Force Spectroscopy Reveals Multiple “Closed States” of the Muscle Thin Filament

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Tropomyosin (Tm) plays a critical role in regulating the contraction of striated muscle. The three-state model of activation posits that Tm exists in three positions on the thin filament: “blocked” in the absence of calcium when myosin cannot bind, “closed” when calcium binds tropolin and Tm partially covers the myosin binding site, and “open” after myosin binding forces Tm completely off neighboring sites. However, we recently showed that actin filaments decorated with phosphorylated Tm are driven by myosin with greater force than bare actin filaments. This result cannot be explained by simple steric hindrance and suggests that Tm may have additional effects on actin-myosin interactions. We therefore tested the hypothesis that Tm and its phosphorylation state affect the rate at which single actin-myosin bonds form and rupture. Using a laser trap, we measured the time necessary for the first bond to form between actin and rigor heavy meromyosin and the load-dependent durations of those bonds. Measurements were repeated in the presence of subsaturating myosin-S1 to force Tm from the closed to the open state. Maximum bond lifetimes increased in the open state, but only when Tm was phosphorylated. While the frequency with which bonds formed was extremely low in the closed state, when a bond did form it took significantly less time to do so than with bare actin. These data suggest there are at least two closed states of the thin filament, and that Tm provides additional points of contact for myosin.

The actin-binding protein tropomyosin (Tm) is thought to operate in opposing roles as an inhibitor or an activator in the cross-bridge cycle by sterically hindering myosin binding to actin in the absence of calcium and, conversely, promoting the cooperative relief of inhibition over the span of one regulatory unit when an initial myosin bins (1–3). Data suggest that the transition of Tm from an inhibitory to a permissive state proceeds through at least one intermediate step. The three-state model of cooperative activation suggests that Tm exists in three positions on the thin filament: 1) blocked, in the absence of calcium when myosin cannot bind; 2) closed, when calcium binds tropolin and Tm still partially covers the myosin binding site; and 3) open, after initial myosin binding forces Tm completely off neighboring myosin binding sites (4, 5).

These state changes are thought to be propagated to neighboring regulatory units on the thin filament. Tm polymerizes in a head-to-tail manner with neighboring Tm dimers (6), and evidence suggests that Tm can transmit conformational changes over long distances via end-to-end interactions (7–9). In the α isoform of Tm found in striated muscle, there is a phosphorylation site near the carboxyl terminus at Ser-283 (10). This phosphorylation site is in the head-to-tail overlap between neighboring Tm dimers, and we showed previously that Tm phosphorylation is necessary to extend cooperative activation beyond one regulatory unit (11). We also found that actin filaments decorated with phosphorylated Tm (A + Tm.P) produced greater isometric force at intermediate densities of myosin than bare actin filaments or actin filaments decorated with dephosphorylated Tm (A + Tm.DP) (11). This increase in force suggests that phosphorylated Tm may augment actin-myosin interactions.

To test the hypothesis that Tm phosphorylation has effects on binding kinetics at the level of single cross-bridges, we used a laser trap to measure the rate of heavy meromyosin (HMM) binding to actin in the rigor state, as well as the load-dependent bond lifetimes of rigor HMM unbinding from actin. We performed these measurements using bare actin filaments, A + Tm.P, and A + Tm.DP. To control the activation state of the filament, we used myosin-S1 to force Tm from the closed to the open state. Our data suggest that Tm exists in at least two “closed” states, and that it provides additional points of contact for myosin on the thin filament that modify both the rate of actin-myosin bond formation and the intrinsic lifetime of the bond.

EXPERIMENTAL PROCEDURES

Proteins—HMM was prepared from rat skeletal muscle as described in Guo and Guilford (12). Myosin subfragment 1 (S1) was prepared according to Margossian and Lowey (13) and modified with N-ethylmaleimide (NEM). All proteins were stored in liquid nitrogen in small aliquots containing 50% glycerol. F-actin was prepared from rat skeletal muscle according to Pardee and Spudich (14), biotinylated with N-hydroxysuccinimide biotin according to Rao et al. (11), and stabilized with phallolidin. Tm was also prepared from rat hind leg skeletal muscle as described by Smillie (15) and reconstituted with F-actin according to Rao et al. (11). The level of Tm phosphorylation
in both the reconstituted and free state was assumed to reflect
the native level as reported by Rao et al. (11) using a similar
preparation. Free Tm and Tm-decorated actin filaments
were dephosphorylated with calf intestine alkaline phosphatase (Cal-
biochem) as described by Rao et al. (11).

Actin filaments, A + Tm.P, and A + Tm.DP were coupled to
0.97-µm (diameter) streptavidin-coated microspheres (Bangs
Laboratories, Fishers, IN). A suspension containing ~4 × 10^8
beads was combined with a 2 µM final concentration of biotin-
ylated actin filaments with and without Tm.P or Tm.DP (a sig-
nificant excess to occupy all available streptavidin sites) and
allowed to couple with overnight mixing at 4 °C. Beads were
washed and resuspended with actin buffer (25 mM KCl, 25 mM
imidazole, 1 mM EGTA, 4 mM MgCl_2 (pH 7.4)). The actin-
coated beads were blocked with 1 mg/ml BSA to reduce non-
specific interactions. HMM rather than myosin was used for all
experiments to reduce nonspecific interactions and to limit
binding events to those with single heads (16). NEM-S1 was
used to transition Tm from the closed to the open state on actin
filaments.

Site Densities and Contact Areas—Site densities from HMM-
coated pedestals were calculated from previously published val-
ues from the lab (12). The number of available myosin binding
sites on the actin-coated beads was determined fluorescently by
binding saturating concentrations of myosin-S1 modified with
Alexa Fluor 488 C_6-maleimide (Invitrogen). Alexa Fluor 488 C_6
maleimide (AF) was added to S1 at a 10-fold molar excess and
reacted at room temperature in the dark for 1 h. The reaction
was stopped by adding 25 mM DTT to quench the excess AF.
The reaction mixture was then dialyzed (10,000 kDa molecular
mass cutoff) with two buffer changes overnight in 1 liter of actin
buffer. An actin-coated bead population of 1.2 × 10^8
beads/µl was mixed with the AF-S1 over a range of concentra-
tions (150 nM AF-S1 to 1 µM AF-S1), and fluorescence intensity
was measured using a FluoStar Optima plate reader (excitation,
480 nm; emission, 520; BMG, Durham, NC) to determine at
what concentration AF-S1 saturably binds the beads. These fluo-
rescence values were compared with an AF-S1 standard curve
over a range of 1–20 nM to determine the concentration of
AF-S1 bound to a population of actin-coated beads. The density
of myosin binding sites (µm^−2) was subsequently calculated.

Laser Trap—The laser trap used in these studies was described
in detail in Guilford et al. (17). Back focal plane interferometry
was used to measure the position of trapped beads relative to the trap
center, from which measurements of displacement and force were
calculated. The sensitivity of the interferometer and the trap stiff-
ness were calibrated by the step response method (18, 19) and
by fits to the power spectral density (20).

Nitrocellulose-coated coverslips with 3–10-µm pedestals
were prepared as described (16). 20 µg/ml rat skeletal HMM in
actin buffer was incubated in a flow cell for 1 min at room
temperature. The flow cell was then blocked with 1 mg/ml BSA
in actin buffer for 1 min. A suspension of actin-coated,
A + Tm.P, or A + Tm.DP beads was then introduced. For
A + Tm.P and A + Tm.DP, 500 nM free Tm.P or Tm.DP
was included. For time to bond formation and bond lifetime exper-
iments, NEM-S1 was added to the bead suspension at a range of
10–20 nM to force the closed-to-open transition of Tm (Fig.
1D). An oxygen scavenger system (0.125 mg/ml glucose oxida-
dase, 0.0225 mg/ml catalase, 2.87 mg/ml glucose) was also
included in the bead suspension. All solutions contained 10 mM
DTT. For control experiments, 1 mM Na_3P_2O_7 (pyrophos-
phate) was included in the bead suspension (16).

The flow cell was placed onto a piezoelectric microscope
stage. A bead was captured in the laser trap and brought into
contact with an HMM-coated pedestal on the surface. Details
of individual laser trap experiments are given under “Results.”

The contact area between the actin-coated and HMM-
coated beads was estimated as reported previously (21). We
assumed the bond length, l, to be 30.5 nm, assuming 20 nm for
the length of an HMM molecule protruding normally to the
nitrocellulose-coated surface, 7 nm for actin, and 3.5 nm for
streptavidin bound on the microsphere. The mean radius of the
HMM-coated pedestals and the actin-coated beads was 1.5 and
0.485 µm, respectively. The resulting contact areas were
6.9 × 10^−2 µm^2 for HMM-coated pedestals and 7.1 × 10^−2 µm^2
for actin-coated beads.

RESULTS

Shifting Tm from the Closed to the Open State—Myosin bind-
ing to actin is necessary to shift Tm from the closed state to the
open state and completely expose myosin binding sites. We
used NEM-S1, which binds tightly to actin, to shift Tm-deco-
rated actin from the closed to the open state. Thus, the fre-
quency with which bonds form would be expected to increase
with increasing NEM-S1 concentration up to a point and then
decrease as the myosin binding sites on the actin filaments
become fully occupied.

To determine the NEM-S1 concentration necessary for max-
imum HMM binding to occur, we measured the frequency of
bond formation for single HMM-actin interactions as a func-
tion of NEM-S1 concentration. Actin-coated beads were cap-
tured in a laser trap and brought into contact with an HMM-
coated target (Fig. 1A). Whether or not a bond had formed was
determined by moving the laser trap away from the HMM-
coated target (Fig. 1B). If a bond had formed, the trapped bead
would remain in contact with the target until the actin-myosin
bond ruptured (Fig. 1C). Bond frequency was defined as the
ratio of the number of bonds formed to the total number of
trapped- and target-bead contacts. Bond frequency was mea-
sured as a function of NEM-S1 concentration. Other factors
such as contact area, protein density, and contact time were not
included because they were constant throughout the experi-
ments (22).

The results of these experiments are shown in Fig. 2. As
expected, bare actin-coated beads exhibited the highest binding
frequency (70 ± 1%) when no NEM-S1 was present and
declined in binding frequency because of competitive inhibi-
tion of binding as NEM-S1 concentration was increased. Over-
all, bare actin produced the highest binding frequencies at all
concentrations of NEM-S1. In contrast, the binding frequen-
cies of beads coated with A + Tm.P and A + Tm.DP produced
the lowest binding frequencies when no NEM-S1 was present.
This implies steric hindrance by Tm in the absence of any initial
myosin binding. As the concentration of NEM-S1 was in-
creased to ~20 nM, the binding frequencies of the A + Tm.P and
A+Tm.DP beads reached maxima of 50 ± 5% and 40 ± 5% respectively. At higher NEM-S1 concentrations, binding frequencies for both A+Tm.P and A+Tm.DP steadily declined, presumably because of competition for binding sites by NEM-S1 and some remaining steric hindrance by Tm.

The binding frequency between HMM with bare actin-coated beads was modeled as competitive inhibition and was fit by a Hill equation as a function of myosin-S1 concentration

\[
B([S1]) = B_{\text{max}} - \frac{B_{\text{max}} \cdot [S1]^n}{K_i + [S1]^n} \quad (\text{Eq. 1})
\]

where \( B \) is the bond frequency, \([S1]\) is the NEM-S1 concentration, \( B_{\text{max}} \) is the bond frequency of HMM when exogenous NEM-S1 is absent, \( K_i \) is \([S1]\) at 50% inhibition, and \( n \) is the Hill coefficient of inhibition. Inhibition of binding with bare actin filaments was non-cooperative with a Hill coefficient of 0.85 ± 0.1 (Fig. 2). This is consistent with previous findings suggesting that myosin binding to actin filaments is non-cooperative (23). 50% inhibition occurred at \([S1] = 99 ± 19 \text{ nm} \).

In the case of A+Tm.P and A+Tm.DP, the addition of NEM-S1 is expected to shift the position of Tm from the closed conformation to the open conformation and expose myosin binding sites. Thus the bond frequency data of A+Tm.P and A+Tm.DP was analyzed as the difference of two Hill equations, one that describes the rise in bond frequency (closed-to-open activation) and one that describes fall in bond frequency (competitive inhibition). Non-zero binding frequency in the absence of NEM-S1 is assumed to arise from incomplete reconstitution of Tm onto the actin filament. In this simple model we do not attempt to model the low rate of myosin binding in the closed state that occurs as Tm diffuses across the actin filament.

\[
B([S1]) = \left( \frac{B_{\text{max}} \cdot [S1]^n}{K_o + [S1]^n} - B_{\text{max}} \right) r + B_{\text{max}} - \frac{B_{\text{max}} \cdot [S1]^n}{K_i + [S1]^n} \quad (\text{Eq. 2})
\]

\( K_o \) is \([S1]\) at 50% activation, \( m \) is the Hill coefficient of activation, and \( r \) is the fraction of filaments regulated by Tm. Fitted values of \( r \) suggest that A+Tm.P and A+Tm.DP filaments were
regulated by 87 and 76%, respectively, as evidenced by the non-zero binding frequencies in the absence of NEM-S1. These data are consistent with the fractional binding of Tm to actin predicted from the binding kinetics of Tm.P and Tm.DP (11) (88 and 95%, respectively). The balance of events without NEM-S1 are presumably because of a small frequency of binding in the closed state.

This simple model also assumes that $B_{\text{max}}$ is the same in both Tm-regulated and unregulated actin filament units. If we fit Equation 2 allowing these $B_{\text{max}}$ to differ between the states, the two fitted estimates of this parameter do not differ significantly from one another (although with large error).

A + Tm.P activation by NEM-S1 was cooperative with a Hill coefficient of activation of $2.3 \pm 0.7$, whereas with A + Tm.DP filaments it was non-cooperative ($n = 1.2 \pm 0.1$). These manifest as bond frequencies that increase rapidly with NEM-S1 in A + Tm.P, whereas the rise in bond frequency in A + Tm.DP is less abrupt (see Fig. 2). These Hill coefficients are consistent with our previous evidence suggesting that phosphorylated Tm extends cooperative activation beyond one thin filament regulatory unit (11).

The Hill coefficients of inhibition for A + Tm.P and A + Tm.DP did not differ significantly from 1.0 and are not suggestive of cooperativity ($n = 1.1 \pm 0.7$ and $1.2 \pm 0.1$ for A + Tm.P and A + Tm.DP, respectively). Therefore, the data were fit using $n = 1$ for A + Tm.P and A + Tm.DP to reduce fitting error. [S1] at 50% inhibition was significantly lower for A + Tm.P compared with actin. This implies that A + Tm.P increases the affinity of the thin filament for myosin compared with bare actin filaments. This is consistent with earlier findings with (24) and without (25) troponin present. In the presence of Ca$^{2+}$ as well, the affinity of S1 for Tm/troponin-decorated filaments is higher than that of bare actin (26).

Tropomyosin Phosphorylation Accelerates Formation of the First Actin-Myosin Bond—To determine whether Tm phosphorylation affects actin-myosin on-rates, we measured the time to bond formation ($t_b$) between single HMM and myosin binding sites on actin-coated beads. Formation of the first bond between actin on the trapped bead and HMM on the target was accompanied by a small shift in bead position and a decrease in Brownian motion of the trapped bead. $t_b$ was defined as time elapsed between bead-target contact and the initial formation of a bond (Fig. 1C). We previously reported this method for studying selectin-ligand bonds (27), and a similar method has been reported using a biomembrane force probe (28). This measurement differs from the frequency of bond formation because $t_b$ is only measured when a bond actually forms.

We used our measured $t_b$ to calculate specific 2D on-rates ($\mu$m$^2$/s) using the equation

$$k_{on} = (t_b m, m A_r)^{-1} \quad \text{(Eq. 3)}$$

where $m_r$ is the site density of HMM molecules on the nitrocellulose-coated pedestals, $m$ is the density of available myosin binding sites on our actin-coated beads, and $A_r$ is the effective contact area between the pedestal and the trapped bead. $m_r$ was determined to be 1255 heads/\mu m$^2$ using previously published data collected using the NH$_2$-ATPase method (12). An $m_r$ of 637 myosin binding sites/\mu m$^2$ of bead surface was determined by binding AF-NEM-S1 to the actin-coated beads and calculating the available myosin binding sites. $A_r$ between the HMM-coated pedestal and actin-coated bead was estimated (21) at 0.14 \mu m$^2$.

Specific 2D on-rates for each experimental condition are shown in Fig. 3A. To test how $t_b$ changed as Tm is shifted from the closed to the open state, NEM-S1 was applied at subsaturating concentrations of 10 and 20 nM. 20 nM was chosen because it corresponds to the highest degree of activation of the reconstituted filament (Fig. 2). Surprisingly, when no NEM-S1 was present (the presumed closed state of Tm) the on-rates of both A + Tm.P and A + Tm.DP were ~3 times and ~2 times greater than bare actin, respectively. As Tm shifted from the closed state to the open state, on-rates decreased significantly in A + Tm.P ($p = 0.04$) and converged toward that of bare actin filaments (see Fig. 3A). Thus, Tm in the closed state accelerates the stereospecific binding of myosin to the filament. Moreover, the degree of acceleration of actin-myosin binding by Tm seems to be dependent on phosphorylation, as $k_{on}$ is highest in A + Tm.P at all concentrations of NEM-S1. While it is tempting to argue that the addition of NEM-S1 simply blocks available myosin binding sites resulting in lower on-rates, half-maximal

![Figure 3](image-url)
inhibition of bare actin occurs at much higher concentrations (50–100 nM NEM-S1).

While specific 2D on-rate results cannot be directly compared with the rate of S1 association with actin as measured by classical methods such as stopped-flow, certain characteristics of myosin-S1 binding to actin still hold true. Consistent with previous studies using stop flow techniques (29), our data show that in the presence of increasing concentrations of myosin-S1, the binding rates of bare actin, actin-Tm.P, and actin-Tm.DP are essentially the same. However, our results are the first to demonstrate that actin-myosin rates are significantly increased when Tm is in the closed state in both the phosphorylated and dephosphorylated state.

We validated our 2D on-rate measurements using an alternate technique in which we estimated 2D on-rates by measuring the probability of bond formation as a function of fixed contact times between the actin-coated bead and the HMM-coated pedestal. Similar to Chesla et al. (22), we determined adhesion probability as a function of contact time (p(t)), between two surfaces by repeatedly stepping an actin-coated bead into contact with a stationary HMM-coated pedestal and counting the number of bonds formed for a fixed number of contacts. This process was repeated over a range of contact times (5–250 ms), and adhesion probabilities were plotted as a function of contact time (Fig. 3B). The resulting data were fitted as in Chesla et al. (22) to determine a k_on for bare actin beads of 2.6 ± 0.6 × 10⁻⁴ μm²/s. This value is in close agreement with our time to bond formation data (2.2 ± 0.2 × 10⁻⁴ μm²/s) under the same experimental conditions.

**Tropomyosin Phosphorylation Increases the Lifetime of the Actin-Myosin Rigor Bond**—A range of step loads (2.5–31.5 pN) was applied to actin-HMM bonds using the laser trap, and the bond lifetimes were measured as illustrated in Fig. 1, B and C. All loading experiments were performed with rigor HMM (no nucleotide present), and data were collected with either 0 or 20 nM NEM-S1. The actin-HMM bond behaved as a catch-slip bond under all experimental conditions, first increasing in bond lifetime to a maximum and subsequently decreasing in bond lifetime with increasing force. The critical force (f_c) at which bond lifetime is maximal was consistent with previous studies (16) and ranged from 6.5–8.9 pN, with the lowest f_c values occurring in A + Tm.P, to the highest f_c values occurring in A + Tm.DP. This corresponds roughly to the maximum unitary force produced by a single myosin head.

In the absence of NEM-S1, the load-dependent bond lifetimes were similar for actin, A + Tm.P, and A + Tm.DP (Fig. 4A). However, at 20 nM NEM-S1, which shifts Tm to the open conformation, the maximum bond lifetime of A + Tm.P-HMM bonds, and only these bonds, increased significantly by about 2-fold (Fig. 4B). These data show that phosphorylated Tm changes the load-dependent dissociation rate of the A + Tm-HMM bond, but only when Tm is in the open state. This implies a structural difference at the A + Tm.P-myosin binding interface as compared with actin alone. Control measurements were performed in the absence of HMM and also in the presence of 1 mM pyrophosphate, which locks myosin in a weak binding state. In both cases, bond lifetimes were low and independent of force (Fig. 4A). Nonspecific bonds in the absence of HMM formed with a 13% frequency. Bonds in the presence of pyrophosphate, however, formed with 2-fold greater frequency than blank tar-
Force Spectroscopy of the Thin Filament

![Diagram](attachment:diagram.png)

**FIGURE 5. A revised “three-state” model of thin filament activation.** To explain our experimental data, we propose two closed states of the thin filament, here designated as closed1 and closed2. Closed1 would be characterized by an actin-myosin bond formation rate (k+on) that is below that of bare actin. In contrast, closed2 would be characterized by a rate of bond formation higher than that of bare actin. Bond formation in the open state is indistinguishable in rate from bare actin but the rate of bond rupture (k-off) is lower.

gets, suggesting that while these bonds were short-lived, they were specific in nature.

**DISCUSSION**

Tm phosphorylation has been shown in a variety of models to be associated with increased contractile function (30–33). Our data show that Tm phosphorylation has significant effects on single cross-bridge interactions that are not purely inhibitory. These effects vary depending on the position of Tm on the actin filament, accelerating actin-myosin binding in the closed state, and slowing load-dependent bond rupture in the open state.

**NEM-S1 as a Thin Filament Activator**—The position of Tm on the filament was controlled through NEM-S1, which has a binding constant of 1.4 × 10^6 M^-1 with bare actin and 6.3 × 10^6 M^-1 with Tm-actin (34) and should result in 11% of the binding sites on the Tm-actin filament being occupied at 20 μM NEM-S1. This value corresponds to about 1 regulatory unit per bound NEM-S1. To explain the cooperativity evident in A++Tm.P (Fig. 2), this suggests that when phosphorylated, Tm polymerizes with at least one neighboring dimer, allowing cooperative activation.

**More Than One Closed State**—The data in Figs. 2 and 3 seem at odds. With no added NEM-S1, Tm begins in the closed state, and the frequency with which bonds form is extremely low. This is exactly what one would intuit from the three-state model. However, when a bond does form, it does so at a considerably higher rate than with bare actin.

The obvious interpretation of these data is that there is more than one closed state. Consider a pair of closed states in equilibrium with one another as illustrated in Fig. 5. One (closed1) is more likely to be the blocked state that in the on-rate for myosin is low relative to bare actin, whereas in the second closed state (closed2), it is high relative to bare actin. This may be envisioned as the Tm molecule diffusing across the myosin binding sites on actin within a range of angles defining the closed state. More than half of the time it is in state 1, and myosin binding is reduced. For the fraction of time Tm is in state 2, Tm becomes a “guide” to myosin binding on actin, enhancing the on-rate. Thus, two closed states explain both the reduced frequency of bond formation and the higher rate with which an initial bond forms. That we observe these two distinct states experimentally tells us something about the timescale of the motion of Tm motion. Tm must be diffusing at such a rate that the two closed states are discernable by HMM. If Tm was diffusing faster, the two states would appear as one indistinct range of positions, and myosin would be unable to bind to the actin-Tm complex. The observed time to bond formation of actin and HMM in the presence of Tm suggests an upper limit to the time scale of diffusion of the Tm between the two states is 50–100 s^-1. When the filament reaches the open state, myosin binds at rates characteristic of bare actin.

This model appears to be consistent with the work of Craig and Lehman (35), showing that actively cycling myosin heads bind to a different location on the thin filament than do rigor heads. Although our data were collected solely with rigor myosin, it may capture the functional manifestations of the binding sites of Craig and Lehman. Structural studies have also shown that the position of Tm on actin is not static but rather remains in dynamic equilibrium with subpopulations of Tm in the closed state in both calcium-containing and calcium-free buffers (36).

Tm in the closed state partially blocks critical amino acid residues on actin that are a part of the myosin-binding site (35, 37). However, sterical hindrance in and of itself cannot explain the force enhancements (11, 23) and increased ATPase rates (38) that have been observed with the addition of Tm. Thus Tm must have other effects on single cross-bridge kinetics to account for those changes and the data presented here. Our model explains this as Tm providing additional binding interfaces for myosin. As a result, the rate with which the actin-myosin bond is formed is increased (in the proposed closed2 state), as is the intrinsic duration of the actin-myosin bond (in the open state). The rate of bond formation is presumably the more relevant of the two because attached times at cellular [ATP] are much shorter than the intrinsic duration of the actin-myosin bond.

One might argue that the disconnect between the rate of bond formation and the probability of bond formation could be the result of a mixed population of beads, some with actin and some without. This is clearly not the case. In two-dimensional bond kinetics there is a positive relationship between the density of binding sites and the frequency of bond formation and also between the density of binding sites and the apparent on-rate (22, 27, 28). However, with bare actin, our measured rate of bond formation remained constant even while the bond frequency decreased with increasing NEM-S1 concentration. Thus, the observed changes must be attributed with factors other than the density of actin on beads.

**Effects of Tm Phosphorylation**—Effects on bond lifetime were observed only when Tm was phosphorylated. However, we argue that the effects on single cross-bridge interactions by Tm that we observed are phosphorylation-independent at the single cross-bridge level. The simplest interpretation is that Tm phosphorylation causes a greater fraction of Tm to shift to the open state when myosin binds, this shift being driven by long-
range transmission of the closed-to-open transition in Tm through enhanced end-to-end interactions. As a result, bond lifetimes on average are longer when Tm is phosphorylated because on average myosin (or NEM-S1) binding shifts more Tm dimers to the open state. Enhanced end-to-end interactions may also be responsible for maintaining a greater amount of Tm in the closed state in the absence of myosin binding, leading to faster on-rates when Tm is phosphorylated.

Myosin as an Allosteric Catch Bond—A number of models have been proposed to explain catch-slip bonds, but the allosteric model (39) (two bound states with different bond dissociation rates, the ratio between them being dependent on applied force (40, 41)) is particularly attractive for myosin. Integrins, for example, undergo an allosteric shift that is characterized by a shift from a bent to a straightened conformation with differing ligand affinities (42–44). Further, the hinge between the epidermal growth factor and lectin domains of selectins (45, 46), and between the lectin and pilin domains of FimH (41, 47) have been shown to be vital for catch-bond behavior (Fig. 4). We also see that phosphorylated Tm changes the behavior of actin-myosin allosteric bond lifetimes. Taken as a whole, though, our data suggest that Tm provides additional points of interaction for myosin on the thin filament.

REFERENCES

1. Greene, L. E., and Eisenberg, E. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 2616–2620
2. Hill, T. L., Eisenberg, E., and Greene, L. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 3186–3190
3. Bremel, R. D., and Weber, A. (1972) J. Biol. Chem. 247, 157–166
4. McKillop, D. F., and Geeves, M. A. (1993) Biochemistry 32, 9844–9849
5. Guo, B., and Guilford, W. H. (2000) Cell Motil. Cytoskeleton 50, 264–272
6. Margossian, S. S., and Lowey, S. (1982) Methods Enzymol. 85, 55–71
7. Pardee, J. D., and Spudich, J. A. (1982) Methods Enzymol. 85, 164–181
8. Suvorova, K., and Block, S. M. (1994) Annu. Rev. Biophys. Biomol. Struct. 23, 247–288
9. Allersma, M. W., Gittes, F., deCastro, M. J., Stewart, R. J., and Schmidt, C. F. (1998) Biophys. J. 74, 1074–1085
10. Rinko, L. I., Lawrence, M. B., and Guilford, W. H. (2004) Biophys. J. 86, 544–554
11. Rao, V. S., Marongelli, E. N., and Guilford, W. H. (2009) Proc. Natl. Acad. Sci. U.S.A. 106, 2616–2620
12. Trybus, K. M., and Taylor, E. W. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 7209–7213
13. Heeley, D. H., Watson, M. H., Mak, A. S., Dubord, P., and S chimlie, L. B. (1989) J. Biol. Chem. 264, 2424–2430
14. Houle, F., Rousseau, S., Morrice, N. L., Luc, M., Mongrain, S., Turner, C. E., Tanaka, S., Moreau, P., and Huot, J. (2003) Mol. Cell. Cell. 14, 1418–1432
15. Maimon, L. D., and Bertozzi, C. R. (1999) Science 283, 7823–7833
16. Rinko, L. I., Lawrence, M. B., and Guilford, W. H. (2004) J. Mol. Biol. 346, 761–772
17. Vibert, P., Craig, R., and Lehman, W. (1997) J. Mol. Biol. 266, 8–14
18. Lehrer, S. S., and Morris, E. P. (1984) J. Biol. Chem. 259, 2070–2072
19. Takagi, J., Petre, B. S., Wang, J. H., and Springer, T. A. (2004) Cell 110, 599–611
20. Xiao, T., Takagi, J., Coller, B. S., Wang, J. H., and Springer, T. A. (2004) Nature 432, 59–67
21. Vinogradova, O., Velyvis, A., Velyviene, A., Hu, B., Haas, T., Plow, E., and Qin, J. (2002) Cell 109, 587–597
22. Phan, U. T., Waldron, T. T., and Springer, T. A. (2006) Nat. Immunol. 7, 883–889
23. Bremel, R. D., and Weber, A. (1972) Nat. New Biol. 238, 97–101
24. McKillop, D. F., and Geeves, M. A. (1993) Biochemistry 32, 9844–9849