Thin Filament Regulation and Ionic Interactions between the N-terminal Region in Actin and Troponin

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ABSTRACT The N-terminal region in actin has been shown to interact with both myosin and troponin (Tn) during the cross-bridge cycle and in regulation. To study the role of this region in regulation, we used yeast actin mutants with increased and decreased numbers of acidic residues. The mutants included D24A/D25A, with Asp^{24} and Asp^{25} replaced with alanines; DNEQ, with the substitution of Asp^{2} and Glu^{4} with their amide analogs; and 4Ac, with Glu^{3} and Asp^{4} inserted in lieu of Ser^{3}. In the in vitro motility assay, using reconstituted regulated thin filaments, the sliding speeds of DNEQ, D24A/D25A, and 4Ac were similar at all pCa values. Thus, Ca^{2+}-sensitivity of the thin filaments and the inhibitory function of TnI appear to be insensitive to changes in charge (±2) at the N-terminus of actin, suggesting little, if any, role of that actin region in regulation. A Ca^{2+}-independent conformational change in that region was detected upon troponin binding to actin-Tm via an increase in the fluorescence of a pyrene probe attached to another yeast actin mutant that we used (Cys^{1}).

INTRODUCTION

In Ca^{2+}-dependent regulation of the cross-bridge cycle, a series of direct and allosteric interactions between actin, tropomyosin (Tm), troponin (Tn), and myosin mediate the activation of the thin filament (reviewed in Leavis and Gergely, 1984; Zot and Potter, 1987; Farah and Reinach, 1995; Tobacman, 1996). In the three-state model proposed by McKillop and Geeves (1993), Tm moves across F-actin as calcium and myosin bind to, and induce conformational changes in, the thin filament. In this scheme, the regulatory complex equilibrates among the *blocked*, *closed*, and *open* states. Structural studies (Lehman et al., 1994; Narita et al., 2001) and kinetic measurements (McKillop and Geeves, 1993; Geeves and Lehrer, 1994) support a repositioning of Tm on the actin filament upon Ca^{2+} addition. The recent higher resolution three-dimensional reconstruction of the thin filament (Lehman et al., 2001), that also includes the first mapping of the Tn positions on actin, is consistent with the main tenets of the McKillop and Geeves' three-state regulation model.

In the muscle thin filaments, Tn binds to actin-tropomyosin with a stoichiometry of 1 Tn to 1 Tm to 7 actin subunits (Ebashi et al., 1969; Potter and Gergely, 1974). In the absence of Ca^{2+}, the Tm–Tn complex inhibits actomyosin interactions by predominantly occupying the blocked state, sterically restricting the access of myosin to weak-binding sites on actin (reviewed in Lehrer and Geeves, 1998; Squire and Morris, 1998). In this mechanism, the inhibitory function of TnI (the inhibitory subunit of Tn) is maintained through contacts to actin and TnC (the calcium binding subunit of Tn). Another region of TnI interacts with TnT (tropomyosin binding subunit of Tn), relaying perhaps conformational changes to Tm (reviewed in Perry, 1999). Upon Ca^{2+}-activation, Tn undergoes conformational changes that result in a shift of Tm–Tn to the closed state, in which the myosin weak-binding sites are exposed (Lehman et al., 2001).

It has been hypothesized that the interaction between TnI and actin is maintained by electrostatic contacts, and that this mode of interaction is important in Tn-dependent regulation of actomyosin in the presence of Tm. The basis for this hypothesis rests with the use of synthetic peptide analogs of the inhibitory region of TnI, i.e., that part of the protein which, by itself, inhibits actin-activated myosin ATPase (Talbot and Hodges, 1981; Levine et al., 1988). Van Eyk and Hodges (1988) identified lysine and arginine residues on TnI that are essential for the maximal inhibition of acto-S1 ATPase. However, other explanations for these results are also possible.

Chemical cross-linking results (Gergely et al., 1988) and NMR studies (Levine et al., 1988) imply a direct interaction between actin N-terminal acidic residues and TnI. In addition, the recent electron microscopy-based reconstituted thin actin filament model shows Tn in close contact with the N-terminal residues 1–4 and 23–27 on actin (Lehman et al., 2001). However, the functional importance of these interactions in the regulation of actomyosin ATPase with fully reconstituted thin filaments has never been established or even tested. Questions about Tm and Tn binding sites on actin can also be raised regarding the Tm-based, cooperative and allosteric model for thin-filament regulation (Lehrer and Geeves, 1998). The role of Tn in this model is that of an allosteric inhibitor of S1 binding to the thin filament and switching it into an activated form. Clearly, it is essential to map at a high resolution the Tm and Tn contacts on actin and determine the importance of the ionic actin–Tn inter-

Submitted March 19, 2002 and accepted for publication July 17, 2002.

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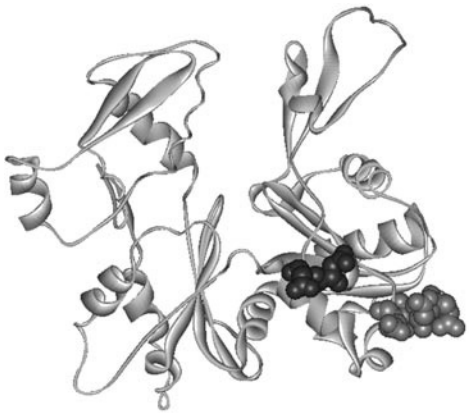

FIGURE 1 Ribbon representation of the G-actin structure (Kabsch et al., 1990) with the mutated amino acid residues in the N-terminal region depicted in a space-filling form. Residues 1–4, *light gray*, have been mutated in the individual yeast actins used in this study: Cys-1 = cysteine introduced at the N-terminus; DNEQ = Asp^2 to *asn* and Glu^4 to *gln* mutations; 4Ac = *asp* and *glu* inserted in lieu of Ser^3. Residues 24/25, *dark gray*, indicate the site where alanines have been substituted for residues Asp^{24} and Asp^{25}.

action if we are to understand the molecular basis of the control of muscle contraction.

To this end, we used in this work yeast actin mutants with Asp^{24} and Asp^{25} residues replaced with alanines (D24A/D25A); with two residues substituted (Asp^2 to *asn* and Glu^4 to *gln*), neutralized at the N-terminus (DNEQ); or with *asp* and *glu* in lieu of Ser^3 added to the N-terminus (4Ac) in subdomain 1 of actin (Fig. 1). These mutants have been used previously to assess the role of actin N-terminal acidic residues in the formation of the weak and strong binding states with myosin subfragment-1 (Miller et al., 1996; Wong et al., 1999). Here, we use them to evaluate the importance of these residues on the ability of Tn, together with Tm, to regulate the sliding of actin filaments.

MATERIALS AND METHODS

Reagents

Adenosine triphosphate (ATP), dithiothreitol (DTT), phalloidin, phenylmethylsulfonyl fluoride, dextrose, 2-{[tris(hydroxymethyl)methyl]amino}-1-ethane sulfonic acid (TES), ethylene glycol-bis(β-aminoethyl ether)*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), Tris-HCl, and β-mercaptoethanol were purchased from Sigma (St. Louis, MO). Yeast extract and tryptone were purchased from Difco (Detroit, MI). DNase I column materials were purchased from Worthington Biochemical Co. (Lakewood, NJ; DNase I) and Bio-Rad (Hercules, CA; Affigel-10). The fluorescent labels rhodamine phalloidin and *N*-(1-pyrene)-maleimide were purchased from Molecular Probes (Eugene, OR).

Preparation of proteins

Actin mutants were expressed in *Saccharomyces cerevisiae* yeast strain cultures (described previously in Wertman et al., 1992; Cook et al., 1993; Hansen et al., 2000) grown at 25°C; wild-type yeast was purchased from a commercial bakery. Yeast actins were isolated from their respective strains using the DNase I affinity chromatography as described previously (Cook et al., 1992). All yeast actins were used within two weeks of purification. The concentrations of the mutant actins were determined using the Bradford protein assay, and rabbit actin as a standard. Rabbit actin and myosin were prepared from rabbit skeletal muscle according to the methods of Spudich and Watt (1971) and Godfrey and Harrington (1970), respectively. Myosin subfragment 1 (S1) was prepared by chymotryptic digestion, according to the method of Weeds and Pope (1977). The concentrations of rabbit actin and S1 were determined spectrophotometrically using extinction coefficients of $\epsilon_{280} = 7.5\ cm^{-1}$ and $\epsilon_{292} = 11.5\ cm^{-1}$ for 1% protein solutions, respectively. Bovine cardiac Tm and bovine cardiac Tn were generous gifts from Dr. L. Tobacman (Univ. of Iowa).

Pyrene labeling of M1C/C374A (Cys-1) actin

Following the standard protocol for yeast actin preparation, the elutant was dialyzed overnight against G-buffer (10 mM Tris-HCl, pH 7.5; 0.2 mM $CaCl_2$, 0.2 mM ATP, pH 7.0) that did not contain DTT. After determining its actin concentration, actin was incubated at room temperature for 90 min with a 2.5 molar ratio of pyrene maleimide (dissolved in dimethyl formamide) and 4 mM $MgCl_2$. The labeling reaction was quenched with 1.0 mM DTT and spun in a Beckman XT tabletop ultracentrifuge at 140,000 $\times$ *g* for 50 min. The supernatant was discarded; the pellet was resuspended in G-buffer, and then dialyzed overnight. After a final ultracentrifugation, the collected supernatant actin contained the labeled G-actin. The degree of pyrene-modification was determined by comparing the actin concentration (as measured by the Bradford protein assay) with the concentration of pyrene label, which was determined using the extinction coefficient of 22 $mM^{-1}cm^{-1}$ at 344 nm. The degree of labeling was typically ~100%.

Fluorescence measurements with pyrenyl Cys-1 actin

Fluorescence emission spectra for the pyrene-labeled Cys-1 actin were recorded at 23°C in a SPEX Fluorolog (SPEX Industries Inc., Edison, NJ) using an excitation wavelength of 344 nm. The concentrations of actin, Tm, and Tn were 4.0, 2.0, and 1.0 μM, respectively, with equimolar amounts of phalloidin added to stabilize the F-actin (prepolymerized with 3.0 mM $MgCl_2$). The buffer consisted of 45 mM TES, pH 7.5, 50 mM NaCl, 3 mM $MgCl_2$, 0.2 mM ATP, 0.2 mM $CaCl_2$, and 1 mM DTT. EGTA was added to 1.0 mM to create "$-Ca^{2+}$" conditions. Troponin titrations of actin–Tm were carried out $\pm Ca^{2+}$ using the same conditions as above. An excitation wavelength of 344 nm and an emission wavelength of 394 nm were used, and the concentration of Tn was increased in increments of ~0.15 μM until the final 1.0 μM concentration was reached.

ATPase regulation of Cys-1 yeast actin

The rates of S1 Mg-ATPase activated by regulated pyrene-labeled Cys-1 actin in the presence and absence of Ca^{2+} were obtained as described previously (Gerson et al., 1999), by using light scattering to monitor the clearing time of regulated F-acto-S1 solutions. Thin filaments were reconstituted using bovine cardiac Tn, and bovine cardiac Tm and the pyrene-labeled actin. The concentrations of actin, Tm, Tn, and S1 were 4.0, 2.0, 1.0, and 1.0 μM, respectively. Experiments were carried out at 23°C using a Mg-ATP concentration of 0.1 mM and either 1.0 mM EGTA or 0.2 mM $CaCl_2$. The time of Mg-ATP hydrolysis was monitored by measuring the light scattering at 350 nm from the above solutions in a SPEX Fluorolog.

In vitro motility assays

The motility assays were performed as described previously (Homsher et al., 1996). The temperature was maintained at 25°C for all assays. Heavy

meromyosin (HMM) was prepared as described by Kron et al. (1991). To remove ATP-insensitive heads, HMM was centrifuged with 0.15 mg/ml rabbit F-actin in a solution containing 25 mM MOPS, 25 mM KCl, 1.0 mM $MgCl_2$, 10 mM DTT, and 4.0 mM ATP at pH 7.4 for 30 min at 130,000 $\times$ *g*. The supernatant was applied to nitrocellulose-treated coverslips at an HMM concentration of 0.3 mg/ml. Sliding speeds at various [Ca^{2+}] were measured as previously described (Homsher et al., 1992). Briefly, a solution containing rhodamine phalloidin-labeled actin filaments (10 nM), together with skeletal Tm and bovine cardiac Tn, each at a concentration of 0.5 μM, was applied to the coverslip. After a 1-min incubation period, the unbound filaments were washed away with the assay buffer at an ionic strength = 50 mM (25 mM KCl, 1 mM EGTA, 2 mM $MgCl_2$, 10 mM DTT, and 10 mM imidazole at pH 7.4). The viscosity of the assay buffer was enhanced with 0.2% methylcellulose. Movement was initiated with the assay buffer containing 1.0 mM ATP and 0.1 μM each of the regulatory proteins, with an oxygen-scavenging system. Quantification of the sliding velocities was carried out with an Expertvision system (Motion Analysis, Santa Rosa, CA). The velocities of individual filaments with SD of less than half of the average velocity were used for statistical analysis (Homsher et al., 1992), and these filaments were considered to move smoothly in the assay system. The sliding speeds at various pCa were fitted to a form of the Hill equation (Homsher et al., 1992),

$$y = \frac{y_{max}}{1 + 10^{n(pCa - pCa50)}}, \quad (1)$$

where y is filament speed, y_{max} is the maximum filament speed, pCa_{50} is the pCa value at which $y = 0.5y_{max}$, and n is the Hill coefficient. The data were fitted to $r^2 > 0.99$.

RESULTS AND DISCUSSION

Experimental considerations

The goal of this work was to test the role of the ionic interactions between Tn and actin subdomain-1 in the regulation of thin filaments. Although previous biochemical studies have used purified Tn subunits (and Tm), particularly for mapping their binding sites on actin, such measurements are open to questions regarding the physiological significance of information derived from the partially reconstructed filaments. For example, it is frequently difficult to reproduce the precise stoichiometry of the actin–Tm–Tn complex (7:1:1) when working with actin–Tm and TnI only. Indeed, in our experiments, the binding of TnI to actin–Tm did not saturate at 1:7 molar ratio of TnI:actin (data not shown). In addition, other investigators have reported on different classes of TnI binding to actin–Tm (Zhou et al., 2000), and on experimental difficulties related to the tendency of TnI to aggregate (Perry, 1999; Geeves et al., 2000). This complicates any attempts to assess the functional role of specific sites on subdomain 1 of actin in TnI binding in partially reconstituted thin filaments. Consequently, this study has been confined to measurements on the fully reconstituted actin–Tm–Tn complexes.

The measurement of TnI binding to actin cannot be done with a fully reconstituted actin–Tm–Tn system because it does not allow for release of the unbound TnI into solution. Therefore, the evaluation of proposed TnI binding to acidic residues in the N-terminal region of actin (residues 1–4 and 24, 25) is based in this work on testing of charge deletions or additions on thin filament regulation. If the proposed electrostatic contacts are indeed important in the blocked state of the thin filament (Levine et al., 1988; Van Eyk and Hodges, 1988; Tripet et al., 1997; Lehman et al., 2001), then charge deletion mutations in subdomain 1 of actin should destabilize this state. This would lower the barrier for the transition to the closed and open states (McKillop and Geeves, 1993) and would thus increase the Ca^{2+} sensitivity of the regulated actin. This prediction provided the experimental framework for testing the role of acidic residues in subdomain 1 of actin in Tm/Tn-based regulation with the help of yeast actin mutants.

Yeast actin is 87% identical (in sequence) with muscle actin (Ng and Abelson, 1980). It activates myosin ATPase, moves over muscle myosin in the in vitro motility assay, and displays the same type of regulation by Tm–Tn as does muscle actin (Gerson et al., 1999; Korman and Tobacman, 1999; Korman et al., 2000; Morris et al., 2001). Although there are some quantitative differences in the rates of polymerization and nucleotide exchange between yeast and muscle actins (Kim et al., 1996; Chen et al., 1995), neither of these properties was pertinent to our studies. Gerson et al. (1999) have previously used yeast actins to study calcium-dependent regulation in the in vitro motility assays. This and the acto-S1 ATPase regulation assays of Korman and Tobacman (1999), Korman et al. (2000), and Yao and Rubenstein (2001) established yeast actin as an attractive system for probing the mechanism of actin regulation.

The choice of regulation assay

Actin regulation by Tm–Tn can be assayed as a function of pCa in acto-S1 ATPase activity and in vitro motility measurements. In the case of yeast actin mutants used in this work (DNEQ and D24A/D25A), which have fewer putative sites for electrostatic interaction with TnI than the wild-type actin ($\Delta = -2$), only the latter option is open. This constraint is due to the low activation of S1 ATPase by the above mutants (Miller and Reisler, 1995; Miller et al., 1996). As documented in these prior studies, the low acto-S1 ATPase activities were due to the reduced weak binding of S1 to DNEQ and D24A/D25A mutant actins. In the in vitro motility assays, methylcellulose (a viscosity-enhancing agent) compensates for the reduced myosin binding (in the presence of ATP) to these actin mutants by inhibiting their diffusion away from the HMM adsorbed to the cover glass. This restores their sliding to that of wild type actin (Miller and Reisler, 1995; Miller et al., 1996; Wong et al., 1999; Doyle and Reisler, 2002), enabling the testing of their regulation by Tm–Tn. The sliding speeds of actin filaments in such assays are not tightly coupled to the V_{max} of actomyosin ATPase. For example, rabbit skeletal α-actin and wild type and 4Ac yeast actin filaments move at similar speeds in the motility assays despite the different

V_{max} values of their corresponding acto-S1 ATPase activities (Cook et al., 1993; Miller et al., 1996; Doyle and Reisler, 2002).

pCa Dependence of regulated actin sliding in the in vitro motility assays

The in vitro motility assays were carried out in the presence of methylcellulose and at an ionic strength of 50 mM, i.e., under conditions at which the mutants and wild-type actin slide at similar speeds over myosin. Figure 2, *A* and *B*, shows that the D24A/D25A and DNEQ actins, each with a net decrease of two acidic residues, had sliding speeds similar to wild type actin at all pCa values. The pCa_{50} values of these mutant and wild-type actin pairs were similar to within ± 0.1 pCa units. The sliding speeds of the 4Ac mutant, that has a net increase of two acidic residues, were also similar to wild-type actin at all pCa values (Fig. 2 *C*), as were the pCa_{50} values (7.0 ± 0.3 and 6.8 ± 0.1, respectively). These results show that the inhibition of filament movement by the Tn–Tm complex in this assay does not appear to be altered by the number of charged residues (± 2) in subdomain 1 of actin.

Our results do not reveal any significant destabilization of the blocked state of the regulated thin filament, or changes in the Ca^{2+}-sensitivity of regulation when using the DNEQ, D24A/D25A, and 4Ac actins. Thus, charge alteration in the putative ionic contacts between TnI and actin subdomain 1 does not affect the overall Tn-regulation of the thin filament. This apparent tolerance in the regulation of actin to charge density changes is inconsistent with the previously proposed binding of TnI to the N-terminal region on actin. Thus, the N-terminal charged residues on actin play only a small role, if any, in the TnI-mediated inhibition of actomyosin. For similar reasons, our results also rule out the possibility that the acidic sites in subdomain 1 could be involved in the binding of TnT or Tm to the blocked-state actin. Clearly, none of the above precludes the possibility that other electrostatic contacts may contribute to the TnI-actin binding. The results of Bing et al. (1998) showed that the Tm-based regulation of E93K actin from *Drosophila melanogaster* was severely affected by a more drastic mutational change, i.e., charge reversal.

Pyrene fluorescence of Cys-1-labeled actin

Pyrene maleimide attached to a cysteine residue introduced at the N-terminus of actin (Cys^1) was used for probing Tn (or Tm) interactions with that region on actin. Previous work has shown that at this position the pyrene probe is sensitive to S1 binding and responds differently to the weakly and strongly bound S1 states (Hansen et al., 2000). The activation of S1 ATPase activity by the pyrenyl Cys-1 actin was similar to that by Cys-1 actin, indicating that the

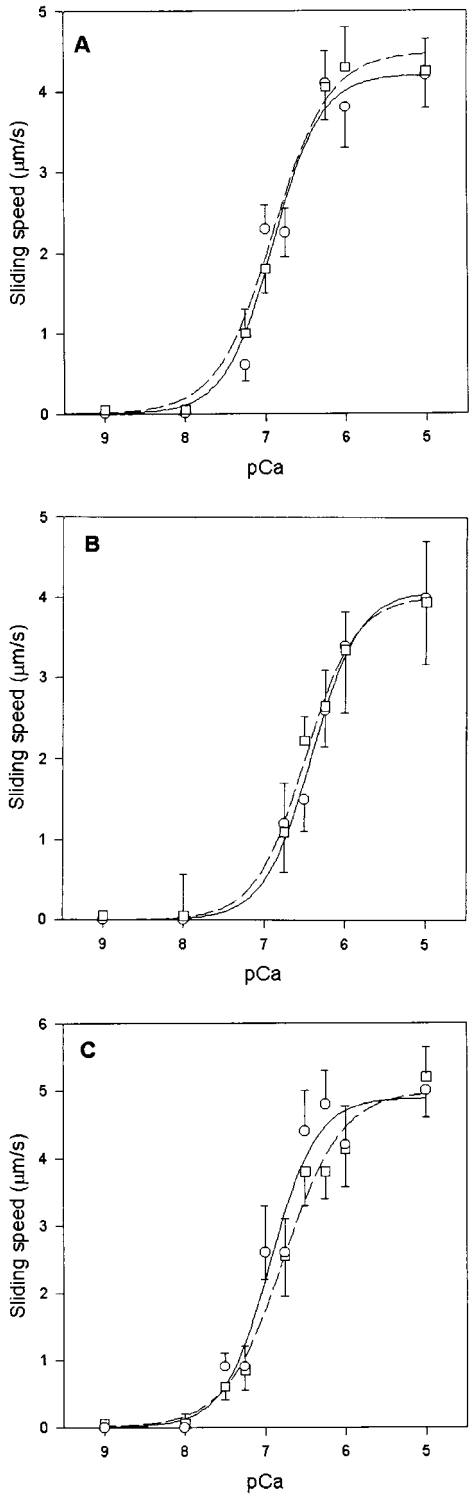

FIGURE 2 Sliding speeds of reconstituted, regulated thin filaments as a function of pCa. (*A*) Wild-type (○) and D24A/D25A yeast actins (□). (*B*) Wild-type (○) and DNEQ yeast actins (□). (*C*) Wild-type (○) and 4Ac (□) yeast actins. The curves were determined by least-squares regression fits to the individual data points, using a modified Hill equation (Eq. 1). In each plot, the solid curve represents wild-type actin, and the dashed curve represents the mutant actin. At least 100 filaments were analyzed for each sample. Error bars represent the standard deviation of the mean values of the smoothly moving filaments.

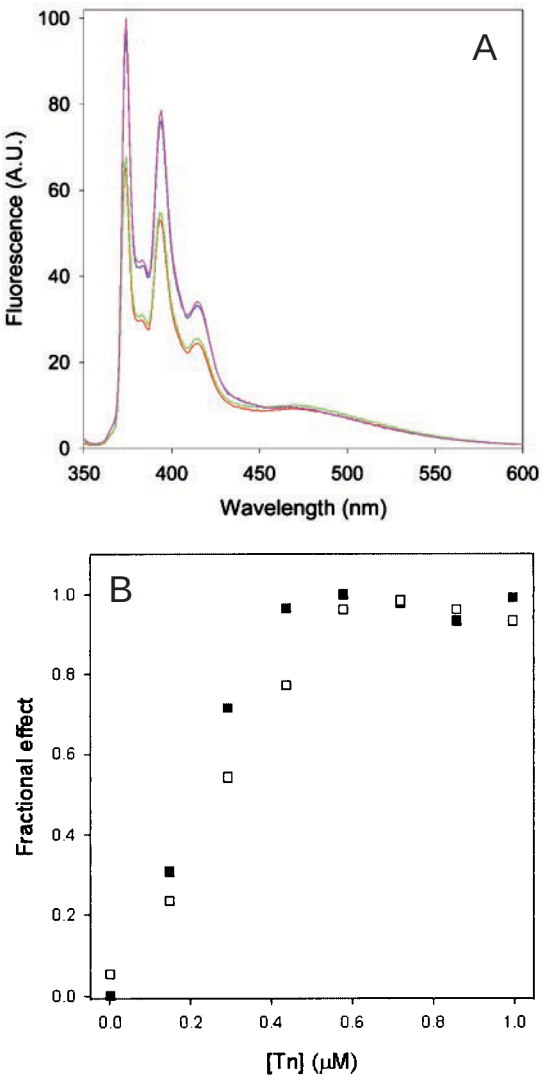

FIGURE 3 Emission spectra of pyrenyl Cys-1 F-actin filaments. (*A*) *Red trace*, 4.0 μM pyrenyl Cys-1 F-actin containing 0.2 mM Ca^{2+}, stabilized by an equimolar amount of phalloidin (λ_{ex} = 344 nm). *Green trace*, the same F-actin after the addition of 2.0 μM Tm. The red and green traces overlap over most of their spectral regions. *Blue trace*, after the addition of 1.0 μM Tn to the pyrenyl Cys-1 actin–Tm filaments. *Pink trace*, the spectrum of the reconstituted thin filaments after the addition of 1.0 mM EGTA. The pink and blue traces overlap over most of their spectral regions. (*B*) Comparison of the effects of Tn on the fluorescence of pyrenyl Cys-1 F-actin–Tm in the presence and absence of Ca^{2+}. The fractional increase in pyrene fluorescence was calculated for each data point after adding Tn (■) or Tn and 1.0 mM EGTA (□) to pyrenyl Cys-l-actin-Tm filaments. Tn was added to the cuvette in increments of ~0.15 μM, to a maximum of 1.0 μM. All the data were normalized against the data measured in the presence of calcium.

modification did not alter actin function (Hansen et al., 2000). We have verified now that pyrenyl Cys-1 actin is fully regulated by Tm–Tn, yielding an approximately tenfold Ca^{2+}-induced activation of acto-S1 ATPase, as measured by the ratio of Mg-ATPase activities in the presence of 0.2 mM Ca^{2+} and 1.0 mM EGTA.

The spectrum of pyrenyl Cys-1 F-actin was unchanged by addition of Tm, but the fluorescence intensity increased by ~35% with the binding of Tn to actin–Tm (Fig. 3 *A*). Virtually identical fluorescence enhancement was observed in the presence and absence of Ca^{2+} (same increase for both, Fig. 3 *A*, *blue* and *pink traces*). Tn binding to actin–Tm, but not the Ca^{2+} activation of regulated filaments, appears to induce conformational changes in the N-terminal region of actin. This suggests that conformational changes at this site are not coupled to the activation of thin filaments. Such a result was unexpected in the context of the perceived biding of TnI to the N-terminus of actin (Perry, 1999) and its observed shift away from actin upon Ca^{2+}-binding to Tn (Tao et al., 1990). To confirm the specificity of Tn binding, the modified actin–Tm was titrated to saturation with Tn both in the presence and absence of calcium (Fig. 3 *B*, ■ and □, respectively). The fluorescence increases plateau in both cases at an ~1:7 molar ratio of Tn to actin, regardless of the presence or absence of Ca^{2+}.

It is tempting to speculate that the change in pyrene fluorescence is related to the improvement of the strong S1–actin binding in the presence of regulatory proteins, which is also insensitive to changes in Ca^{2+} concentration (Korman et al., 2000). This improved recruitment of S1 may be connected to the increase in the isometric force exerted by myosin on regulated compared to unregulated actin (Homsher et al., 2000), and may be the result of allosteric changes induced in F-actin by Tm–Tn, or a direct S1 interaction with Tm. In the former scenario, the Ca^{2+}-insensitive, Tn-induced changes in the N-terminus of actin may be related to the allosteric transitions that improve S1 binding to actin.

In summary, our results show that electrostatic interactions of Tn (and of other components of the regulatory complex) with the acidic residues in the N-terminal region of actin are not essential for the regulation of actomyosin function by Tm–Tn. This negative result is important for ruling out the previously proposed model of TnI interaction with actin and emphasizes the need for mapping the binding sites for regulatory proteins on actin. Finally, the perturbation of the N-terminus of actin by Tm–Tn may be related to the effect these proteins have on the binding of S1 to actin.

We thank Larry S. Tobacman (Univ. of Iowa) for his generous gift of bovine cardiac Tm and Tn.

This work was supported by grants from the United States Public Health Service (AR-22031) and the National Science Foundation (MCB 9904599) to E.R. and from the United States Public Health Service (GM-33689) to P.A.R.

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