In all cases the agreement between experimental and fitted values was within the experimental accuracy of the measurement. As shown in Table 1 the value of K_T obtained from the fits varies considerably between titrations under different conditions. In the absence of calcium the values vary from 0.013 to 0.18. However the fits are not particularly sensitive to the value of K_T as under these conditions the occupancy of the open conformation in the absence of bound S1 is small (≤2%). Any value of $K_T < 0.2$ will give a good fit to the titration. In the presence of calcium the fitted value of K_T varies from 0.03 to 0.6 and again the fitted value of K_T is not well defined if K_2 is more than 100 times the value of K_T. Thus the most reliable values of K_T are from the two titrations when ADP was present. Using the values of K_T of 0.25 and 0.09 defined above gives a good fit to all the titrations as judged by eye.

The uncertainty of the value of K_T in the absence of calcium leaves undefined the calcium sensitivity of K_T. The data presented can be fitted with very little change in K_T with calcium binding. However, the calcium sensitivity of the acto·S1 ATPase cannot be accounted for without a significant calcium induced change in K_T. This is particularly true under conditions which suggest that occupancy of the blocked state can be reduced (i.e., $K_B > 1$ in the absence of calcium) and still leave a significantly calcium-sensitive acto·S1 ATPase reaction (see Discussion).

The results presented here are compatible with the three-state model where K_B switches from a value of 0.25 to ≥ 16 on binding calcium to the thin filament, i.e., 76% of the thin filament is in the blocked state in the absence of calcium and this is reduced to no significant occupancy on binding calcium. In contrast, if the assignment of K_B is correct, then K_T appears to be less affected by calcium. A value of 0.25 and 0.09 being compatible with the data in the presence and absence of calcium. A change of more than a factor of 10 in K_T on calcium binding remains possible.

Previous studies have shown that tropomyosin alone will produce cooperative binding of S1 to actin and that the A_7·Tm complex behaves in a similar way to A_7·Tm·Tn in the presence of calcium (27, 28). Thus it is possible that in the absence of any ancillary proteins actin filaments are completely in the open conformation. The presence of tropomyosin allows occupancy of the open and closed states and troponin is required for the blocked state. In the presence of calcium, troponin has little influence on actin-myosin interaction. Thus tropomyosin itself has an important role in influencing actin myosin interactions, and this will have implications for regulation mechanisms in smooth muscle and in nonmuscle actin motor systems.

Having proposed and argued for the particular form of the model presented here, it is appropriate to consider the effect of challenging some of the assumptions of the model and alternative interpretations. We have shown that the cooperativity in fluorescence titrations can be accounted for by tropomyosin controlling the A to R isomerization of the acto·S1 complex. The kinetics of association and the light scattering titrations can be accounted for by introducing the blocked state. We have assumed no effect of regulation on step 1. Allowing K_1 to be calcium-dependent would provide a way of fitting the light scattering titration of Fig. 3 *b* without introducing the blocked state (i.e., the two-state model). However, the kinetic data still require a blocked state. The key observation is that in the presence of calcium the rate of association is identical to that seen in the absence of Tm/Tn, yet the titration data suggest that 80% of the thin filament is in the closed state. Thus the rate of S1 binding to the closed state does not appear to be slower that the rate of binding to free actin sites. This is true when the experiment was performed with either S1 or actin is in excess. Therefore the rate of binding is the same for the first S1 to bind to a thin filament (when 80% of the thin filament cooperative units will be in the closed state) and for the last S1 to bind to a fully occupied thin filament (when the filament will be in the open state).

We assume that the cooperative unit is the seven actins bridged by a single Tm. We have continued to avoid building into the model any influence of end-to-end interactions between tropomyosins as the data does not require such interactions. If these interactions are included their effect would be to increase the apparent size of the cooperative unit and result in slightly smaller values of K_B and K_T (see Discussion of McKillop and Geeves (13)). Another alternative is that the cooperativity arises from nearest neighbor interactions between actins mediated by tropomyosin (29). In this type of model the removal of calcium results in the blocking of 76% of actin sites not 76% of A_7Tm·Tn units. A possibility suggested by K. Holmes (personal communication) is that rather than producing a dynamic equilibrium between the blocked and closed states so that 76% of the actin is always unavailable the troponin complex could directly block a fraction of the actin sites in the absence of calcium. To be compatible with the data presented here Tn would have to block five of the seven actin sites in each A_7·Tm·Tn unit.

The model presented here is compatible with the time resolved x-ray studies (4). In the absence of calcium and in the presence of ATP the thin filament would be predominantly in the off state. Binding of calcium would cause a change in the conformation of the thin filament from the blocked state to the closed state. This allows access of myosin heads to the actin sites and would result therefore in an increased binding of myosin heads which would then lead to occupancy of the R state giving a fully turned on thin filament and force generation.

Models of the regulation of muscle contraction which require more than two states of the thin filament have been proposed previously. Lehrer and Morris (6) suggested that the dependence of the acto·S1 ATPase rate on S1 and actin concentration require more than two states of the thin filament. Babu and Gulati (32) from studies of the effects of TnC extraction on calcium sensitivity of force and stiffness in single fibers suggested two independent sites of calcium regulation. One regulating the formation of "weak" bridges and the other the transition from "weak" to "strong" bridges. This muscle fiber study clearly parallels the proposals here. In addition the studies of HMM binding to thin filaments (33) and the kinetic studies of Rosenfeld and Taylor (16) have all suggested that a simple two-state model of the thin filament may not be adequate to account for all of the data.

One problem which remains unresolved is the original work of Chalovich and Eisenberg (8) which led to a serious challenge of the original steric blocking model. Their experimental data at very low ionic strengths (μ = 0.01 M) showed that the equilibrium binding of S1 to actin·Tm·Tn was not influenced by calcium, although the ATPase reaction was regulated. The model presented here predicts that a significant fraction of the actin sites would be unavailable to S1 whatever nucleotide were bound to the S1. A repeat of the experiment shown in Fig. 4 *b*. at an ionic strength of 0.015 M showed that the rate of the association reaction was independent of calcium even though the ATPase reaction was calcium-sensitive. This suggests that K_B is ionic strength-dependent such that K_B is greater than one at this ionic strength. Thus the regulation of actomyosin interactions must be operating primarily through K_T at these low ionic strengths. At the present time there is no information on the ionic strength dependence of K_T. This observation is compatible with the view of Babu and Gulati (32) that in single muscle fibers the calcium regulation of the formation of weak crossbridges (which in our model is controlled through K_B) is abolished at low ionic strengths.

The observations of Chalovich and Eisenberg were repeated by El Saleh and Potter (7) at higher ionic strength using heavy meromyosin (HMM) in place of S1. The use of HMM can introduce additional complications due to interactions between the two heads in binding to actin. However, they do suggest that there is no large effect of calcium on the equilibrium (or more strictly steady state) binding of HMM to actin. However kinetic regulation cannot be eliminated by these studies.

An alternative view is that the kinetics of binding of S1·ATP is fundamentally different from that of either the S1 or S1·ADP like complexes examined here. The equilibrium binding of S1·ATP to actin is much weaker than the complexes examined here but the kinetics of this reaction has not been examined in detail. A kinetic study of S1·ATP binding to actin presents formidable practical problems but would be worth attempting.

The model proposed defines three conformations of the thin filament which are in dynamic equilibrium. The open conformation which predominates in the absence of Tm·Tn or in the presence of Tm·Tn when strongly bound (R state) crossbridges are present. The closed state, predominant in the absence of strongly bound crossbridges and the presence of calcium and also the state found in the presence of Tm and absence of Tn. The blocked state, predominant in the absence of both calcium and strongly bound crossbridges. Regulation of the myosin ATPase and contraction occurs because only the open conformation of the thin filament is compatible with the formation of the strongly bound force generating crossbridges. Acceleration of P_i and nucleotide release from the myosin active site and force generation only occurs in the strongly bound R conformation. Thus regulation operates by controlling whether or not crossbridges can bind to actin and whether or not having bound the crossbridges can pass into the force holding R conformation.

The model will allow predictions on the effects of changes in calcium concentration on the fraction of tightly bound crossbridges for any given nucleotide state. Predictions of the effects of changes in calcium concentration or changes in nucleotide or phosphate concentrations on the level of force produced by a muscle fiber will require knowledge of both the behavior of HMM in place of S1 and of the fraction of tightly bound crossbridges in the steady state.

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