Mutations in Actin Subdomain 3 That Impair Thin Filament Regulation by Troponin and Tropomyosin*

(Received for publication, November 20, 1998, and in revised form, April 15, 1999)

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Thin filament-mediated regulation of striated muscle contraction involves conformational switching among a few quaternary structures, with transitions induced by binding of Ca2+ and myosin. We establish and exploit Saccharomyces cerevisiae actin as a model system to investigate this process. Ca2+-sensitive tropomin-tropomyosin binding affinities for wild type yeast actin are seen to closely resemble those for muscle actin, and these hybrid thin filaments produce Ca2+-sensitive regulation of the myosin S-1 MgATPase rate. Yeast actin filament inner domain mutant K315A/E316A depresses Ca2+ activation of the MgATPase rate, producing a 4-fold weakening of the apparent Ca2+ affinity and a 50% decrease in the MgATPase rate at saturating Ca2+ concentration. Observed destabilization of troponin-tropomyosin binding to actin in the presence of Ca2+, a 1.4-fold effect, provides a partial explanation. Despite the decrease in apparent MgATPase Ca2+ affinity, there was no detectable change in the true Ca2+ affinity of the thin filament, measured using fluorophore-labeled tropomyosin. Another inner domain mutant, E311A/R312A, decreased the MgATPase rate but did not change the apparent Ca2+ affinity. These results suggest that charged residues on the surface of the actin inner domain are important in Ca2+- and myosin-induced thin filament activation.

Cardiac and striated muscle contraction are regulated by conformational changes in the thin filament, which comprises F-actin, tropomyosin, and troponin. Troponin has three subunits: troponin C, which serves as the Ca2+ sensor for the system; troponin I, which inhibits myosin cycling; and troponin T, which binds to tropomyosin, troponin I, troponin C, and F-actin (reviewed in Refs. 1–3). Structural and biochemical evidence favor a three-state model for the regulation of muscle contraction (4–11). The inhibited or blocked state occurs in the absence of Ca2+ and allows tropomyosin and troponin to inhibit actin-myosin binding and cycling (4, 6, 10). The Ca2+ state, alternatively viewed as closed or permissive, occurs upon Ca2+ binding to troponin C. It involves tropomyosin movement and possibly other conformational changes, and results in improved myosin binding to the thin filament. The active or open state corresponds to a final shift in the position of the regulatory proteins, which allows myosin to bind strongly to F-actin. This state appears to be required for cross-bridge cycling (4, 5, 10, 12, 13). Currently there is little detail concerning where on the actin filament the regulatory proteins bind for each of the different states. Average tropomyosin positions relative to actin have been proposed based on three-dimensional reconstructions using electron micrographs of thin filaments or x-ray diffraction of oriented filaments (6–8, 14). Only indirect methods such as cross-linking and peptide NMR have been able to suggest where troponin might interact with F-actin (15).

Three model systems have been used to generate actin mutants to examine the interactions of actin with myosin and with the regulatory proteins. Actin from the indirect flight muscle of Drosophila provides useful in vivo information as well as the potential for genetically-derived insights (16–19). Only a limited amount of actin can be purified from this system, but this was used to investigate tropomyosin function in one study (17). The other two model systems that have been used to investigate muscle regulation and actin-myosin interactions are Dictyostelium and Saccharomyces cerevisiae (20–27). Both Dictyostelium and S. cerevisiae can yield mg quantities of actin. Although S. cerevisiae actin has the lowest homology to mammalian muscle actin of these two systems, it is still a relatively high 88% (28, 29). Yeast actin has been used to study myosin and actin interactions (20–25), and one report using in vitro motility assays demonstrated that movement is Ca2+-dependent in the presence of the regulatory proteins (26). In a study of in vivo effects of mutagenesis, Wertman et al. (30) conducted a “clustered charged-to-alanine scanning mutagenesis” in S. cerevisiae, systematically changing groups of two or more charged residues in the actin sequence to alanines. Previous studies suggest that charged residue interactions are important for tropomyosin and troponin binding to actin (8, 15), and increasing ionic strength weakens tropomyosin-troponin binding to F-actin (31). Therefore, this series of mutants provides a method to test the actin surface for sites of interaction with the regulatory proteins.

The present paper employs yeast actin to examine tropomin-tropomyosin function. Despite the sequence differences between yeast actin and mammalian muscle actin, tropomyosin and troponin are shown both to bind to yeast actin and to regulate the actin-myosin subfragment 1 (S-1)1 ATPase rates in a Ca2+-sensitive manner, very similar to previous results for muscle actin. Charged actin side chains between residues 309 and 326 located in subdomain 3 have been hypothesized by Lorenz et al. (8) to interact with tropomyosin. Using the

* This work was supported by National Institutes of Health Grant HL-38834 (to L. T.) and American Heart Association IA-97-SA-3 (to V. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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charged residue to alanine actin mutants, the present work shows this region to be important in tropomyosin-troponin function.

**MATERIALS AND METHODS**

**Protein Purification**—Bovine cardiac tropomyosin, whole troponin as well as the separate subunits troponin C, troponin I, and troponin T were all purified from ether powder using the method of Tobacman and Lee (32). Myosin S-1 was obtained from rabbit fast skeletal muscle using the method of Weeds and Taylor (33). Rabbit fast skeletal muscle tropomyosin-troponin was purified from ether powder using the method as described (34). Recombinant α-tropomyosin, which is unacyethylated, was obtained according to Willadsen, et al. (35). Mutant yeast strains were obtained as a gift from Dr. Drubin’s laboratory and were grown to stationary phase in YPD medium at 30 °C for mutant K315A/E316A or 25 °C for mutant E311A/R312A (30). Wild type actin was obtained from a bakery as cakes of Saccharomyces cerevisiae, since its sequence is identical to the S. cerevisiae actin and it can be obtained in large quantities (20). Actin was purified from the wild type and mutant strains using the method from Cook et al. (20). G-actin was polymerized by adding either 3 mM MgCl₂ or 2 mM MgCl₂ with 50 mM KCl. Polymerization by either solution gave the same results.

**Cosedimentation Actin-binding Assay**—Bovine cardiac tropomyosin or recombinant α-tropomyosin was labeled at Cys-190 with 125Iiodoacetic acid (36). Conditions and protein concentrations are described in figure legends. Samples were incubated for 30 min at 25 °C and then centrifuged at 35,000 rpm using a TLA100 rotor (Beckman) for 30 min at 25 °C. The radioactivity of the supernatant was compared with the total radioactivity of the reaction mixture before centrifugation. The McGee-von Hippel equation (34, 37), which describes the binding of an extended ligand to a linear lattice, was used to fit the data, Kₐ represents the affinity of one ligand for an isolated binding site, and Y is a measure of the cooperativity that arises when two ligands bind to adjacent sites. Kₐ is given by the product KᵥY, with 50% saturation of actin occurring when the tropomyosin or tropomyosin-troponin concentration equals 1/Kᵥ.

**MgATPase Activity**—Myosin S-1 ATPase activity was measured using the method described by Pollard and Korn (38). The conditions used were: 25 °C, 7 mM F-actin (either wild type or mutant), 1 mM rabbit fast skeletal muscle myosin S-1, 1.2 mM bovine cardiac tropomyosin, 1.2 mM bovine cardiac troponin, 5 mM imidazole (pH 7.5), 3.5 mM MgCl₂, 1 mM DTT, and 0.5 mM of either 5,5'-dibromo-BAPTA or 5'-nitro-BAPTA (Molecular Probes). As the concentration of CaCl₂ was varied to alter the Ca²⁺ concentration, a linear plot was used to fit the data. Kᵥ represents the affinity of one ligand for an isolated binding site, and Y is a measure of the cooperativity that arises when two ligands bind to adjacent sites. Kᵥ is given by the product KᵥY, with 50% saturation of actin occurring when the tropomyosin or tropomyosin-troponin concentration equals 1/Kᵥ.

**RESULTS**

**Yeast Actin Mutants** K315A/E316A and E311A/R312A Alter MgATPase Rate—The Lorenz model suggests that several residues in the third and fourth subdomain of actin, including residues from 309 to 336, are positioned so they may interact with tropomyosin in the absence of troponin (8). Two of the yeast actin mutants, E311A/R312A and K315A/E316A, which are altered at residues 311 and 312, respectively, were used to examine the effect of tropomyosin-troponin interaction on myosin ATPase activity.

In the presence of tropomyosin-troponin, the MgATPase activity of wild type yeast actin was decreased by 30% (Fig. 1). This decrease in MgATPase activity was not observed in the presence of either nonmuscle tropomyosin or tropomysin-troponin from Drosophila melanogaster (39). The MgATPase activity of the yeast actin mutants, E311A/R312A and K315A/E316A, was also decreased by 30% in the presence of tropomyosin-troponin, indicating that the decrease in MgATPase activity was not specific to the wild type yeast actin.

**Ca²⁺ Titrations** of the Thin Filament with Rabbit Skeletal Tropomyosin-Troponin—Bovine cardiac tropomyosin-troponin was labeled with 4-(N-iodoacetoyethyl-n-methyl)-7-nitrobenzo-2-oxa-1,3-diazole (IANBD, Molecular Probes) following the procedure by Trybus and Taylor (40). Steady state fluorescence Ca²⁺ titrations were performed under the following conditions: 25 °C, 7.5 mM F-actin (either wild type or mutant), 0.47 mM rabbit skeletal tropomyosin-troponin IANBD, 20 mM imidazole (pH 7.06), 3.5 mM MgCl₂, 6.5 mM KCl, 1 mM DTT, and 0.5 mM EGTA. The fluorescence samples were excited at 436 nm, and the emission intensity was monitored at 535 nm. The titrations were performed by sequential additions of CaCl₂ to 1.6-mL samples. The free Ca²⁺ concentration was calculated as described elsewhere (39), taking into account the CaCl₂ already contained in the actin buffer. Fluorescence data were corrected for dilution and fit to Equation 12 from Ref. 39.

**Ca²⁺ Titration of the Thin Filament with Bovine Cardiac Tropomyosin Labeled with IAANS**—Bovine cardiac tropomyosin C was labeled with 2-(4'-iodoacetamidoanilino)naphthalene-6-sulfonic acid, sodium salt (IAANS, Molecular Probes) and reconstituted with troponin I and troponin T following the procedure by Tobacman and Sawyer (39). Steady state fluorescence Ca²⁺ titrations were performed under the following conditions: 25 °C, 7 mM F-actin (either wild type or mutant), 2 mM bovine cardiac troponin, 0.6 mM bovine cardiac troponin labeled with IAANS, 5 mM imidazole (pH 7.5), 3.5 mM MgCl₂, 1 mM DTT, 7.5 mM KCl, and 0.5 mM 5,5'-dibromo-BAPTA. Fluorescence measurements employed a SLM 8000 spectrophotometer outfitted with a stirred water jacket sample holder. The samples were excited at 392 nm, and the emission intensity was monitored at 503 nm. The titrations were fit to a binding equation with a single dissociation constant, Kᵥ, with 50% saturation of actin occurring when the calcium concentration was calculated using the dissociation constant, 6.5 M, was used to determine the free Ca²⁺ concentration, which facilitates exploration of a broader range of Ca²⁺ concentrations. The dissociation constant for 5'-nitro-BAPTA was determined by spectrophotometric titration at 25 °C using the above buffer conditions by monitoring the increase in absorbance at 340 nm upon the addition of Ca²⁺. The data from each titration were fit to a binding equation with a single dissociation constant. The titration was repeated four times, and the average dissociation constant, 6.5 ± 0.4 μM, was used to determine the free Ca²⁺ in the ATPase assays, under these buffer conditions. Using either of the [1,2-bis(o-aminophenoxyl)ethane-N,N,N',N'-tetraacetic acid]-derived chelators gave similar results. The free Ca²⁺ concentration was also corrected for the CaCl₂ already included in the actin buffer. The MgATPase data were fit to Equation 12 derived in Tobacman and Sawyer (39), which describes cooperative Ca²⁺ binding to a linear array of sites.

**MgATPase Rates**—The Lorenz model suggests that several residues in the third and fourth subdomain of actin, including residues from 309 to 336, are positioned so they may interact with tropomyosin in the absence of troponin (8). Two of the yeast actin mutants, E311A/R312A and K315A/E316A, which are altered at residues 311 and 312, respectively, were used to examine the effect of tropomyosin-troponin interaction on myosin ATPase activity.

In the presence of tropomyosin-troponin, the MgATPase activity of wild type yeast actin was decreased by 30% (Fig. 1). This decrease in MgATPase activity was not observed in the presence of either nonmuscle tropomyosin or tropomysin-troponin from Drosophila melanogaster (39). The MgATPase activity of the yeast actin mutants, E311A/R312A and K315A/E316A, was also decreased by 30% in the presence of tropomyosin-troponin, indicating that the MgATPase activity was not specific to the wild type yeast actin. The MgATPase activity of the yeast actin mutants, E311A/R312A and K315A/E316A, was also decreased by 30% in the presence of tropomyosin-troponin, indicating that the MgATPase activity was not specific to the wild type yeast actin.
absence of Ca\(^{2+}\) the actin-activated rate was only 7% of the rate seen at saturating Ca\(^{2+}\). In the presence of saturating Ca\(^{2+}\), regulated thin filaments containing either E311A/R312A actin or K315A/E316A actin produced only 50% of the thin filament-myosin S-1 MgATPase rate observed with wild type actin. The apparent Ca\(^{2+}\) affinity was 2.6 \(\times\) 10\(^5\) M\(^{-1}\) for the wild type filament, very similar to results seen with muscle actin (2.4 \(\times\) 10\(^5\) M\(^{-1}\); Ref. 9). Also, this apparent Ca\(^{2+}\) affinity was not significantly different from results seen for E311A/R312A actin (3.1 \(\times\) 10\(^5\) M\(^{-1}\)). However, it was 4-fold higher than the apparent Ca\(^{2+}\) affinity seen for K315A/E316A actin: 6.5 \(\times\) 10\(^4\) M\(^{-1}\). Given these observations, we chose to focus our studies on mutant actin K315A/E316A.

Fig. 1 shows that the thin filament-myosin S-1 MgATPase rate in the presence of saturating Ca\(^{2+}\) concentrations was significantly reduced when K315A/E316A actin was utilized. Importantly, other experiments differing from these by the absence of troponin-tropomyosin showed that the myosin S-1 ATPase rates using wild type F-actin and K315A/E316A F-actin were equivalent, 0.23 \(\pm\) 0.02 s\(^{-1}\) for wild type and 0.24 \(\pm\) 0.03 s\(^{-1}\) for the mutant (average \(\pm\) standard error, n = 4). Therefore, the decreased MgATPase rate occurs specifically when the regulatory proteins are present.

Ca\(^{2+}\) has a cooperatively activating effect on the thin filament-myosin S-1 MgATPase rate (39, 41), and this is also true when yeast actin is used (Y > 1, Fig. 1; Y \(\sim\) n\(_1\)n\(_2\); Ref. 39). For unclear reasons, the cooperativity was too variable to reliably evaluate any effect of the actin mutations on Ca\(^{2+}\)-mediated cooperativity. However, the higher thin filament-myosin S-1 MgATPase rate for wild type actin (in the presence of saturating Ca\(^{2+}\)) was not due to a greater cooperative activation by myosin. Rather, myosin S-1 was not acting cooperatively under these conditions. This conclusion is based upon control data (data not shown) indicating that the MgATPase rate was linear with myosin S-1 concentrations up to 6 \(\mu\)M, much higher than the 1 \(\mu\)M used in Fig. 1. This parallels results with muscle actin under similar conditions (9).

Effects of K315A/E316A Actin Mutation on the Affinity of Ca\(^{2+}\) for the TnC Regulatory Sites—We further evaluated the lower apparent Ca\(^{2+}\) affinity by employing IAANS-labeled cardiac troponin to follow the binding of Ca\(^{2+}\) to the regulatory site of troponin C within the thin filament (39, 42). Fluorescence titrations indicated that the actin mutation had no effect on troponin Ca\(^{2+}\) affinity (6.5 \(\pm\) 0.4 \(\times\) 10\(^5\) M\(^{-1}\) for wild type versus 6.8 \(\pm\) 0.2 \(\times\) 10\(^5\) M\(^{-1}\) for K315A/E316A actin) (Fig. 2A). However, this result was not definitive, because the IAANS labeling causes a severalfold increase in thin filament regulatory site affinity (39), and this may have obscured the effect of the actin mutation. Therefore, rabbit skeletal tropomyosin-troponin was labeled with IANBD, since the ATPase regulation was unaffected by labeling (40, 43). The fluorescence titration again, showed that the apparent Ca\(^{2+}\) affinity was similar for regulated thin filaments comprising either wild type or K315A/E316A F-actin, \(K_{\text{app}}\) = 1.6 \(\pm\) 0.1 \(\times\) 10\(^6\) M\(^{-1}\) and \(K_{\text{app}}\) = 1.5 \(\pm\) 0.1 \(\times\) 10\(^6\) M\(^{-1}\), respectively (see Fig. 2B). The wild type actin result is similar to data reported previously for muscle actin (40). In a control MgATPase rate versus pCa experiment using skeletal muscle tropomyosin-troponin IANBD, the actin mutation weakened the Ca\(^{2+}\) \(K_{\text{app}}\); \(K_{\text{app}}\) = 1.8 \(\pm\) 1.0 \(\times\) 10\(^5\) M\(^{-1}\) for wild type actin and \(K_{\text{app}}\) = 2.1 \(\pm\) 1.3 \(\times\) 10\(^5\) M\(^{-1}\) when K315A/E316A actin was present (data not shown). The errors are large because the ATPase rates, which are always lower with yeast actin (Fig. 1 and Ref. 21), were too low for accurate measurement. In contrast to results with cardiac regulatory proteins (44), skeletal muscle tropomyosin-troponin-Ca\(^{2+}\) decreases the ATPase rate as much as 80% compared with actin alone (45).

Effect of the Actin Mutation on Thin Filament Assembly—Thin filament assembly analysis was used to measure any changes in the interactions with the regulatory proteins that would be detectable by a change in their affinities for the mutant actin when compared with wild type actin. Fig. 3 shows the binding of the tropomyosin-troponin complex to either type of yeast actin in either the presence or absence of Ca\(^{2+}\). The figure shows that, at pCa 8, the tropomyosin-troponin complex binds equally tightly to both wild type (\(\Delta\)) and K315A/E316A (+) F-actin (8.3 \(\pm\) 0.3 versus 7.8 \(\pm\) 0.7 \(\times\) 10\(^6\) M\(^{-1}\)). The addition of Ca\(^{2+}\) causes an approximately 2-fold decrease in affinity of the regulatory complex for F-actin, a similar decrease to that seen for muscle actin (11, 34). Upon the addition of Ca\(^{2+}\), the affinity of the complex for wild type F-actin is \(K_{\text{app}}\) = 4.0 \(\pm\) 0.1 \(\times\) 10\(^6\) M\(^{-1}\), which is slightly greater than the affinity seen for the mutant, \(K_{\text{app}}\) = 2.8 \(\pm\) 0.1 \(\times\) 10\(^6\) M\(^{-1}\). In other words, the charged residues at 315 and 316 (normally present), increase...
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FIG. 3. The binding of the bovine cardiac tropomyosin-tropomyosin complex to either wild type or K315A/E316A yeast actin. Varying concentrations of bovine cardiac tropomyosin-tropomyosin complex were incubated with 7 μM F-actin, 10 mM Tris (pH 7.5), 3 mM MgCl₂, 300 mM KCl, 2 mM DTT, 0.3 mg/ml BSA, and 0.5 mM EGTA. For experiments with Ca²⁺, 0.6 mM CaCl₂ was added to the reaction mix. The figure is a composite of three or more data sets for each yeast actin under both conditions. The solid lines are the best fit theoretical curves. In the absence of Ca²⁺, the wild type yeast actin (∆) data indicate Kₐpp = 8.3 ± 0.3 × 10⁵ M⁻¹ and γ = 67 ± 22 and the mutant actin (+) data indicate Kₐpp = 7.8 ± 0.7 × 10⁵ M⁻¹ and γ = 39 ± 25. In the presence of Ca²⁺, wild type yeast actin (∆) data imply Kₐpp = 4.0 ± 0.1 × 10⁶ M⁻¹ and γ = 82 ± 18 and the mutant actin (+) data imply Kₐpp = 2.8 ± 0.06 × 10⁶ M⁻¹ and γ = 78 ± 13. The tendency of tropomyosin to bind end-to-end on actin is proportional to γ (37). Note this is not the same as the term γ in Figs. 1 and 2, which is approximately the square of the Hill coefficient (39).

tropomyosin-tropomyosin binding affinity for actin very slightly, by only 1.4-fold. This is smaller than the 4-fold increase in the apparent Ca²⁺ affinity seen in the ATPase data (Fig. 1). The change in protein-protein binding is insufficient to explain the change in Ca²⁺ affinity.

The wild type data in Fig. 3 data also suggest that yeast actin retains the tropominin-troponin-interaction sites present in muscle, despite the absence of troponin in S. cerevisiae. Cardiac tropomyosin-troponin affinity for yeast actin is comparable to values determined by the same methods using muscle actin (in contemporaneous data, Kₐpp = 6.9 × 10⁵ M⁻¹ in the absence of Ca²⁺, and 2.7 × 10⁶ M⁻¹ in the presence of Ca²⁺ (Ref. 46); in older data, Kₐpp = 3.5 × 10⁵ M⁻¹ (Ref. 47)).

The Effects of Ionic Strength on the Relative Binding Affinity of Regulatory Complexes to Both Types of Yeast Actin—The ATPase assays are conducted at low ionic strength to produce a measurable ATPase rate, where the protein-protein binding studies must be done at high ionic strength to prevent unbound tropomyosin-troponin from polymerizing in a way that would preclude binding measurements (36). It is possible that this high ionic strength decreases the effect of charged actin residues 315 and 316 on tropomyosin-troponin binding to actin, and therefore diminishes the effect of the mutation. To explore the effect of ionic strength on the relative binding affinities of tropomyosin-troponin to wild type versus mutant yeast actin, we varied the KCl concentration (Fig. 4). Unacetylated recombinant α-striated muscle tropomyosin was used instead of cardiac tropomyosin, since it is unable to polymerize and therefore a lower range of ionic strength can be explored (35). (Polymerization causes non-recombinant muscle tropomyosin-troponin to sediment in the absence of actin, complicating binding measurements unless high ionic strength is used to prevent polymerization (34).) In a control experiment (data not shown), substituting unacetylated recombinant α-tropomyosin for cardiac tropomyosin in ATPase assays did not alter the effect of the actin mutation. In general, Fig. 4 shows that troponin-unacetylated tropomyosin bound slightly less tightly to the mutant F-actin than to the wild type actin (solid bars versus open bars). The data also show that the effect of the actin mutation did not increase at lower ionic strength. This is contrary to what one would expect if high ionic strength decreases the effect of the mutation on the binding of the regulatory complex to actin.

The actin affinity of tropomyosin alone is slightly weaker for the K315A/E316A F-actin. A related issue is the effect of the actin mutation on tropomyosin binding to yeast actin filaments in the absence of troponin. This was examined in the presence of 100 mM KCl, because actin-tropomyosin binding is too weak in the absence of troponin to be measured under conditions used in the presence of troponin (Fig. 3, 300 mM KCl). The results (Fig. 5) indicate that the binding of tropomyosin to actin occurs with a Kₐpp of 2.3 ± 0.6 × 10⁶ M⁻¹ for the wild type actin (∆) and a slightly weaker Kₐpp of 1.5 ± 0.1 × 10⁶ M⁻¹ for the K315A/E316A actin filament (+). Although these values indicate only a small effect of the mutation, the ratio of the two affinities (wild type:mutant) equals 1.5, which is the same magnitude found in the presence of troponin and Ca²⁺ (Fig. 3). Notably, three-dimensional reconstructions of muscle thin filaments show the position of tropomyosin on the actin filament to be the same under these two conditions (7, 8, 14).

As in the case of muscle actin (36), the presence of myosin S-1 causes tropomyosin to bind very tightly to yeast actin filaments. For both types of yeast actin, the binding of tropomyosin is nearly stoichiometric (+, ∆, Fig. 5). Because binding is very strong, we were unable to measure the effects of the mutation on tropomyosin-actin affinity under these conditions.

DISCUSSION

An emerging but incomplete (48) consensus from three-dimensional reconstructions and biochemical studies is that regulation of striated and cardiac muscle contraction can be described by a three-state model for the thin filament (4–11). Actin mutants provide one method to probe the actin surface for residues that interact with the regulatory proteins and
the regulatory complex without \( \text{Ca}^{2+} \)

Finally, regulated thin filaments have the same \( \text{Ca}^{2+} \) affinity for regulated thin filaments containing K315A/E316A that was 25% of the apparent \( \text{Ca}^{2+} \) affinity of wild type thin filaments. However, in thin filament assembly studies, there was only a slight decrease; the affinity of the tropomyosin-troponin complex at \( p \gamma \) for the mutant actin was 70% of the affinity for the wild type actin. Initially, a plausible reason for this discrepancy was the difference in the ionic strength between the two assays. The high ionic strength needed for the binding assay might diminish the effects of the two charged actin residues. This hypothesis was tested by examining how ionic conditions affected the binding of troponin-uncetylated tropomyosin to actin, which can be examined under a broader range of conditions than when (acetylated) muscle tropomyosin is used. The results indicate that lower ionic strength does not increase the discrepancy in the binding affinities for the wild type actin filament versus the mutant actin filament.

Fig. 6 shows the linkage relationship between tropomyosin-troponin and \( \text{Ca}^{2+} \) binding to F-actin. If \( K_a \) corresponded to the \( \text{Ca}^{2+} \) titration of the actin-activated myosin S-1 ATPase rates, altered 4-fold by the mutation, then there would be a compensatory change in the other association constants, \( K_3 \) and/or \( K_5 \). (Actin mutations obviously cannot alter \( K_3 \)). However, Fig. 3 shows that \( K_1 \) is unchanged and \( K_4 \) is only slightly changed. Therefore, the linkage relationships suggest that the actin mutation has only a slight effect on \( \text{Ca}^{2+} \) binding to the thin filament (this is experimentally confirmed in Fig. 2), and the larger effect on the \( \text{Ca}^{2+} \) \( K_{\text{app}} \) of the ATPase titration implies an effect of the mutation on an additional process. An unproven but plausible possibility for such a process is suggested by previous studies in which a nonlinear relationship was found between myosin S-1 MgATPase activity and troponin saturation with \( \text{Ca}^{2+} \) (9). This nonlinear relationship indicated that (when using muscle actin) \( \text{Ca}^{2+} \) binding to two adjacent troponins was necessary to activate the myosin S-1 MgATPase rate. This non-linear behavior may be exaggerated in the presence of the actin mutation.

It is significant that the decreased myosin S-1 ATPase rates observed with K315A/E316A actin occurred only when the regulatory proteins were present. We suggest that these charged residues in actin subdomain 3 interact weakly with the regulatory proteins in the presence of \( \text{Ca}^{2+} \) (Fig. 3). Further, we suggest that they more strongly influence the back and forth transitions between the \( \text{Ca}^{2+} \) state and the myosin-induced state of the thin filament, and that access to the myosin-induced state is required for cross-bridge cycling. Similarly, the neutralization of the charge on actin residues 311 and 312 might also alter the ability of the regulatory complex to control actin-myosin interactions. A more specific possibility is that residues 311 and 312 normally impede the transition from the myosin-induced state to the \( \text{Ca}^{2+} \) state of the thin filament. Mutation to Ala could then promote reversal of strong cross-bridge binding. This would explain not only the current MgATPase data (reversal before product release), but also a preliminary report using \textit{in vitro} motility to examine the yeast actin mutant E311A/R312A (49). These studies showed that motility rates were similar for E311A/R312A F-actin and wild type F-actin alone, but in the presence of the regulatory proteins the sliding speed was increased for the E311A/R312A thin filaments when compared with the wild type, implying faster cross-bridge detachment.

The present studies are not intended to suggest that troponin and tropomyosin interact exclusively with subdomain 3 of actin. Thin filament reconstructions suggest that tropomyosin may contact the other subdomains, and the interactions of troponin with actin remain obscure. Furthermore, two previous actin mutagenesis studies are relevant. Saeki et al. (27) used
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Dictyostelium actin mutants to propose that subdomain 4 interacts with tropomyosin in the “on” state of the thin filament. Bing et al. (17) used the limited amount of actin that could be purified from Drosophila to observe that actin subdomain 2 mutant E93K has abnormal in vitro motility in the presence of tropomyosin (17). The combined results of our data and these earlier studies suggest that several areas of actin participate in interactions with the regulatory proteins.

In summary, yeast actin interacts with the regulatory proteins in a manner similar to muscle actin and thus can serve as a model system for muscle regulation. Neutralizing the charge on the actin residues 315 and 316 or 311 and 312 by mutation to alanine inhibits myosin activity when the regulatory proteins are present. For the former mutant, there is a 4-fold decrease in the apparent Ca$^{2+}$ affinity as well as a 50% lower MgATPase activity in the presence of saturating Ca$^{2+}$. Also, the K315A/E316A mutation slightly weakens tropomyosin binding to actin, specifically under conditions when it is normally found on the inner domain of F-actin (tropomyosin alone; tropomyosin-troponin-Ca$^{2+}$). These results suggest that charged residues on the surface of the actin inner domain are important for thin filament activation.

Acknowledgments—We thank Dr. David Drubin for the gift of both the K315A/E316A and E311A/R312A yeast actins. We also thank Dr. Emil Reisler for discussions and for critiquing an earlier version of this manuscript.

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