Dynamics of the Muscle Thin Filament Regulatory Switch: The Size of the Cooperative Unit

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ABSTRACT Actin thin filaments containing bound tropomyosin (Tm) or tropomyosin.troponin (Tm.Tn) exist in two states ("off" and "on") with different affinities for myosin heads (S1), which results in the cooperative binding of S1. The rate of S1 binding to, and dissociating from, actin, Tm.actin, and Tm.Tn.actin, monitored by light scattering (LS), was compared with the rate of change in state, monitored by the excimer fluorescence (FI) of a pyrene label attached to Tm. The ATP-induced S1 dissociation showed similar exponential decreases in LS for actin.S1, Tm.actin.S1, and Tm.Tn.actin.S1 ± Ca^{2+}. The FI change, however, showed a delay that was greater for Tm.Tn.actin than Tm.actin, independent of Ca^{2+}. The S1 binding kinetics gave observed rate constants for the S1-induced change in state that were 5–6 times the observed rate constants of S1 binding to Tm.actin, which were increased to 10–12 for Tm.Tn.actin, independent of Ca^{2+}. The rate of the FI signals showed that the on/off states were in rapid equilibrium. These data indicate that the apparent cooperative unit for Tm.actin is 5–6 actin subunits rather than the minimum structural unit size of 7, and is increased to 10–12 subunits for Tm.Tn.actin, independent of the presence of Ca^{2+}. Thus, Tm appears semi-flexible, and Tn increases communication between neighboring structural units. A general model for the dynamic transitions involved in muscle regulation is presented.

INTRODUCTION

The regulation of striated muscle contraction involves a Ca^{2+}-sensitive change of state of the muscle thin filament, composed of actin, tropomyosin, and troponin (actin.Tm.Tn), which affects the interaction between actin subunits of the thin filament and myosin heads (S1) of the thick filament (see reviews by Chalovich, 1993; Leavis and Gergely, 1984; Zot and Potter, 1987). A wide range of solution studies have shown that the equilibrium binding of myosin heads to thin filaments in solution is cooperative and that Ca^{2+} is an allosteric regulator of this binding (Greene and Eisenberg, 1980; Hill et al., 1980; Geeves and Halsall, 1987). Thus, it appears that the thin filament can exist in two states, "on" and "off", and operates as an allosteric/cooperative binding system analogous to many globular proteins (Koshland et al., 1966; Monod et al., 1965; Weber, 1992; Wyman and Gill, 1990). Pure actin filaments exist completely in the on-state, and the presence of Tm alone is sufficient to produce an equilibrium between the on and off states of the thin filament with the equilibrium predominantly towards the off-state (Lehrer and Morris, 1982; Williams et al., 1988). The major role of Tn is to introduce a Ca^{2+}-sensitive modulation of the two states that exist in the actin.Tm filament. Thus, by binding to Tn, Ca^{2+} modulates myosin binding to the thin filament similar to the manner that, e.g., 2,3-diphosphoglycerate modulates oxygen binding to hemoglobin (Benesch and Benesch, 1967).

Questions remain regarding the relationship between the Tm.Tn-controlled on/off state change of the thin filament as modulated by Ca^{2+} and the binding of myosin heads to actin, which occurs in two steps (Geeves and Halsall, 1987). Equilibrium studies have provided a framework in which to formulate these relationships (Hill et al., 1980; Geeves and Halsall, 1987; McKillop and Geeves, 1991), and estimates of all of the equilibrium and most of the rate constants needed for a complete description are available from previous studies.

Although the thin filament behaves in an analogous manner to other allosteric/cooperative binding systems, there is a major difference. In contrast to globular protein systems where the cooperative unit is clearly defined, the thin filament is a continuous structure of repeating units $(actin_7.Tm.Tn)_x$; i.e., a 400 A coiled-coil Tm molecule in a complex with Tn interacts with 7 actin subunits, with $x \approx 26$ in rabbit skeletal muscle fibers. This structural unit consisting of 7 actin subunits has been used as the cooperative unit in calculations with the above model. However, because of the flexibility of Tm and of its contiguous end-to-end interactions, it is not clear whether the number of actin monomers involved in the off/on transition (i.e., the cooperative unit) is the same as the structural unit.

Previous studies have shown that fluorescence labels at Cys-190 of Tm can report directly the change in state of the actin.Tm or actin.Tm.Tn filament. Fluorescence probes that monitored the change in environment (Ishii and Lehrer, 1985), relative Tm-actin movement (Lehrer and Ishii, 1988), and Tm conformation (Ishii and Lehrer, 1990) have all been used. The excimer fluorescence of

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Tm labeled at Cys-190 with pyrene iodoacetamide (Tm*) (Ishii and Lehrer, 1990) has been particularly useful in directly monitoring the equilibrium change in state of reconstituted thin filaments and correlating it with the S1 binding profile.

We report here a detailed study of the kinetics of both ATP-induced dissociation of actin.S1 complexes and the association of S1 with actin in the presence of bound Tm* and Tm*.Tn. These studies follow the rate of the state change, monitored by Tm* fluorescence and the rate of the association/dissociation of S1 with the actin filament, monitored by light scattering (White and Taylor, 1976). The results presented here demonstrate that the allosteric/cooperative model used provides an accurate description of both the equilibrium and kinetics of thin-filament S1 interactions. This study also provides estimates for the size of the cooperative unit for actin.Tm and actin Tm.Tn that differ from 7 actin subunits. In addition, we verify recent studies that show that there is a Ca^{2+}-dependent substate of the off-state (McKillop and Geeves, 1993). A preliminary study examined the rate of the ATP-induced dissociation of S1 from Tm*.actin.S1 (Ishii and Lehrer, 1993).

THE MODEL

The thin filament, shown in Fig. 1 as a single structural unit, Tm*.actin$_7$, dynamically equilibrates between two states, off and on, defined by an equilibrium constant K_T = [on-states]/[off-states] = [^{on}Tm*.actin$_7$]/[^{off}Tm*.actin$_7$]. In the absence of any bound S1, the filament is predominantly in the off-state with $K_T \approx 0.2$ (Ishii and Lehrer, 1990; Williams et al., 1988). The results of Trybus and Taylor (1980) and the rate at which muscle contraction is activated suggest that the rate of the state change ($k_{+T} + k_{-T}$) is of the order of 500 s^{-1}. As shown by Ishi and Lehrer (1990), the excimer fluorescence of a pyrene label on Cys-190 of Tm (Tm*) senses the on/off state change.

Myosin subfragment 1 binds to actin in two principal steps (the nonspecific collision complex is neglected).

$$A + M \overset{1}{\leftrightarrow} \underset{\text{A-state}}{A\text{-}M} \overset{2}{\leftrightarrow} \underset{\text{R-state}}{A.M}$$

S1 initially binds to form the relatively weakly bound A-state ($k_{+1} = 1\text{–}2 \times 10^6\ M^{-1}\ s^{-1}$, $K_1 = 6 \times 10^4\ M^{-1}$; (Coates et al., 1985; Geeves and Halsall, 1987; McKillop and Geeves,

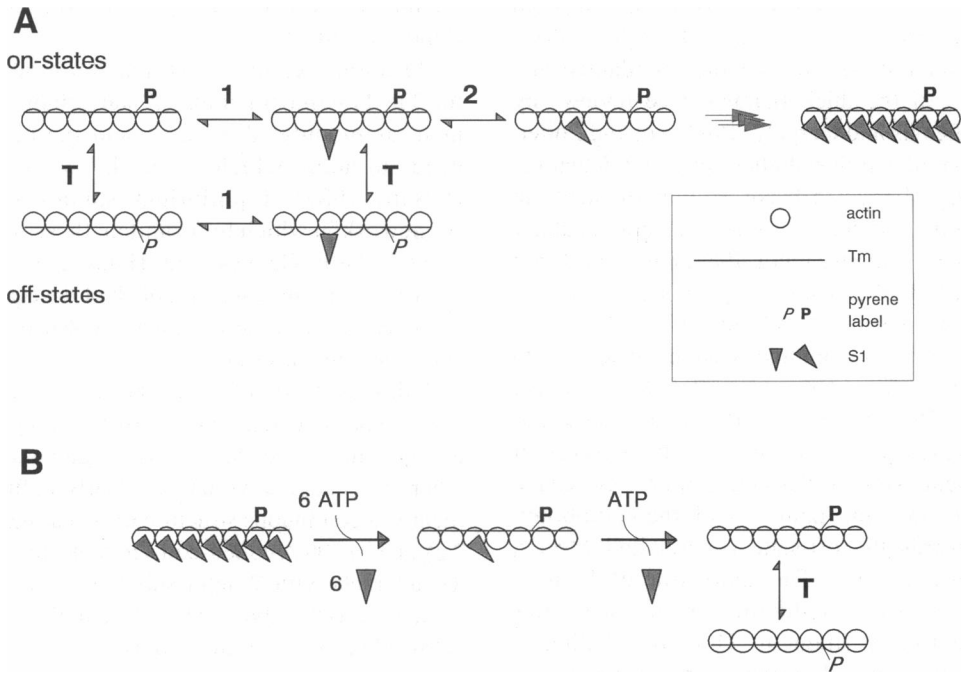

FIGURE 1 Schematic model of the S1-induced off-to-on transition of Tm.actin (*A*) and the ATP-induced on-to-off transition of Tm.actin.S1 (*B*) for a cooperative unit consisting of seven actin subunits. Step T, off/on equilibrium of the thin filament (rate constants k_{+T}, k_{-T}, and equilibrium constant K_T), monitored by the increase in pyrene excimer fluorescence from a low value (P *pointing down*) to a greater value (P *pointing up*). In the presence of Tm, the thin filament is predominantly in the off-state. Step 1, S1 binding to the initial attached state, *A* (in Fig. oriented at 90°), bound weakly ($K_1 = 6 \times 10^4\ M^{-1}$, $k_{+1} = 1\text{–}2 \times 10^6\ M^{-1}\ s^{-1}$). Step 2, S1 isomerization to the rigor-like state, R (in Fig. oriented at 45°), bound strongly ($K_2 = 200$, $k_{+2} + k_{-2} = 2000\ s^{-1}$). (*A*) S1-induced off-to-on transition of the thin filament. The binding of the S1 in step 1 does not appreciably affect the off-on equilibrium. The following fast isomerization of S1.actin traps the thin filament in the on-state, affecting 7 actin subunits. The fluorescence is changed and the light scattering increases until all actin sites are saturated. For *n* actin subunits in a cooperative unit, if the rate of S1 binding (controlled by $[S1]k_{+1}$) is much slower than the rate of the switch opening ($k_{+T} + k_{-T}$), the observed rate of the fractional fluorescence change (k_{FI}) will be *n* times the observed rate of the fractional light scattering change (k_{LS}). (*B*) ATP-induced on-to-off transition of the thin filament. The random binding of ATP to the S1-saturated Tm.actin thin filament, in the on-state, results in dissociation of S1 via reversal of the isomerization step. The thin filament is maintained in the on-state until the last S1 dissociates from the cooperative unit, resulting in a delay in the fluorescence change compared with the light scattering change. The length of the delay in the fluorescence signal is proportional to the size of the cooperative unit.

1991)), shown with principal axis at 90° to the actin filament in Fig. 1, which then isomerizes to the strongly bound R-state, in a second step, with $K_2 = 200$ and $k_{+2} + k_{-2} = 2000\ s^{-1}$ (Coates et al., 1985), shown at 45° to the actin filament. The essential feature of the Geeves and Halsall (1987) model of Fig. 1 is that in the off-state of the filament, S1 can bind to form the A-state without affecting the on/off equilibrium, but the isomerization to the R-state is prevented, presumably by Tm. However, if the filament can dynamically switch between the off and on states (at a rate controlled by $k_{+T} + k_{-T}$), then a bound S1 can isomerize to the R state during the time the filament is transiently in the on-state. Once the S1 is in the R-state, the filament is trapped in the on-state provided $K_2 \gg 1/K_T$, as is the case here. The equilibrium results of Ishii and Lehrer (1990) show that 1–2 S1s bound per Tm*.actin$_7$ structural unit are sufficient to trap the filament in the on-state.

Two types of kinetic experiments were made on Tm*.actin$_7$ and Tm*.Tn.actin$_7$ filaments: a) The rate of S1 binding to actin subunits, monitored by the change in light scattering, was compared with the rate of the S1-induced change in state, monitored by the change in pyrene excimer fluorescence; b) The rate of the ATP-induced irreversible dissociation of S1 from the thin filament was monitored with the same two signals. The S1-binding and resulting off-on equilibria are illustrated in Fig. 1 *A*. If both the S1.actin isomerization (K_2) and the on-off state change of the filament (step T) are rapid compared with the rate of S1 binding to individual actin monomers, the light scattering signal (LS) will be proportional to the concentration of occupied actin sites. Thus, d(LS)/dt is proportional to $-d[A.M]/dt = k_{+1}[A][M] - k_{-1}[A\text{-}M]$. Assuming that the back reaction is negligible, ($k_{-1}[A\text{-}M] = 0$), and that pseudo-first-order conditions apply ($[M] = [M]_o \gg [A]$), $LS = [A] = [A]_o \exp(-t[M]_o k_{+1})$. Therefore, the observed rate constant for the light scattering change, $k_{LS} = [M]_o k_{+1}$. The fluorescence (Fl) signal is proportional to the number of cooperative units binding 1 or more myosin heads. If a cooperative unit contains n actin subunits then $Fl = [A_n] = [A_n]_o \exp(-nt[M]_o k_{+1})$. Thus, $k_{LS} = nk_{Fl}$. The ATP-induced dissociation of S1 and on-off equilibria are illustrated in Fig. 1 *B*. The filament is initially trapped in the on-state by the bound myosin heads. ATP binds randomly to individual S1 causing a rapid reversal of the A/R isomerization and rapid dissociation. Both the reversal of the isomerization and dissociation steps are much faster than the rate of ATP binding provided [ATP] < 1 mM (Geeves, 1991; Millar and Geeves, 1983) and are not shown explicitly in the figure. As ATP binds and dissociates the bound S1s, the light scattering will fall but the Tm*.actin unit will remain in the on-state as long as a single S1 remains bound within the cooperative unit. Thus, while the light scattering signal will show a single exponential decay with k_{LS} defined by the rate of ATP binding, the fluorescence will remain constant until the last S1 dissociates and then will change rapidly as the filament switches to the off-state. Therefore, in the S1 binding experiment, the observed rate constant of the fluorescence change will be n times theobserved rate constant for the light scattering change; in the ATP-induced dissociation experiment, a delay in the fluorescence signal is expected, the magnitude of which should depend upon the size of the cooperative unit.

MATERIALS AND METHODS

S1 was prepared by chymotryptic digestion of rabbit muscle myosin (Weeds et al., 1977) and stored as a powder obtained by freeze-drying in the presence of 1 mg of sucrose per mg of protein. Actin was freshly prepared from an acetone powder of rabbit muscle and purified by two cycles of polymerization depolymerization (Lehrer and Kerwar, 1972). Tm and Tn were prepared from rabbit skeletal muscle, and Tm was labeled at Cys groups with *N*-(1-pyrenyl) iodoacetamide (Molecular Probes, Eugene, OR) as described earlier (Ishii and Lehrer, 1990). The experimental buffer used throughout was 0.14 M KCl, 5 mM $MgCl_2$, and 20 mM MOPS, pH 7.0, 20°C, unless otherwise stated.

Light scattering (LS) and fluorescence (Fl) kinetic signals were obtained using a Hi-Tech Scientific (Salisbury, England) SF-51 stopped-flow spectrophotometer equipped with a 100 W Xe/Hg lamp and monochromator. Excimer fluorescence, excited at 365 nm, was detected after passing through a GG 455 nm cut-on filter to remove light scattering and monomer fluorescence. Light scattering was observed at 90° to the incident light using a narrow band interference filter centered at 405 nm. 500 12-bit data points were collected using a DAS 50 A to D converter in a Viglen 486 computer with Hi-Tech software. 3 to 6 traces of LS and Fl data were collected with the same stock solutions and were averaged and fitted to an exponentials using a nonlinear least-squares fitting routine.

RESULTS

Tm.actin.S1: ATP-induced S1 dissociation and the on-off change in state

When ATP was rapidly mixed with Tm*.actin.S1 it bound to, and caused dissociation of, S1 from the thin filament complex (Fig. 2). Under these pseudo-first-order conditions (constant [ATP]), the light scattering signal decreased exponentially. The observed rate constant (k_{LS}) was linearly dependent upon ATP concentration until the reaction became too fast to observe in the stopped-flow equipment (Fig. 2 *C*). Under the experimental conditions, the reaction could be observed to [ATP] = 500 μM. The plot of [ATP] against k_{obs} yielded a value of $1.05 \times 10^6\ M^{-1}\ s^{-1}$ for the second-order rate constant. Previous studies have shown that the kinetic events after ATP binding (ATP induced R-to-A isomerization of the complex and dissociation of S1 from the complex) are at least $500\ s^{-1}$ and could be as fast as $5000\ s^{-1}$ (Geeves et al., 1986; Millar and Geeves, 1983) and, therefore, the observed dissociation rate constant can be associated with ATP binding.

In contrast to the light scattering signal, the pyrene fluorescence signal showed little change until the scattering was more than 70% complete and then rapidly decreased (Fig. 2). Because of the inital delay, a simple kinetic analysis over the complete time course was not possible. An estimate of the rate of the fluorescence change was made, however, by fitting a single exponential to the last 50% of the amplitude change. A plot of this apparent rate constant for the fluorescence change (k_{Fl}) versus [ATP] shows that k_{Fl} remained

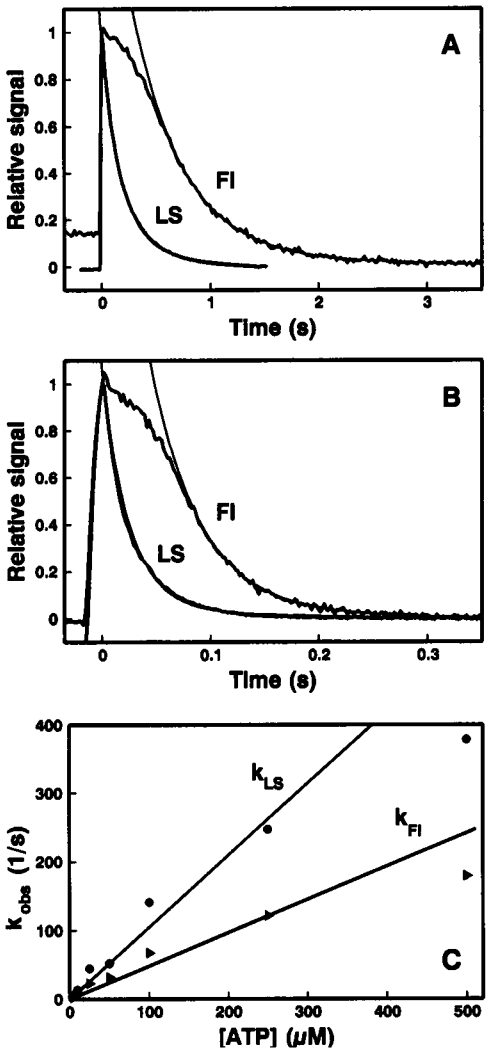

FIGURE 2 Comparison of the rates of ATP-induced dissociation of S1 from Tm*.actin.S1 (LS) with the on-to-off change of thin filament state (Fl). The ATP induced dissociation of 1 μM S1 from 0.15 μM Tm*, 1 μM actin in 140 mM KCl, 5 mM $MgCl_2$, 20 mM MOPS, pH 7.0 at 20°C. The signals were scaled to the same amplitude and superimposed. Three to five transients were averaged, and the best-fit single exponential to the trace was superimposed. For light scattering the exponential was fitted to the whole transient; for fluorescence the exponential was fitted to the last 50% of the amplitude. (*A*) 5 μM ATP, $k_{LS} = 5.61\ s^{-1}$, $k_{Fl} = 2.11\ s^{-1}$. (*B*) 25 μM ATP $k_{LS} = 44.4\ s^{-1}$, $k_{Fl} = 22.6\ s^{-1}$. (*C*) Plot of k_{obs} versus ATP concentration slopes; LS = $1.05 \times 10^6\ M^{-1}\ s^{-1}$, Fl = $0.48 \times 10^6\ M^{-1}\ s^{-1}$.

approximately half that of k_{LS} over the accessible range of ATP concentrations (Fig. 2 *C*). Also, the relative delay between the two signals (or the lag in the fluorescence signal) remained approximately constant (Fig. 2, *A* and *B*). At [ATP] > 500 μM, the lag appeared to be reduced, but this was due to the loss of signal in the dead time of the apparatus. The non-zero slope during the initial delay appears to be caused by the contribution of a process that follows the light scattering. A similar phenomenon was observed in the S1 binding kinetics and is described below. These results demonstrate that the rate of the thin filament switching to the off-state after loss of S1 from the complex is not limiting the observed signal and, therefore, the rate of switching off (controlled by $k_T + k_{-T}$) is much greater than 500 s^{-1}.

To obtain further evidence for the model, the ATP-induced dissociation experiment was repeated at different levels of saturation of the actin filament with S1 (Fig. 3 *A*). ATP at 5 μM was used to dissociate S1 from Tm*.actin.S1 at S1:actin ratios of 1:10, 1:4, and 1:1. In each case, the light scattering signal gave an observed rate constant of 4.5 s^{-1}, and the amplitude scaled with the concentration of S1 (data not shown). The fluorescence signals, in contrast, did not scale simply with [S1] and had relative amplitudes of 0.27:0.57:1.0 for the respective ratios of S1:actin (Fig. 3 *A*). The size of the lag in the fluorescence signal, however, did decrease with S1 and at an S1:actin ratio of 1:10, the lag had almost disappeared. At this low level of actin occupancy, if S1 binds randomly to the filament, few units will have more than one bound S1 per Tm*.actin$_7$ and, at the limit as [S1]→0, the two signals should coincide. At low S1:actin ratios, the normalized light scattering and fluorescence signals are seen to coincide approximately without significant delay between them (Fig. 3 *B*). These results provide clear evidence for random binding of S1 to Tm.actin and, more generally, for the prediction of the model (Fig. 1 *B*): that the fluorescence signal changes when the filament switches off during the dissociation of the last S1 from the Tm.actin cooperative unit.

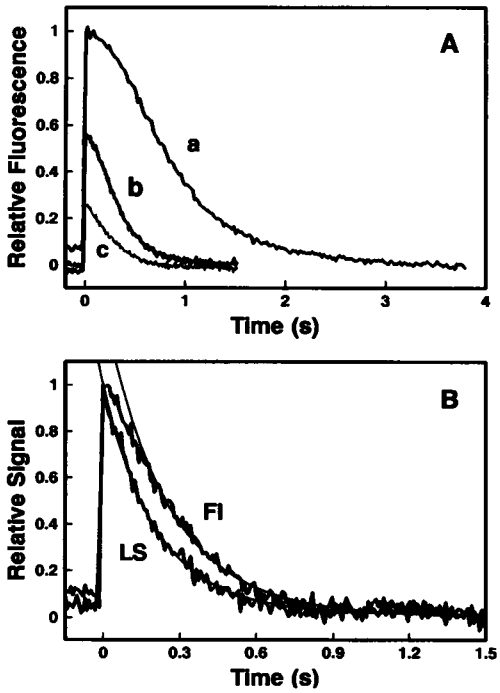

FIGURE 3 The delay in on-off state change (Fl) on ATP-induced dissociation of S1 (LS) from Tm*.actin.S1 at different levels of saturation with S1. Conditions as for Fig 2 except 5 μM [ATP], 1 μM actin, 0.15 μM Tm*, and [S1]: a) 1 μM, b) 0.25 μM, c) 0.1 μM. (*A*) Fluorescence transients: the amplitudes are all scaled relative to the amplitude at an actin:S1 ratio of 1:1. (*B*) The light scattering (LS) and fluorescence transients (Fl) for *c* scaled to the same amplitude and superimposed. The best-fit single exponentials are fitted and superimposed on each transient as for Fig 1.

Tm.actin: S1 binding and the off-on change in state

When S1 was rapidly mixed with Tm*.actin, the light scattering showed a single exponential increase (Fig. 4, *A* and *B*). The reaction was pseudo-first-order with respect to S1, and the observed rate constant (k_{LS}) was linearly dependent upon S1 concentration to [S1] = 40 μM. The second-order rate constant for the association reaction (k_{+1} in Fig. 1 *A*) given by the slope of the plot of k_{obs} versus [S1] is 1.3×10^6 M^{-1} s^{-1} (Fig. 4 *C*). The second-order rate constants were similar to those observed for actin alone and also as had been reported previously for actin.Tm.Tn in the presence of Ca^{2+} (Geeves and Halsall, 1986; Trybus and Taylor, 1980). However, at lower ionic strengths (<60 mM) the rate constant in the presence of Tm was about 40% greater than actin alone. The reason for this difference in behavior with ionic strength is not known.

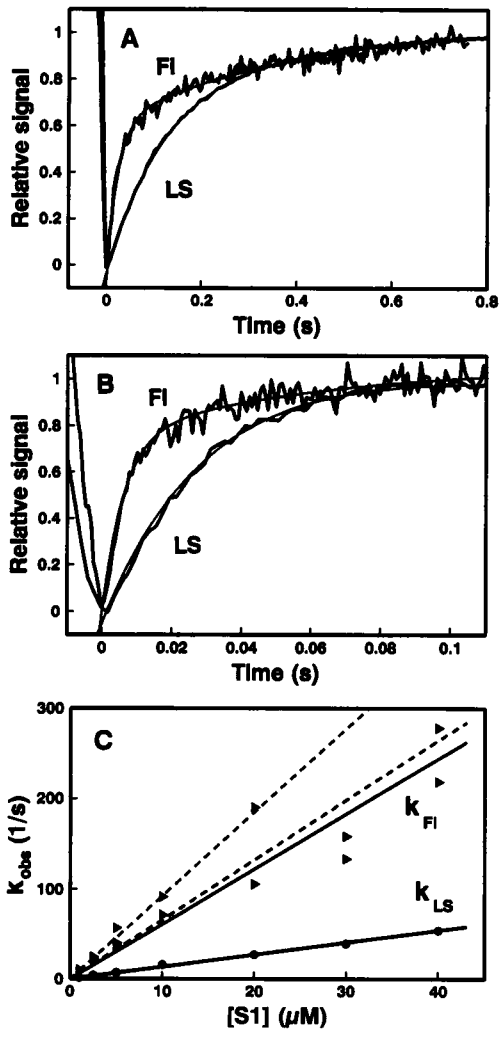

FIGURE 4 The rate of S1 binding (LS) to Tm*.actin.compared with off-to-on state change (Fl). S1 mixed with 0.15 μM Tm* and 1 μM actin. (*A*) 5 μM S1. k_{LS} = 6.7 s^{-1}, k_{Fl} = 44.1, and 3.8 s^{-1}. (*B*) 30 μM S1. k_{LS}= 38.9 s^{-1}, k_{Fl} = 158 and 16.2 s^{-1}. (*C*) k_{obs} versus [S1]. The two sets of points are for two different fitting routines. The least-squares fit to a line gave slopes; LS = 1.32 $^{-1}$ s^{-1}, Fl = 6.09 M^{-1} s^{-1}. The dashed lines are the slopes for 5 and 7 times the light scattering slope.

The fluorescence signal was composed of two kinetic components that were fitted by the sum of two exponentials. The poor signal-to-noise ratio resulted in relatively large errors in the fitted rate constants, but the faster component had the largest amplitude (60–70%) and the observed rate constants from both components were linearly dependent upon S1 concentration. The observation that the slower component had an observed rate similar to the LS signal (within a factor of 2) indicates that it originates from one or more of the following sources: i) a contribution from the much stronger light scattering signal; ii) a specific fluorescence change caused by S1 binding to the actin subunit close to the label on Tm; and/or iii) some S1-induced binding of Tm to actin because S1 is known to increase the affinity of Tm for actin. A plot of the S1 concentration dependence of the observed rate constant of the faster component (k_{Fl}) demonstrated that k_{Fl} was linearly dependent on [S1] over the range 1–40 μM (Fig. 4 *C*). Thus, S1 binding is the rate-limiting step, and the rate of the off-to-on switch, $k_{+T} + k_{-T}$ (which gives rise to the fluorescence change) after S1 binding is as fast or faster than the largest observed value of k_{Fl}, i.e., $k_{+T} + k_{-T} \geq 250$ s^{-1}. In addition, k_{Fl} is 5–6 times faster than k_{LS} over the concentration range studied (Fig. 4 *C*). These data, therefore, are consistent with the model of Fig. 1 *A* for a cooperative unit of 5–6 actins. Thus, it appears that the cooperative unit size is slightly but significantly less than the structural unit size of 7 actins.

The difference between k_{LS} and k_{Fl} for the data shown in Fig. 4 and predicted with the model of Fig. 1 is caused by the two signals monitoring different processes, both limited by the rate of S1 binding. The light scattering signal monitors the rate of S1 binding to each actin monomer (controlled by k_{+1}[S1]); the fluorescence signal monitors the rate of S1 binding to any one of the monomers of a cooperative unit (controlled by nk_{+1}[S1]). With this reasoning, if the experiment is repeated with [Tm*.actin$_7$] in excess of [S1], the two observed rate constants should be the same (controlled by k_{+1}[A]). This experiment is illustrated in Fig. 5, where 0.35 μM Tm* and 2.5 μM actin (i.e., 0.35 μM Tm*.actin$_7$) was mixed with 0.1 μM S1 at an ionic strength of approximately 45 mM. (The lower ionic strength is used to increase the affinity of S1 for actin to allow the experiment to be performed at reasonably low actin concentrations.) The occupancy of actin sites by S1 is very low, resulting in small amplitudes for both signals and a large, slow light scattering artifact. However, the observed exponential component of the light scattering gave k_{LS} = 37.6 s^{-1}. For the fluorescence signal, the transient was again described by a double exponential with the faster component comprising more than 70% of the amplitude and k_{Fl} = 33.4 and 5.7 s^{-1}. Thus, for excess Tm.actin, k_{Fl} for the major component was very similar to k_{LS}, as predicted. Under these lower ionic strength conditions, the second-order rate constants for S1 binding (light scattering)

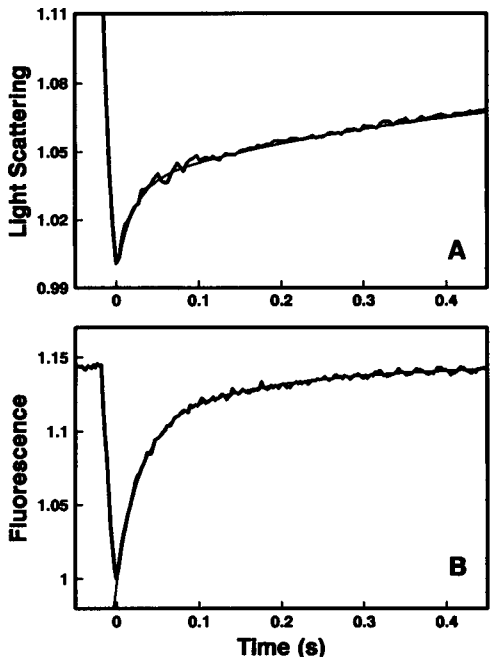

FIGURE 5 Comparison of the rate of S1 binding (LS) excess Tm*.actin with the rate of the state change (Fl). 0.1 μM S1 binding to 0.35 μM Tm* and 2.5 μM actin. (*A*) Light scattering with fit to a single exponential and sloping end point superimposed, k_{LS} = 37.6 s^{-1}. (*B*) Fluorescence with fit to double exponential superimposed k_{Fl} = 35.4 and 5.7 s^{-1}, amplitudes 10.6 and 4.2%, respectively. Conditions: 30 mM KCl, 5 mM $MgCl_2$, 20 mM HEPES, pH 7.5, 20°C.

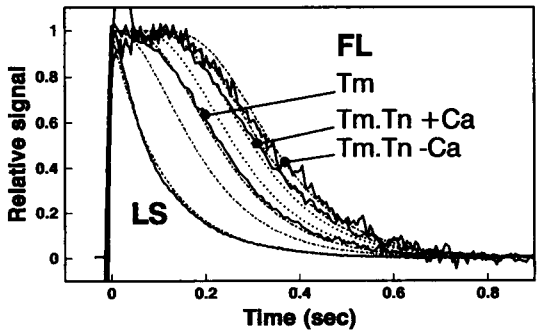

FIGURE 6 Comparison of the kinetics of ATP-induced dissociation of S1 (LS) with the on-to-off change in thin filament state (Fl). 10 μM ATP was mixed with 1 μM S1, 1 μM F-actin and 0.15 μM Tm*, 0.15 mM Tm*, 0.2 mM Tn, 0.2 mM Ca^{2+}, 0.15 mM Tm*, 0.2 mM Tn, and 1 mM EGTA ($-Ca^{2+}$). All three light scattering traces superimposed (k_{LS} = 9.0 s^{-1}); for clarity only a single trace is shown. The fluorescence traces with Tn $\pm$ Ca^{2+} are also almost superimposible. The dotted lines are the computer-simulated curves for the fraction of the cooperative unit in the on-state (f_{on}), $f_{on} = 1 - (1 - \exp(-k_{LS}t))^n$, where the rate of S1 binding (k_{LS}) is that observed experimentally and n is the size of the cooperative unit. The curves shown are those for n = 1, 3, 5, 7, 10, 12, and 15. All other conditions as in Fig. 2.

and the major component of Tm* fluorescence change (both measured for excess S1 binding at 2.5 μM to Tm*actin as in Fig. 4) were 11.6 and 66.9 $\times$ 10^6 M^{-1} s^{-1}, respectively. Thus, the expected observed rate constant of S1 binding for the excess actin.Tm experiment is 11.6 μM^{-1} s^{-1} $\times$ 2.5 μM S1 = 29 s^{-1}, in close agreement to the above values for k_{LS} and k_{Fl}.

All of the data presented for Tm*.actin filaments, therefore, are consistent with the model proposed in Fig. 1 where i) random binding of single S1s to cooperative units, consisting of 5–6 actin subunits, is sufficient to result in switching the cooperative unit into the on-state and ii) the rate constant of the switching ($k_T + k_{-T}$) is more than several hundred per second.

Tm.Tn.actin.S1: ATP-induced S1 dissociation and the on-off change in state

The effects of Tn and Ca^{2+} on the ATP-induced dissociation of S1 from the Tm.actin filament are shown in Fig. 6. Single exponential transients were obtained for the light scattering signals for all three actin-containing filament types (Tm*, Tm*.Tn + Ca^{2+}, and Tm*.Tn $-$ Ca^{2+}), and the fitted rate constants were indistinguishable. (For clarity, only a single light scattering transient is shown in Fig. 6.) The observed rate constants were linearly dependent upon [ATP] up to 500 μM (data not shown). Thus, ATP binds to actin.S1 at the same rate irrespective of whether Tm or Tm.Tn are present.

The fluorescence signal of all three thin filament types showed significant lags before changing. The lag was clearly greater in the presence of Tn (Fig. 6). Although this data set showed a small further increase in the lag in the absence of Ca^{2+}, the difference between the $\pm Ca^{2+}$ traces were generally within the experimental noise. Fitting the latter half of the transient to a single exponential gave smaller apparent rate constants when Tn was present than in its absence, but the ratio k_{LS}/k_{Fl} remained constant for each filament type over the range of ATP concentrations studied.

The greater delay of the fluorescence signal behind the light scattering signal when Tn is additionally bound to actin.Tm can be interpreted in terms of the model (Fig. 1). Because the light scattering transient is the same and k_{Fl} remains linearly dependent upon [ATP], no contribution to the lags come from slower events after ATP binding, i.e., ATP binding is rate limiting. A greater lag, therefore, is consistent with a greater number of actin subunits being maintained in the on-state by the last S1 to dissociate from the cooperative unit, i.e., a greater size of the cooperative unit. The superimposed fits to the data shown in Fig. 6 are computer-modeled predictions for the expected rate of switching off of a cooperative system when the last S1 dissociates at the rate shown by the light scattering signal for cooperative units of different sizes. It can be seen that the fluorescent transient can be fit with an apparent cooperative unit of 5–6 actin subunits for Tm.actin and 10–12 actin subunits for Tm.Tn.actin. It should also be noted that the apparent cooperative unit is independent of Ca^{2+}.

Tm.Tn.actin: S1 binding and the off-on change in state

When S1 was rapidly mixed with Tm*.Tn actin + Ca^{2+}, the light scattering signals were very similar to the absence of Tn

(Fig. 7 *A*). The data can be described by single exponentials, and the observed rate constants were linearly dependent on [S1] over the accessible range. For Tm*.Tn + Ca^{2+} as observed for Tm*, the fluorescence transients can be described by two exponentials with the faster component having the major amplitude and both observed rates linearly dependent upon [S1]. The k_{LS} and k_{FI} (for the faster component) values obtained in the experiment shown in Fig. 7 are listed in Table 1. As already demonstrated in Fig. 4 for Tm.actin, k_{FI} is 5–6 times k_{LS}. However, for Tm.Tn.actin, k_{FI} is 10–12 times k_{LS} (and, therefore, k_{FI} for TmTn is almost twice k_{FI} for Tm, k_{LS} being similar for both). Thus, these results are consistent with those of the ATP dissociation reaction and suggest that the presence of Tn and Ca^{2+} increases the apparent size of the cooperative unit from 5–6 to 10–12.

In the absence of Ca^{2+}, the kinetics are more complex. The light scattering transient shows an initial lag followed by an approximately exponential phase (Fig. 7 *A*, curve *c*). Such traces have been interpreted in terms of an additional state of the thin filament that inhibits the initial binding of S1, the blocked-state (Geeves and McKillop, 1993; Trybus and Taylor, 1980). The fluorescence transient, in the absence of Ca^{2+} (Fig. 7 *B*, curve *c*), in contrast, shows a single exponential change with an observed rate constant approximately one-third of that observed in the presence of Ca^{2+} (Fig. 7 *B*, curve *b*). In the presence of Ca^{2+}, the pyrene signal has been shown to respond to the filament state change induced by the first S1 to bind to a cooperative unit. In the absence of Ca^{2+}, the first S1 binds to the filament at approximately one-third the rate in the presence of Ca^{2+} (McKillop and Geeves, 1993), i.e., during the lag in light scattering, the binding rate of S1 should be 7.8 $s^{-1}/3 = 2.6\ s^{-1}$, where 7.8 s^{-1} is the rate in the presence of Ca^{2+}. Thus, the ratio of k_{FI} ($-Ca^{2+}$) to the predicted observed rate constant for S1 binding is 32 $s^{-1}/2.6\ s^{-1} = 12.3$, similar to the $-Ca^{2+}$ ratio. The observed rate of the fluorescence change, therefore, is consistent with the presence of the blocked-state of McKillop and Geeves (1993) and further indicates that even in the absence of Ca^{2+}, the binding of a single S1 is sufficient to switch on the cooperative unit of 10–12 actin subunits.

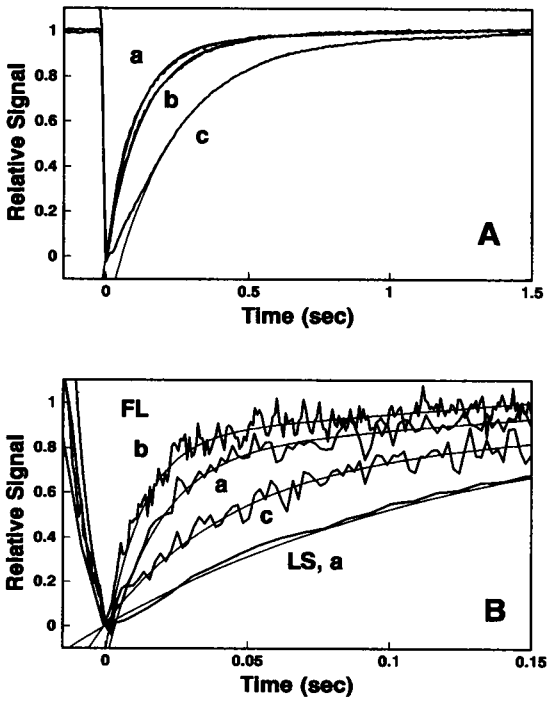

FIGURE 7 Comparison of the rates of S1-binding with the S1-induced off-to-on change of thin filament state. (*A*) Light scattering. (*B*) Fluorescence compared with light scattering at short times. 5 μM S1 binds to 1 μM F-actin and (*a*) 0.15 μM Tm*, (*b*) 0.15 μM Tm*, and (*c*) 0.2 μM Tn and either 0.2 mM $CaCl_2$ or 1 mM EGTA. Conditions as for Fig. 2.

TABLE 1 Comparison of the rate of S1 binding, k_{LS}, and thin filament state change, k_{FI}, obtained from the data in Fig. 7

System	k_{LS} (s^{-1})	k_{FI} (s^{-1})	k_{FI}/k_{LS}
Actin	9.0		–
Tm*.actin	8.8	56	6.4
Tm*.Tn.actin + Ca^{2+}	7.8	80	10.2
Tm*.Tn.actin − Ca^{2+}	4.2*	32	–

The observed rates for light scattering (LS) and fluorescence (FI) on mixing 5 μM S1 with 1 μM actin, and Tm* and Tn are as described in Fig. 4.
* In the absence of Ca^{2+} the transient is not an exponential, and k_{LS} is obtained by fitting an exponential to the last 30% of the amplitude.

DISCUSSION

The results presented here are in broad agreement with the predictions of the model of Fig. 1. In the absence of myosin heads, the Tm.actin and the Tn.Tm.actin thin filament are predominantly in the off-state. S1 binds to an actin site within the cooperative unit in a rate-limiting association to the A-state, followed by a rapid equilibration between A- and R-states of the actin.S1 complex and between the off- and on-states of the cooperative unit of the filament. Because $K_2 \gg 1/K_T$, most of the bound S1 quickly isomerizes to the R-state, and the cooperative unit is switched on. Subsequent S1s bind to the unit in the on-state. No difference is seen between the rate at which the first S1 binds and later S1s bind because both are limited by k_{+1}. The rate of S1 binding to actin.Tm* was similar to that in the absence of Tm and only slightly slower than actin.Tn.Tm* (Table 1), indicating that the initial kinetics of binding of S1 to actin (k_{+1}) was not greatly affected by Tm or Tn in the presence of Ca^{2+}. The good agreement between the experimental results and the model based on previous estimates of the four equilibrium and eight rate constants support the prediction that acto-S1 can make the transition to the R-state only if the cooperative unit is switched on. A limitation of the test of the model provided here is that steps T and 2 are both in rapid equilibrium and, thus, the model is insensitive to any influence of the rates of the two steps on each other. A more rigorous test will come from similar experiments in the presence of ADP, where the rapid equilibrium assumption for step 2 is no longer valid (Geeves, 1991; Geeves and Halsall, 1987). Under those conditions, the simple analysis used here would not be valid and modeling of all 47 interme-

diates in the kinetic pathway is required. Such a study is currently underway.

For actin.Tm, n, the apparent cooperative unit size determined in this study, is smaller than 7 actin subunits, the structural unit size. Possible reasons for $n < 7$ are i) more than one S1 bound is required to switch on the cooperative unit, ii) not all of the actin subunits are fully regulated because of incomplete binding of Tm, or iii) Tm is flexible such that not all of the actin subunits of the structural unit are switched to the on-state. In terms of the model, the first possibility seems unlikely because both steps T and 2 are rapid equilibrium steps, and with $K_2 = 200$ and $K_T = 0.2$, the fraction of units remaining in the off-state after binding one S1/unit is $1/(1 + K_T + K_2) = 1/41$. Thus, after binding a single S1, only 2% of the cooperative units would remain in the off-state. The second possibility, whereby S1 increases the affinity of Tm for actin, also seems unlikely because both the off-to-on and the on-to-off kinetic experiments gave the same value of n. In addition, n was independent of both [Tm] and ionic strength from 0.05 to 0.17 M, over which range the affinity of Tm for actin varies. The third possibility, based on considerable evidence, is that Tm acts as a semi-flexible rod (Flicker et al., 1982; Graceffa and Lehrer, 1980; Hvidt et al., 1983; Mabuchi, 1990; Phillips et al., 1986; Ueno, 1984). Thus, it appears that only part of the Tm molecule switches between the on/off states rather than the entire molecule. It does not appear probable that the presence of the pyrene probe produced the flexibility in view of the previous results with two other probes that showed the same equilibrium behavior, whereby 1–2 S1s/7 actin subunits produced the off-on shift (Ishii and Lehrer, 1985; Lehrer and Ishii, 1988).

In the presence of Tn, the apparent cooperative unit increased to 10–12 actin subunits, independent of Ca^{2+}, greater than the minimum structural unit size of 7. Previous studies have provided estimates for n. Titrations that monitored ATPase activity with binding of NEM.S1 (which binds predominantly in the R-state even in the presence of ATP) gave $n = 11$ and 14 for Tm.actin and Tm.Tn.actin, respectively, and were interpreted as involving both strands of the 2-start actin helix (Nagashima and Asakura, 1982). Mechanical studies on muscle fibers were modeled as $n = 26 \times 7$ (Brandt et al., 1987) or $n = 14$–21 (Moss et al., 1986). The latter estimate appears closer to our value in the presence of Tn. Previous S1 titrations of Tm*.actin agreed with a random binding model consistent with 1–2 S1s/7 actin subunits or a stoichiometry of $n \approx 5$ actin subunits/S1 (Ishii and Lehrer, 1985). Tn stabilizes Tm principally via the TnT component, with the N-terminus of TnT interacting with the C-terminus of Tm and overlapping several N-terminal residues of the adjacent Tm molecule (Brisson et al., 1986; Heeley et al., 1987). Despite this evidence for Tn interacting with the ends of Tm, Tn does not appear to facilitate interactions between Tm molecules on binding to actin in the absence of S1 (Hill et al., 1992). This apparent disagreement with the present findings can perhaps be resolved if it is noted that Tm binds predominantly to actin in the off-state (in the absence of S1) but that Tn may preferentially strengthen end-to-end interactions in the on-state.

A simple interpretation of the size of the cooperative unit can be made in terms of the model in which Tm sterically blocks the binding of S1 to actin in the R-configuration and, therefore, must physically move to uncover adjacent binding sites for S1 (see below). Thus, for Tm.Tn, the binding of an S1 to an actin subunit distorts the Tm.Tn filament on actin such that, on average, 5 actin subunits on either side are uncovered. For Tm alone, the information is transmitted, on average, to 2–3 actins on either side. It may be too simplistic to expect that the continuous Tm filament is so uniform that the efficiency of the structural transition would be the same in the middle of a Tm molecule compared with its overlap region at the ends. Clearly, more data are needed to determine whether the number of actin subunits affected by a bound S1 depends upon its location within the structural unit. Information on the nature of the transmission between Tm.Tn. actin units might come from studies of Tms with modified N- and C-termini and Tms from different species.

For Tm*.Tn.actin in the absence of Ca^{2+} there was a lag in the light scattering, which was consistent with the presence of a state that inhibits the initial binding of S1 (the blocked-state; McKillop and Geeves, 1993). No lag was apparent in the fluorescence transient, but k_{FI} ($-Ca^{2+}$) was approximately one-third of k_{FI} ($+Ca^{2+}$). This is consistent with the proposal that two-thirds of the actin subunits are unable to bind S1 significantly, and the lower rate is the result of binding to one-third of the subunits that are in rapid equilibrium with blocked units. (The experimental data, however, cannot distinguish between the availability of one-third of actin sites or one-third of cooperative units). The observation that the fluorescence does increase shows that the filament is being switched on by the S1 binding at this slower rate. The estimated ratio k_{FI}/k_{LS} during this early phase remains 10–12. Thus, the data in the absence of Ca^{2+} are consistent with the presence of a subset of the off-state, which inhibits the initial binding of S1 but which does not change the size of the cooperative unit. In the original steric-blocking model of regulation, Tm blocks a myosin head binding site on actin and Tm moves to a new position, allowing myosin heads to bind (Haselgrove, 1972; Huxley, 1972; Parry and Squire, 1973). Our results can be incorporated into a modified steric-blocking model in which Tm in the off-state sterically prevents the isomerization of the actin.S1 complex (Geeves and Halsall, 1987; McKillop and Geeves, 1993), probably by competing with the binding of the R-configuration of S1. actin to a common site on actin. Thus, the off-state prevents the isomerization, thereby inhibiting the loss of phosphate in the ATPase pathway and blocking force generation. The blocked sub-state of the thin filament state, which prevents the initial weaker binding of S1 (A-state) in the absence of Ca^{2+}, could involve Tm in a third position on actin without much change in excimer fluorescence. An alternate possibility is steric-blocking of initial binding of S1 by the TnI component of Tn (with possible involvement of the TnT component). TnI is known to inhibit acto-S1 ATPase activity

(Syska et al., 1976), presumably by competing with binding of S1-ATP to actin (Grabarek and Gergely, 1987; Levine et al., 1988). Recent fluorescence studies indicate that Ca^{2+} dissociates TnI from actin (Miki and Iio, 1993; Tao et al., 1990), which is in agreement with the removal of the binding inhibition by Ca^{2+}. If Tn increases the cooperative unit size by affecting Tm in the on-state of the thin filament rather than the off-state, as suggested above, the Ca^{2+}-dependent blocked-to-off transition must involve blocking by Tn rather than Tm.

Two classes of models have been proposed for the general phenomenon of cooperative/allosteric binding (Weber, 1992; Wyman and Gill, 1990), the MWC two-state concerted model (Monod et al., 1965) and the KNF induced-fit or sequential model (Koshland et al., 1966). The two were combined in a more general description (Hammes and Wu, 1971). The model outlined in Fig. 1 has elements of both the concerted and induced-fit formalisms because the thin filament equilibrates between two states in the absence of S1, yet it is the independent S1-induced isomerization of individual actin.S1 complexes which is coupled to the $Tm.actin_n$ state change. The model of Fig. 1 assumes that the intrinsic values of K_T and K_B are independent of S1 binding, and the model gives an adequate description of the data. However, as $K_2 \gg 1/K_T$, the model is not sensitve to moderate changes in K_T with increased binding of S1 to the actin sites in a cooperative unit. Similarly, because steps T and 2 are both very rapid compared with the rate of S1 binding, the model is not sensitive to changes in $k_{+T} + k_{-T}$ caused by S1 binding or to changes in $k_{+2} + k_{-2}$ caused by the thin filament state change. As mentioned above, this can be tested by using S1 with ADP bound, which reduces both K_2 and $k_{+2} + k_{-2}$ by more than a factor of 10 (Geeves, 1989, 1991).

These studies confirm and extend the concept that the Tm-actin thin filament can exist in two activity states, off and on, and that the cooperative binding of S1 to actin (in the Rigor-like state) is associated with the "turning-on" of 5–6 actin subunits/bound S1 in the cooperative unit. The presence of Tn increases the size of the cooperative unit to 10–12, independent of Ca^{2+}. In the presence of Ca^{2+}, Tn does not significantly alter the equilibrium between off and on states. For actin.Tn.Tm. in the absence of Ca^{2+}, however, a substate of the off-state exists (blocked-state) that inhibits the initial weak binding of S1. Thus, in the Tm.Tn.actin thin filament, Ca^{2+} regulates contraction by releasing an interaction (TnI-actin) that affects two processes: the unblocking of a state that inhibits the initial binding of S1, and the allosteric facilitation of the switch (transition) by altering the on/off equilibrium constant. However, more studies will be necessary to clarify all of the structural changes that occur on the thin filament associated with shifts in the A/R, blocked/off/on, and $\pm Ca^{2+}$ states.

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