Regulatory Proteins Alter Nucleotide Binding to Acto-Myosin of Sliding Filaments in Motility Assays

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ABSTRACT The sliding speed of unregulated thin filaments in motility assays is only about half that of the unloaded shortening velocity of muscle fibers. The addition of regulatory proteins, troponin and tropomyosin, is known to increase the sliding speed of thin filaments in the in vitro motility assay. To learn if this effect is related to the rate of MgADP dissociation from the acto-S1 cross-bridge head, the effects of regulatory proteins on nucleotide binding and release in motility assays were measured in the presence and absence of regulatory proteins. The apparent affinity of acto-heavy meromyosin (acto-HMM) for MgATP was reduced by the presence of regulatory proteins. Similarly, the regulatory proteins increase the concentration of MgADP required to inhibit sliding. These results suggest that regulatory proteins either accelerate the rate of MgADP release from acto-HMM-MgADP or slow its binding to acto-HMM. The reduction of temperature also altered the relationship between thin filament sliding speed and the regulatory proteins. At lower temperatures, the regulatory proteins lost their ability to increase thin filament sliding speed above that of unregulated thin filaments. It is hypothesized that structural changes in the actin portion of the acto-myosin interface are induced by regulatory protein binding to actin.

INTRODUCTION

The role of the regulatory proteins tropomyosin (Tm) and troponin (Tn) in muscle contraction has been viewed as controlling only the access of the cross-bridge (control of the rate of strong cross-bridge binding) to the thin filament (Huxley, 1972). This ''steric blocking'' mechanism is supported by the following facts: 1), The position of Tm/Tn on the thin filament limits subfragment 1 (S1) access to strong binding sites on actin (Xu et al., 1999). 2), The isometric force is proportional to the thick and thin filament overlap (Edman, 1979; Gordon et al., 1966a,b) and a monotonic function of the calcium bound to the thin filaments (Moss, 1992). 3), The unloaded shortening velocity, V_u, in intact muscle is independent of sarcomere length from 1.7–3.1 μm (Gordon et al., 1966b). These results support A. F. Huxley's cross-bridge model that predicts that V_u is independent of the number of cross-bridges attached to the thin filament but is limited by a drag imposed by strongly bound cross-bridges (Huxley, 1957). The fact that regulatory proteins exert little or no effect on the acto-myosin S1 ATPase V_{max} in solution, when Ca^{2+} is bound to troponin, also supports this view (Williams et al., 1988; Tobacman et al., 2002).

However, other data suggest that regulatory proteins can modulate the size of the power stroke, the duration of cross-bridge attachment (t_{on}), and/or the rate of ADP release. Strongly bound cross-bridges (e.g., N-ethylmaleimde S1) at subsaturating [Ca^{2+}] increase V_u in skinned muscle fibers (Swartz and Moss, 2001) and increase the affinity of the thin filament for the Tm complex by 10^4 (Tobacman and Butters, 2000). These data show that consequent to S1 binding to the thin filament, a major change in Tm and actin interaction occurs. Variation of myofibrillar [Ca^{2+}] alters not only isometric force and rate of force redevelopment (k_{tr}), but also V_u in skinned single muscle fibers (Brenner, 1988; Julian, 1971). Addition of regulatory proteins to unregulated actin thin filaments increases thin filament in vitro motility sliding speed, V_o, by as much as 100% (Fraser and Marston, 1995; Bing et al., 1997; Gordon et al., 1998; Homsher et al., 1996, 2000) and isometric force by 30–70% (Homsher et al., 2000; Fujita et al., 2002). These results imply that the interaction of Tm/Tn with the thin filament modifies both maximal force and V_o. These effects could be produced by Tm/Tn modulation of the size of the force and/or displacement produced by the power stroke, the rate of ADP release from, and/or the rate of ATP binding to attached crossbridges.

In the experiments described below we tested the hypothesis that ADP release may be hastened in the presence of regulatory proteins by measuring the effects of regulatory proteins on the rate of thin filament sliding at saturating calcium concentrations at various concentrations of ATP and ADP. Further we measured the effects of regulatory proteins on V_o at temperatures ranging from 7° to 25°C to learn if the potentiation of V_o by regulatory proteins is temperature dependent. The results showed that regulatory proteins reduced the binding affinity of both ATP and ADP for acto-HMM. However, as the temperature was lowered, the potentiating effects of regulatory proteins on unloaded sliding speed were lost. Reduction of temperature may reposition Tm/Tn on the actin surface, or otherwise alter indirect interactions between myosin and troponin-tropomyosin, thus preventing the potentiating effects of regulatory proteins on the unloaded sliding speed.

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MATERIALS AND METHODS

Proteins

Isolation and purification of rabbit skeletal myosin or HMM, rabbit skeletal actin, bovine cardiac troponin (BVCTn), bovine cardiac TnC, and bovine ventricular tropomyosin (BVCTm) were as previously described (Gordon et al., 1998; Homsher et al., 1996, 2000).

Motility measurements and analysis

Rhodamine-phalloidin labeled F-actin was prepared as described previously (Homsher et al., 1996) and used within 2 weeks. Nitrocellulose-coated cover slips and flow cells were prepared as previously described (Homsher et al., 1996). An HMM solution (300 μg/ml) was injected into the flow cell. Two minutes after adding the HMM, the solution was replaced by 50 μl of 0.5 mg/ml bovine serum albumin in assay buffer (25 mM MOPS, 25 mM KCl, 2 mM $MgCl_2$, 2 mM EGTA (pCa 9), 1 mM DTT, pH 7.4). One minute later 50 μl of 20 nM RPh-actin was introduced into the chamber and allowed to incubate for 2 min. Excess actin was washed off by several 50 μl aliquots of assay buffer and the solution was replaced by an assay buffer containing 0.5 μM cTm and 0.5 μM cTn and allowed to incubate for 7 min followed by several washes of assay buffer (containing 100 nM cTm and 100 nM cTn) and then replaced by a motility solution. This method of reconstituting regulated thin filaments already bound to the motility surface prevents thin filament bundling which sometimes occurs during incubation of 2 μM actin with 0.5 μM Tm and 0.5 μM Tn previously used (Homsher et al., 1996; Lin et al., 1996). It produces thin filaments that are completely regulated (total absence of movement at pCa 9), gives a sliding speed and calcium sensitivity identical to the incubation method, and has been used successfully in previous work (Homsher et al., 2000; Karibe et al., 2001). The motility solution contained 120 mM MOPS/KOH (pH 7.4), 2 mM $MgCl_2$, 2 mM CaEGTA (pCa 5), 0.05–2 mM MgATP, 0–5 mM MgADP, 100 nM cTm, 100 nM cTn, 0.2% methyl cellulose, and 10 mM DTT. Photobleaching protective agents (Kron et al., 1991), 14 mM glucose, 240 units of glucose oxidase/ml, and 9×10^3 units of catalase/ml, were also added. The ionic strength was 91 mM. In experiments in which the temperature was altered the motility solution contained 25 mM KCl, 25 mM MOPS/KOH (pH 7.4 at 25°C), 2 mM CaEGTA, 2 mM $MgCl_2$, 1 mM MgATP, and 10 mM DTT and 100 nM Tm/100 nM Tn along with the photobleaching protective agents. This solution's ionic strength was 58 mM. The pK_a for MOPS at 25°C is 7.2 and the slope of MOPS pK_a with respect to temperature is -0.013/°C (Sankar and Bates, 1978). At 7°C, the pH is 7.63 (instead of 7.4 in the experiments at 25°C), and the pCa is 5.2 (instead of the pCa 5.0 at 25°C).

The motility chamber was mounted on a temperature-controlled microscope stage and objective so that temperature could be set at any value between 7° and 30°C. Motility was viewed under fluorescence illumination through a 100× objective and the thin filament movement recorded using a Dage MSIT camera and Panasonic tape recorder. Quantification of the thin filament sliding speed was performed using a Motion Analysis system (Santa Rosa, CA). Data were acquired and analyzed as previously described (Homsher et al., 1992, 1996) and are expressed as the mean ± SE. In these analyses, filaments not moving at a uniform sliding speed were rejected. However, the results were qualitatively the same if, instead, all filaments were averaged, including those moving at nonuniform speeds.

RESULTS

To assess the effect of ADP release on the thin filament sliding speed, the effect of [ATP] on the rate of thin filament sliding at pCa 9 and 5 in the presence and absence of cardiac regulatory proteins was first measured. In control experiments at 25°C and pCa 9 in the absence of regulatory proteins, actin filaments moved over the HMM-coated surface at the same speed as at pCa 5. Between 80 and 90% of the filaments on the surface moved at a uniform speed. However, at pCa 9, regulated thin filaments bound to the surface, but <1% of them moved (and those that did moved at speeds <0.5 μm s^{-1}). This means that the filaments were well regulated. Fig. 1 *A* shows the unregulated thin filament sliding speeds as a function of [MgATP] at pCa 5, and Fig. 1 *B* is the same for regulated thin filaments. Each point in the figures represents the mean ± SE for 100–200 filaments. The data show that the results are well fitted by a hyperbolic function in which the thin filament sliding speed, V_o, at infinite [MgATP], is 5.3 ± 0.2 μm s^{-1} and is 9.4 ± 0.5 μm s^{-1} (mean ± SE) for regulated thin filaments. The increased thin filament sliding speed at 1 mM [MgATP] in the presence of regulatory proteins is similar to that previously reported (Fraser and Marston, 1995; Bing et al., 1997; Gordon et al., 1998; Homsher et al., 1996, 2000). For unregulated thin

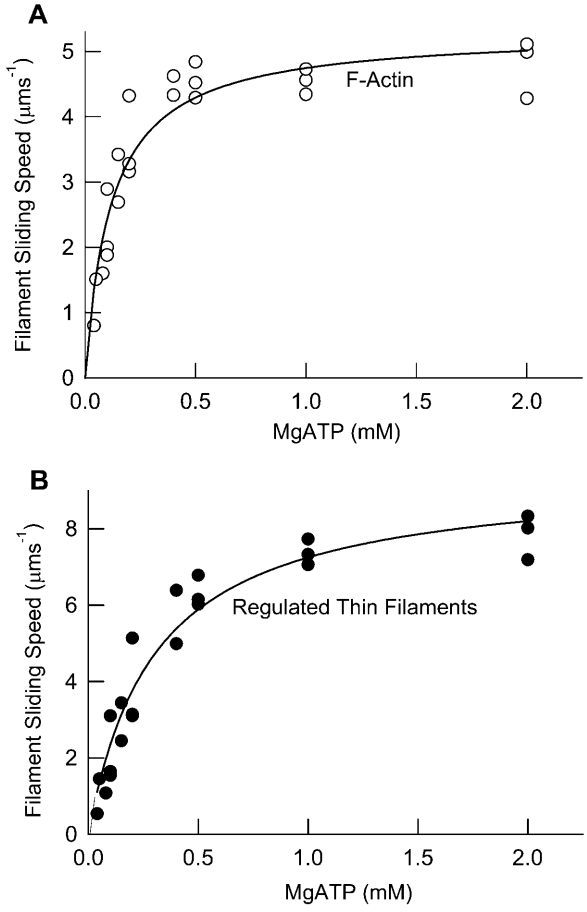

FIGURE 1 The effect of ATP on the sliding speed of thin filaments in the absence (*A*) and (*B*) presence of troponin and tropomyosin at 25°C. The solid line in each case is a least squares regression of the thin filament sliding speed on the [MgATP] for Eq. 1. $V = V_o\ (K_m/(K_m+[MgATP]))$ (1) The results of the fits in *A* are $V_o = 5.3 \pm 0.2$ μm s^{-1} and $K_m = 118 \pm 18$ μM (mean ± SE). The results of the fits in *B* are $V_o = 9.4 \pm 0.5$ μm s^{-1} and $K_m = 301 \pm 47$ μM (mean ± SE).

filaments K_m for MgATP was 118 ± 18 μM while that for regulated thin filaments was 301 ± 47 μM. (The observed sliding speeds at 2 mM MgATP for the unregulated thin filaments was 89.8% of the V_o (compared to a value of 94.4% predicted by the regression) and for regulated thin filaments was 83.5% of the V_o (compared to a value of 86.9% predicted by the regression).) These K_m values are significantly different ($p < 0.01$). The results show that either MgATP binds more tightly to the unregulated acto-HMM or MgATP binds with the same affinity, but a forward rate constant subsequent to binding is slower in unregulated acto-HMM, resulting in a tighter apparent affinity.

Fig. 2 shows a plot of the thin filament shortening as a function of [MgADP] at 2 mM [MgATP]. The data were fit by the equation:

$$V = V_o/((K_m/[\text{MgATP}]) \times (1 + ([\text{MgADP}]/K_i)) + 1), \quad (1)$$

where V is the observed thin filament sliding speed, V_o is the sliding speed at infinite [MgATP], and K_m and K_i are the Michaelis and inhibition constant, respectively. The data in Fig. 2 were fitted using the K_m values measured in Fig. 1, *A* and *B*. The regressions yield a V_o of 5.8 ± 0.2 and 9.7 ± 0.2 μm s^{-1} (mean ± SE) for unregulated and regulated thin filaments, respectively. These values are not different from those estimated in Fig. 1. More importantly, MgADP binds to regulated acto-HMM less strongly (217 ± 20 μM) than it does to unregulated acto-HMM (131 ± 17 μM). The difference in K_i is significant at $p < 0.01$. K_i is equal to the ratio of the rate of MgADP release/rate of MgADP binding. Thus, elevated K_i for regulated thin filaments may be a result of a faster dissociation rate of MgADP from acto-HMM-MgADP. Such behavior could account for the increased speed of unloaded sliding seen in regulated thin filaments. If this is the case, it implies that the binding of the regulatory proteins to actin modifies actin structure so that its interaction with MgADP-S1 promotes a more rapid MgADP release.

Ideally this hypothesis could be tested using stopped flow measurements of ADP dissociation from acto-HMM-MgADP. However, at room temperature the rate of MgADP release from acto-myosin is so fast that it is difficult to accurately measure it in the absence of regulatory proteins (Weiss et al., 2001). It could be that the hypothesis can be tested at a lower temperature. To learn if this was a viable possibility, we measured the unloaded shortening velocity of thin filaments at temperatures ranging from 7° to 25°C. The results are shown in Fig. 3.

Fig. 3 shows that at 25°C and 1 mM ATP the unregulated thin filaments moved over the surface at 3.8 ± 0.5 μm s^{-1} (mean ± SD) while regulated thin filaments moved at 6.58 ± 1.1 μm s^{-1} (mean ± SD) and thus reproduce the effects shown in Figs. 1 and 2. When the temperature was lowered, the unregulated thin filament sliding speed declined, but that of the regulated thin filaments declined more rapidly with temperature. At 7°C the unregulated thin filaments moved at 0.44 ± 0.03 μm s^{-1} (mean ± SD) while the regulated thin filaments moved at 0.36 ± 0.06 μm s^{-1} (mean ± SD). Such data would be obtained if at lower temperature there were reduced calcium binding to the troponin. However, raising [Ca^{2+}] to 1 mM did not alter the thin filament sliding speed

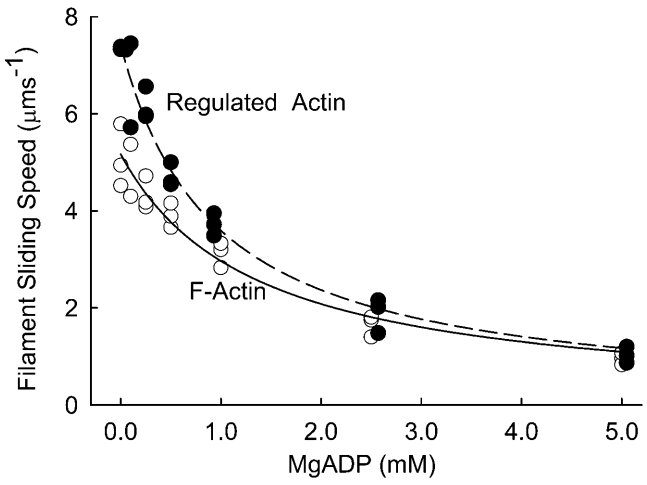

FIGURE 2 The effect of MgADP on the sliding speed of thin filaments in the absence (*open circles*) and presence (*filled circles*) of (Tm/Tn). The *solid line* in each case is the least squares fit of the data to $V = V_o/((K_m/[\text{MgATP}])(1+([\text{MgADP}]/K_i)) + 1)$, where $K_i = k_{\text{ADP off}}/k_{\text{ADP on}}$. The line labeled F-Actin is the fit for unregulated actin and that labeled Regulated Actin is for regulated thin filaments. The K_m MgATP for each condition in Fig. 1 was used for fitting. The results of the fits for unregulated filaments were $V_o = 5.8 \pm 0.0.2$ μm s^{-1} and $K_i = 131 \pm 17$ μM (mean ± SE). The results of the fits for the regulated filaments was $V_o = 9.7 \pm 0.2$ μm s^{-1} and $K_i = 217 \pm 20$ μM (mean ± SE).

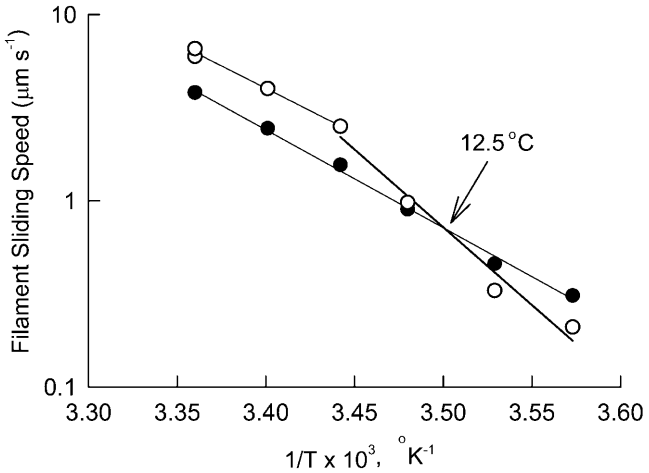

FIGURE 3 An Arrhenius plot of thin filament sliding speed for regulated (*open circles*) and unregulated (*filled circles*) thin filaments. The data from the unregulated filaments are fitted to with a line having an activation energy (E_a) of 100 ± 2 kJ/mol. Fitting of the data from the regulated thin filaments requires two E_a: for temperatures between 17° and 25°C, the E_a is 86 ± 2 kJ/mol while that for temperatures between 7° and 17°C is 159 ± 19 kJ/mol.

(data not shown). It might also be that the rise of pH to 7.63 as temperature was lowered modified the response to regulatory proteins. However, in control experiments in which the pH at 7°C was set at 7.4, thin filaments containing the regulatory proteins moved with a speed which was less than half that of the unregulated thin filaments (a result comparable to that seen at a pH of 7.63). It could also be that at this temperature the increase in K_m for MgATP by regulatory proteins is so great that V_o is significantly less than its maximal value. The activation energy (E_a) for unregulated thin filament sliding is 100 ± 2 kJ/mol and is close to that earlier reported for unregulated thin filaments, 98 kJ/mol (Homsher et al., 1992) and the value of 123 ± 7 kJ/mol fitted to the data of Anson (1992, Fig. 5) over the same temperature range as used here. Fitting the data from the regulated thin filaments requires two values of E_a. For the temperature range from 17°C and above, the data is well fitted by an E_a of 86 ± 2 kJ/mol while for data from 7° to 17°C the E_a is 159 ± 19 kJ/mol.

The fraction of thin filaments sliding at uniform speed at different temperatures is shown in Fig. 4. It is noteworthy that the fraction of thin filaments moving at uniform speed is greatest for the naked thin filaments and does not markedly change with temperature. However, the fraction of uniformly moving regulated thin filaments drops markedly with temperature suggesting that the nature of the interaction between the thin filament and the regulatory proteins depends on the ambient temperature. The crossover point for the sliding speeds is ~12°C. The reason for this change may be related to the fraction of time the regulatory proteins spend in a position on the actin filament which allows the cross-bridges to attach productively.

DISCUSSION

Previous measurements of the K_m for MgATP binding to acto-HMM in motility assays of unregulated thin filaments gave values ranging from 70 to 138 μM (Kron and Spudich, 1986; Harada et al., 1987; Homsher et al., 1992; Regnier et al., 1998; Baker et al., 2002). These values are close to the present data (118 μM). The inhibitory constant, K_i, for MgADP binding to unregulated thin filaments had previously been measured at 120 ± 30 μM (Homsher et al., 1992) and also agrees with the value measured in this work (131 ± 17 μM). Comparable measurements of V_u in skinned single fast rabbit or rat fibers (contracting at 10–15°C) yielded 150–250 μM for K_m and 200 μM for K_i (Chase and Kushmerick, 1995; Cooke and Pate, 1985; Cooke and Bialek, 1979; Regnier et al., 1998). While these values are greater than those from in vitro motility studies, fibers contain regulatory proteins not present in the unregulated thin filaments. A more appropriate comparison would be with the K_m and K_i values from regulated thin filaments which were 301 ± 47 μM and 217 ± 20 μM, respectively. These data better fit the skinned muscle fiber data. Although not an explicit objective of these experiments, an important implication of this data is that the regulatory proteins increase V_o by 30–80% above that of unregulated thin filaments (Lin et al., 1996; Fraser and Marston, 1995; Homsher et al., 1996, 2000). This is important because direct comparisons of V_u from intact muscles and skinned muscle fibers are approximately twofold greater than the V_o measured in vitro motility assays of unregulated thin filaments. For example, over the temperature range 15°–35°C V_u for intact rat type II muscle fibers is 50% greater (Ranatunga, 1998) than the comparable unregulated thin filament sliding speed (Hook and Larsson, 2000). For rabbit type II fibers only data from skinned muscle fibers are available. V_u in these fibers is 4.1 μm s^{-1}/half-sarcomere at 20°C and 5.6 μm s^{-1}/half-sarcomere at 25°C (Pate et al., 1994). The corresponding unregulated thin filament in vitro motility speeds, V_o, are 2.4 μm s^{-1} at 20°C and 4.3 μm s^{-1} at 25°C (Homsher et al., 1992). The experiments illustrated in Fig. 3 give values of 4.0 μm s^{-1} and 6.3 at 20° and 25°C, respectively. These values are also underestimates because the [MgATP] was only 1 mM in the motility assays, correction for the effect of a K_m of 300 μM, raises these values to 5.5 and 8.1 μm s^{-1}, respectively. Thus, to better compare V_o from the in vitro motility assays with V_u in intact and skinned muscle fibers, the motility assays should contain regulatory proteins bound to the thin filaments and an [MgATP] near 5 mM (the physiological concentration used in most skinned muscle studies).

The increase in K_m for MgATP binding to myosin in the presence of regulated proteins is similar to effects earlier reported by Resetar and Chalovich (1995) and Kraft et al. (1992) for MgATP and MgATP-γ-S, respectively. In those reports the K_m in the presence of regulatory proteins and calcium was 6- to10-fold greater than that in the absence of regulatory proteins. These results show that the interaction of regulatory proteins with the thin filament do induce allosteric effects on kinetic steps involving nucleotide interaction with myosin.

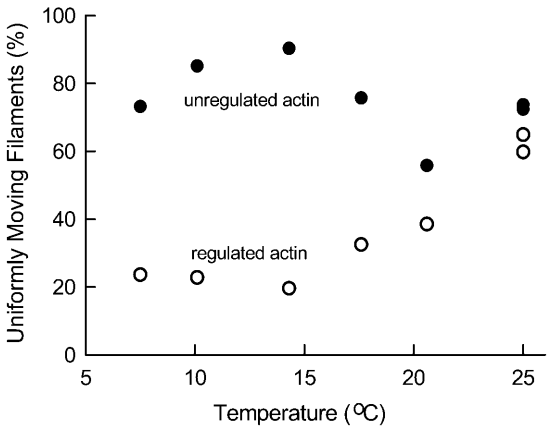

FIGURE 4 A plot of the fraction of thin filaments moving as a function of temperature. The filled circles are the data for regulated thin filaments while the data in open circles are data from unregulated thin filaments.

Is it tropomyosin, troponin, or both that control the effect on thin filament sliding speed? Earlier studies showed that the addition of native skeletal or cardiac tropomyosin alone to thin filaments (propelled by skeletal myosin) does not produce an increase in V_o (Homsher et al., 2000; Fraser and Marston, 1995; VanBuren et al., 2002) although at lower S1 head density, it clearly inhibits thin filament sliding speed. (Smooth muscle Tm markedly potentiates sliding speed of thin filaments propelled by smooth muscle or limulus myosin (Umemoto and Sellers, 1990, Wang et al., 1993). There is smaller increase (~20%) seen using skeletal myosin when an expressed α-Tm containing an alanine-serine addition to the amino terminus is used (Bing et al., 1997)). However, in those studies the concentrations of Tm (100–200 nM) were probably not great enough to produce a saturation of Tm binding to the thin filament (Strand et al., 2001). Strand et al., (2001) also have produced data suggesting that at saturating Tm ($\geq$2 μM) and a high density HMM on the motility surface, the speeds of the thin filaments were elevated by ~40% above those of unregulated actin. Because this effect is crucially dependent on the HMM density on the surface of the motility slide, we cannot say with certainty whether or not Tm directly mediates this response. An increase in thin filament sliding speed does occur in the presence of troponin alone or in the presence of both troponin and tropomyosin. Although troponin C does not bind significantly to actin at concentrations used in the in vitro motility studies, both TnI and TnT do bind to actin (Tobacman and Butters, 2000; Tobacman et al., 2002). High affinity constants are only observed when Tm is also present so it is not clear whether the Tn binding is specific in the absence of Tm. TnT binds to actin particularly well in the region of residues 1–153 of the 284 residue bovine cardiac TnT (Tobacman et al., 2002). The site of this binding to actin is not certain, but electron microscopic reconstruction techniques suggest tropomyosin is positioned onto the same surface of actin to which S1 binds (Tobacman et al., 2002). Cardiac TnI binds to the same surface on actin via residues (141–153) near cysteine-42 of actin and via residues 183–203 to unidentified residues on actin (Van Eyk et al., 1997; Lehman et al., 2001).

What is the mechanism by which regulatory proteins exert these effects? We argue that this occurs because regulatory proteins increase the rate of ADP release. This conclusion seems inconsistent with the results of Rosenfeld and Taylor (1987) who found that the rate of Mg-ε-ADP release from both unregulated and regulated acto-S1 was about the same (450–600 s^{-1}). This rate is much less than that of MgADP (Weiss et al., 2001; Siemankovski et al., 1985). Further in work in our group (Pavlov et al., 2003) we find that V_o in 1–2 mM ε-ATP at 25°C is only ~30% (1.3 μm s^{-1}) of that seen in ATP and V_o is the same in the presence and absence of regulatory proteins. Therefore, the features of acto-myosin interaction that produce the effects seen in MgATP are ineffective in Mg-ε-ATP.

Because the effect of regulatory proteins on thin filament sliding involves changes in the binding of ATP and ADP to the acto-S1, it is likely that regulatory proteins promote changes in the actin sites that bind to S1 and thus influence the structure of the S1 molecule itself. This is an idea previously called allosteric or kinetic regulation of contraction (for an excellent analysis of this idea see Chalovich, 1992). In its original form the kinetic mechanism of regulation hypothesized that regulatory proteins accelerate a weak to strong crossbridge transition associated with product (Pi and ADP) release steps. However, measurement of the effects of Pi on the isometric tension transients implied that neither the force generating step nor the release of Pi were regulated by calcium (Millar and Homsher, 1990). It was instead suggested that calcium controlled a weak to strong crossbridge transition preceding both the force generation and Pi release steps. Subsequently, data was obtained showing that thin filament sliding speed and/or unloaded shortening velocity were increased by point mutations of regulatory proteins (Sweeney et al., 1998; Lin et al., 1996; Bing et al., 1997; Homsher et al., 2000). These data were most simply explained by assuming that the rate of MgADP release from the acto-S1-MgADP complex was accelerated by the regulatory proteins. This notion is reenforced by the facts that the presence of S1 increases the affinity of the regulatory proteins for actin by ~10^4 (Tobacman and Butters, 2000) and the presence of the regulatory proteins increases S1-actin affinity by four- to sevenfold (Geeves and Halsall, 2002; Williams and Greene, 1983; Tobacman and Butters, 2000). Thus, there is abundant evidence for allosteric regulation of nucleotide and S1 binding to actin by regulatory proteins.

How might regulatory thin filaments modulate kinetic steps associated with the cross-bridge cycle? Evidence from both biochemical (McKillop and Geeves, 1993) and structural (Xu et al., 1999) studies indicates that Tm exists in three different positions on the thin filament: the ''blocked'' state (near the external edge of the filament, blocking weak and strong myosin binding sites in the absence of calcium); the closed state (nearer the groove between the two actin strands, partially weak and strong binding sites in the presence of calcium); and the ''open'' state (nearer still to the groove between the two actin strands fully exposing myosin binding sites when the cross-bridges are attached to the actin filament). The activation of cross-bridge attachment to actin thus depends on the positioning of the regulatory proteins and they are believed to occupy these positions dependent on the equilibrium constants between the different states. The important point is that the changing position of the regulatory proteins implies that different actin amino acid residues interact with the thin regulatory proteins. These interactions could produce structural changes in actin, which could influence the interaction of actin-myosin and ADP. An alternative possibility is that the regulatory proteins, by virtue of their positioning on the thin filament, may also

interact with the regions of S1 adjacent to the actin-binding surface. There is little known about the specific actin, tropomyosin, or troponin interaction sites because the structure of the actin-troponin/tropomyosin-S1 form is not known. In any case, the present results establish that the regulatory proteins do modulate actomyosin interaction with its nucleotide, thereby altering filament sliding over a myosin-coated surface in vitro. It is only a modest extrapolation to predict corresponding effects of the regulatory proteins on the mechanical behavior of intact muscle.

The failure of regulatory proteins to potentiate unloaded sliding speed at temperatures lower than ~10°C was an unexpected finding. At least two explanations are possible. One could be that the interaction of Tm/Tn with actin that may accelerate the rate of MgADP release from the actomyosin S1-ADP complex might be altered. Using the idea that the activation of the thin filament depends on the positioning of Tm/Tn on the thin filament, it could be that at temperatures above 10°C the equilibrium between the ''open'' and ''closed'' states is shifted toward the ''closed state'' while at higher temperatures the equilibrium shifts toward the ''open'' state. This implies that the specific interactions between Tm/Tn and actin and/or myosin are different in the two different states. If the free energy change for the transition from the ''closed'' to the ''open'' position is relatively small (Tobacman and Butters, 2000; Tobacman et al., 2002), then as the temperature is reduced, less time will be spent in the open position so that the effective number of cross-bridges pulling on thin filaments will be reduced. On the other hand, this interpretation does not exclude the possibility that at low temperatures myosin and the regulatory proteins affect each other via alterations in actin structure. In fact, the affinity of tropomyosin for actin at 10°C is increased by at least an order of magnitude by addition of myosin S1 and is too tight to readily measure (M. Heller and L. Tobacman, data not shown). Finally, it is well known that as temperature is raised, the force of isometrically contracting muscle fibers increases (Ford et al., 1977). It may be that as temperature rises Tm/Tn complex shifts toward the ''open'' position. An alternative explanation for the temperature dependence of the regulatory protein effects on unloaded sliding speed is that the rate limiting step in the ATPase mechanism changes with temperature so that the effects of the regulatory protein are no longer affecting the step that controls sliding speed.

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