Role of Residues 311/312 in Actin-Tropomyosin Interaction

IN VITRO MOTILITy STUDY USING YEAST ACTIN MUTANT E311A/R312A

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According to the Lorenz et al. (Lorenz, M., Poole, K. J., Popp, D., Rosenbaum, G., and Holmes, K. C. (1995) J. Mol. Biol. 248, 108–119) atomic model of the actin-tropomyosin complex, actin residue Asp-311 (Glu-311 in yeast) is predicted to have a high binding energy contribution to actin-tropomyosin binding. Using the yeast actin mutant E311A/R312A in the in vitro motility assays, we have investigated the role of these residues in such interactions. Wild type (wt) yeast actin, like skeletal α-actin, is fully regulated when complexed with tropomyosin (Tm) and troponin (Tn). Structure-function comparisons of the wt and E311A/R312A actins show no significant differences between them, and the unregulated F-actins slide at similar speeds in the in vitro motility assay. However, in the presence of Tm and Tn, the mutation increases both the sliding speed and the number of moving filaments at high pCa values, shifting the speed-pCa curve nearly 0.5 pCa units to the left. Tm alone (no Tn) inhibits the motilities of both actins at low heavy meromyosin densities but potentiates only the motility of the mutant actin at high heavy meromyosin densities. Actin-Tm binding measurements indicate no significant difference between wt and E311A/R312A actin in Tm binding. These results implicate allosteric effects in the regulation of actomyosin function by tropomyosin.

The contraction of vertebrate striated muscle is regulated by the thin filament-associated proteins tropomyosin (Tm) and troponin (Tn), which modulate the interaction of actin and myosin in a Ca2+-dependent fashion (2). Each Tm molecule associates with one Tn molecule and seven actin monomers. The amino acid sequence of Tm contains a pattern of charged and uncharged amino acids that repeats 14 times along its length (3). As each pair of repeats corresponds to an actin monomer along the actin filament, it has been inferred that the binding of Tm to actin is dominated by electrostatic interactions (1).

Tn is composed of three subunits: TnT, TnI, and TnC. The TnC subunit binds Ca2+ and confers calcium sensitivity to the actin-Tm-Tn system. According to the three-state model of thin filament regulation (4), the actin-Tm-Tn complex can assume three structural states: blocked, closed, and open. In the blocked state there is a very low incidence of myosin binding. When Ca2+ binds to TnC, the Tm-Tn complex shifts to the closed state, uncovering additional myosin weak binding sites on the actin filament. The increase in weak binding and the initial strong binding of myosin induce Tm-Tn to shift from the closed (where it prevents myosin strong binding) to the open state. According to this model, the azimuthal shift of Tm-Tn around the axis of the actin filament sterically regulates myosin binding to actin.

Despite the elegance of the three-state model, it cannot explain the results of earlier acto-S1 ATPase solution studies (5–7). Results from these studies describe the ability of Tm alone to both inhibit, at low S1 concentrations, actin-activated S1 ATPase rates and to potentiate the reaction at intermediate, nonsaturating concentrations of S1. Although inhibition can readily be explained using the steric block model, the potentiation suggests the presence of an allosteric component in actomyosin regulation.

The Ca2+-induced Tm-Tn movement on actin was first indicated by x-ray diffraction studies (8–10). Electron microscopy has also been used to directly visualize this shift in Tm-Tn position (11–16), and the studies of Limulus muscle (17, 18) and vertebrate muscle (19) identified the positions for the Tm-Tn complex on actin in the presence and absence of Ca2+. A high resolution model of the Tm-F-actin complex was proposed by Lorenz et al. (1) on the basis of their x-ray fiber diffraction investigation. According to these authors, Tm alone and Tm-Tn in the presence of Ca2+ reside in the same closed-state orientation on the actin filament. In this study Lorenz et al. (1) predict that 16 actin residues participate in the electrostatic interactions between F-actin and Tm and calculate their ΔG contributions to this interaction. Our choice of residue 311 as a suitable starting point to test the predictions of this model and to gain additional insight into Tm regulatory function was based on two criteria. First, according to Lorenz et al. (1) residue 311 has a relatively high energy contribution to actin-Tm interaction, and secondly, there is a viable yeast actin mutant at this location: E311A/R312A (20) (Fig. 1).

In this study, we compared the regulation and function of wt and E311A/R312A yeast actins, as well as their interaction with Tm, in the in vitro motility assays, equilibrium binding experiments, and acto-S1 ATPase measurements. The results of our work support an allosteric explanation of the role of Tm in the regulation of actomyosin interaction.

MATERIALS AND METHODS

Reagents—ATP, ADP, dextrose, DTT, phalloidin, phenylmethylsulfonyl fluoride, and β-mercaptoethanol were purchased from Sigma. Yeast extract and tryptone were purchased from Difco. DNAse I was purchased from Roche Molecular Biochemicals.

Proteins—Skeletal myosin and actin were prepared from rabbit back muscle according to Godfrey and Harrington (21) and Spudich and Watt (22), respectively. S1 and HMM were prepared from myosin using the

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protocol of Weeds and Pope (23) and Kron et al. (24), respectively. Yeast actins were purified over a DNase I affinity column (25) and were stored on ice in a G-actin buffer (5 mM TES, 0.2 mM CaCl₂, 0.2 mM ATP, 0.5 mM β-mercaptoethanol, pH 7.6). Skeletal tropomyosin was prepared according to a previously reported protocol (26), as was cardiac troponin (27). N-(1-pyrenyl)iodoacetamide-labeled Tm was prepared using the protocol of Iahil and Lehrer (28) with a pyrene-to-Tm labeling ratio of 2.0. The cardiac troponin and tropomyosin were generous gifts from Dr. L. Tobacman. Yeast actin strains DBY6962 and DBY6958 (20), producing the col of Ishii and Lehrer (28) with a pyrene-to-Tm labeling ratio of 2.0. The actins were purified over a DNase I affinity column (25) and were stored in a 5 mM imidazole (pH 7.4) buffer containing 10 mM NaCl at 22 °C. The samples were pelleted as above. The concentrations of N-troponiniodoacetamide-labeled Tm left in the supernatants were determined by fluorescence measurements using a Spex Fluorolog with excitation and emission wavelengths set at 344 and 385 nm, respectively.

In Vitro Motility Assays—The in vitro motility assays were performed according to a previously described protocol (30). The HMM titrations, as a variation of the above protocol in that the HMM was applied to the nitrocellulose-covered coverslips at concentrations ranging from 0.06 and 0.3 mg/ml. Regulated thin filaments were assembled using the yeast actin mutant K315A/E316A mutant actins, respectively, were generous gifts from Drs. D. Botstein and T. C. Doyle.

Actin-activated ATPase—The malachite green assay (29) was used to measure the ATPase activity of actin-activated S1. The procedure was the same as that used by Miller et al. (30). The assays were carried out at 22 °C in a buffer containing 5 mM KCl, 2 mM MgCl₂, and 10 mM imidazole, pH 7.4. The S1 concentration was 0.4 μM, whereas that of the mutant or wt actin ranged between 0 and 35 μM.

Regulated Actin-activated ATPase—Hydrolysis rates of regulated actin-activated S1 MgATPase at pCa values ranging between 5.0 and 9.0 were obtained by using light scattering to monitor the clearing time of regulated F-acto-S1 solutions. Clearing time is defined as the duration of the decrease in light scattering of acto-S1 solutions after the addition of ATP. The light scattering of the solution increases sharply upon the hydrolysis of the added ATP, leading to the determination of clearing time, i.e. the time of ATP hydrolysis. Thin filaments were reconstituted using either wt or E311A/R312A actin, bovine cardiac troponin, and either bovine cardiac or skeletal tropomyosin. The assay buffer was adjusted to 30 mM total ionic strength (including calcium concentrations) using a program written in QuickBasic by Drs. E. Homsher and N. Millar based on the equation of Fabiato and Fabiato (31). This buffer contained 5 mM imidazole (pH 7.5), 13.2 mM KCl, 3 mM MgCl₂ (free), 2 mM EGTA (with varying ratios of Ca²⁺/K⁺), 1 mM EDTA, 4 mM MgCl₂, 10 mM DTT, 0.1 μM of both Tm and Tn (or Tm alone, depending on the nature of the assay), and 10 mM imidazole at pH 7.4. Regulatory proteins were included in the assay buffer to prevent the dissociation of these proteins from regulated actin (32). Movement was initiated by applying the same assay buffer containing 1.0 mM ATP and an oxygen-scavenging system (33). An ExpertVision System (Motion Analysis, Santa Rosa, CA) was used to quantify the sliding speeds of individual filaments. Individual filaments were judged to be moving smoothly and were used for statistical analysis if the standard deviation of their sliding speeds was less than one-third of their average velocity (34).

RESULTS

Ca²⁺ Titration of the Regulated System in the in Vitro Motility Assays—According to Lorenz et al. (1), residue 311 has a considerable electrostatic role in the interaction between actin and Tm in the closed state (~850 cal/mol). If this is the case, replacing the charged glu-311 with an uncharged alanine could partially destabilize the closed state, causing changes in the regulation of the E311A/R312A actin mutant.

To test this possibility we measured the regulated thin filament sliding speed at various pCa values for both the regulated mutant and wt actins. Our results show a definite shift to the left of the pCa curve for the 311/312 mutant, indicating a lowered dependence on calcium to turn on the system (Fig. 2A). We also found a similar trend in the numbers of moving filaments. At pCa 5.0 both actins have approximately the same fraction of motile actin filaments. At lower calcium concentrations, the regulated mutant actin has a larger percentage of moving filaments than does wt and ceases to move at a calcium concentration approximately half a pCa unit higher than that of wt actin (Fig. 2B). Experiments similar to those shown in Fig. 2 were repeated on four separate preparations of actins. In each case the pCa shift of the mutant versus wt complex was clearly defined. Although the midpoints of pCa titrations varied somewhat among the preparations, the shifts between mutant and wt actins were consistently reproduced (0.5 ± 0.1 pCa units). Importantly, we saw no significant difference in sliding speed or percentage of filaments moving between the two actins in the absence of Tm and Tn (Table I).

As an additional control, we also performed motility experiments using the yeast actin mutant K315A/E316A. According
The speeds and numbers of smoothly moving actin-Tm-Tn filaments were measured at 25 °C as they moved over a lawn of HMM adsorbed onto a nitrocellulose-coated surface. The pCa of the motility assay buffer (0.1 μM Tm, 0.1 μM Tn, 1 mM ATP, 10 mM imidazole, pH 7.4, 25 mM KCl, 1 mM EGTA, 4.0 mM MgCl2, and 10 mM DTT) was varied from 7.5 to 5.0. Panel A, the mean speeds of wt (●) and 311/312 mutant (○) actins are plotted versus pCa. Error bars represent standard errors for the mean speeds at each pCa value. The speeds of between 104 and 313 filaments were measured at each pCa in these experiments. Panel B, percentages of wt (black) and 311/312 mutant (white) filaments moving at each of the Ca2+ concentrations used.

Table I
Structure-function comparison of wt and 311/312 mutant actins
Experimental details are given under “Materials and Methods.”

| Assay                  | wt | 311/312 mutant |
|-----------------------|----|---------------|
| Acto-S1 binding       |    |               |
| (strong)              |    |               |
| $K_a$                 | (1.8 ± 0.3) $\times 10^6$ M$^{-1}$ | (1.5 ± 0.2) $\times 10^6$ M$^{-1}$ |
| S1 - ADP binding to   |    |               |
| actin-Tm               |    |               |
| $K_a$                 | (5.5 ± 0.6) $\times 10^6$ M$^{-1}$ | (5.2 ± 0.8) $\times 10^6$ M$^{-1}$ |
| Hill coefficient       | 1.8 ± 0.3 | 1.7 ± 0.3    |
| Acto-S1 ATPase         |    |               |
| $V_{max}$              | 4.8 ± 1.2 s$^{-1}$ | 5.2 ± 1.5 s$^{-1}$ |
| $K_{m}$               | 36.5 ± 13.4 μM | 39.9 ± 16.9 μM |
| In vitro motility      |    |               |
| Actin polymerization   | Same | Same         |
| (rate and extent)      |    |               |
| Far UV CD spectra      | Same | Same         |
| Tryptophan emission    | Same | Same         |

* It may be noted that, as reported before (47), the strong binding constants of S1 to actin are about 10-fold lower for yeast than for rabbit skeletal actin.

To the calculations of Lorenz et al. (1), the electrostatic contribution of residue 315 is about 60% smaller than that of residue 311 (~330 versus ~850 cal/mol). Thus, any changes in the regulation of this mutant actin should be smaller than those observed with E311A/R312A actin. Indeed, the pCa titration results of the 315/316 mutant (both the sliding speeds and percentage of filaments moving) fell between those of the other two actins (data not shown).

In Vitro Motility of Actin-Tropomyosin Complexes—Solution studies have shown that the presence of Tm strongly influences the acto-S1 ATPase rate; at low S1 concentrations Tm inhibits the ATPase reaction, whereas at high S1 concentrations the reaction is potentiated (5-7). According to biochemical and structural studies (1, 4), the actin-Tm complex populates only two states, closed and open, of the three states proposed for the Tm-Tn-actin complex in the McKillop and Geeves (4) model. Clearly, the equilibrium between the closed and open states of actin-Tm is shifted by the binding of S1. When S1 is present at sufficient high concentrations, actin-Tm is switched to the open state, releasing the inhibition of acto-S1 ATPase. To shed light on these transitions and on the change in Ca2+ regulation of the regulated mutant actin, we measured the in vitro motilities of actin-Tm at various densities of HMM on the coverslips used in these assays.

In the case of wt yeast actin we observed a Tm-induced slowing of actin movement at low HMM concentrations (Fig. 3) but did not detect an acceleration of actin sliding by Tm at high HMM concentrations. Tm did not increase the sliding speed of wt thin filaments even when the switching “on” of the actin filaments was facilitated by increasing the HMM concentration to 0.5 mg/ml or by adding N-ethylmaleimide modified myosin S1 to the motility assay buffer. However, our results for mutant actin-Tm thin filaments (Fig. 3B) differ from those for wt actin (Fig. 3A). Binding of Tm to the mutant thin filaments significantly increased their sliding speed over surfaces incubated with HMM at concentrations greater than 0.12 mg/ml. At the highest HMM concentration tested, the mutant actin-Tm complex moved almost 50% faster than the mutant actin alone (5.7 versus 3.8 μm/s). On the other hand, at low applied HMM concentrations (0.06 mg/ml), the mutant actin filaments behaved like their wt counterparts; the 311/312 actin alone was still sliding at speeds of approximately 1.8 μm/s, whereas the actin-Tm complex did not move at all.

As expected, the percentage of filaments moving was high for both types of actin at high applied HMM concentrations (data not shown). On surfaces incubated with low HMM concentrations, however, the addition of Tm significantly reduced the numbers of moving filaments. This effect was especially marked for wt actin, with a significant drop in the numbers of moving filaments at 0.10 mg/ml HMM and no moving filaments noted at the lowest HMM concentration. The impact of Tm on the mutant actin was less pronounced, with a clear decrease in the number of moving filaments noted only at the lowest HMM concentration. Thus, it appears that lower concentrations of HMM are required to release the Tm-induced inhibition of actin sliding in the mutant than in wt actin filaments.

Binding of Tm to Actin and S1-ADP to Actin-Tm—In an effort to relate the observed effects of Tm on actin motility to the binding of Tm to actin, we measured the affinity of Tm for both the mutant and wt actins using cosedimentation assays. The binding of Tm to both actins was cooperative and could be described by sigmoidal curves with Hill coefficients of 2.4 ± 0.4 for wt and 2.1 ± 0.6 for 311/312 actin (Fig. 4). There was no significant difference in the binding coefficients, with $K_a$ values of $(3.2 ± 0.2) \times 10^6$ M$^{-1}$ and $(2.5 ± 0.4) \times 10^6$ M$^{-1}$ for wt and 311/312 actin, respectively. In terms of the Lorenz et al. model (1), the predicted loss of binding energy contribution because of the E311A replacement (~850 cal/mol (1)) should result in an approximately 4-fold decrease in the $K_a$ value of Tm for actin. However, it should be noted that our studies were performed with the double mutant E311A/R312A. Although residue Arg-312 was not credited with having a significant contribution to
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**Fig. 3.** Effect of HMM surface density on the in vitro motility of actin-Tm thin filaments. The density of the HMM-coated surface was varied by applying HMM at concentrations between 0.05 and 0.3 mg/ml to the nitrocellulose-covered surface of the assay coverslip.

Panel A, speeds of wt actin filaments in the presence (○) and absence (●) of Tm. Panel B, speeds of 311/312 actin filaments in the presence (○) and absence (●) of Tm. Between 52 and 174 filaments were monitored at each HMM concentration in these experiments.

**Fig. 4.** Binding of Tm to wt and 311/312 actins. The actin-Tm cosedimentation binding assays were performed using wt (○) and 311/312 mutant (●) actins (5.0 μM) and 0–2.2 μM N-(1-pyrenyl)iodoacetamide-labeled Tm (PIATm) in a 5.0 mM HEPES buffer, pH 7.5, 3.0 mM MgCl₂, 150 mM NaCl at 22 °C. The data were fitted with sigmoidal curves using SigmaPlot 4.0 (Jandel Scientific) and yielded Hill coefficients and binding constants of 2.4 and 0.06 and 2.1 and 0.3 mg/ml to the nitrocellulose-coated surface of the assay coverslip.

The actin-Tm thin filaments. The density of the HMM-coated surface was varied by applying HMM at concentrations between 0.05 and 0.3 mg/ml to the nitrocellulose-covered surface of the assay coverslip. Panel A, speeds of wt actin filaments in the presence (○) and absence (●) of Tm. Panel B, speeds of 311/312 actin filaments in the presence (○) and absence (●) of Tm. Between 52 and 174 filaments were monitored at each HMM concentration in these experiments.

**The interactions of the mutant and wt actins with myosin were compared in acto-S1 MgATPase measurements and in binding assays of the actins to S1 under rigor conditions. The acto-S1 Mg-ATPase activities for wt and 311/312 mutant actins were virtually identical, yielding almost the same Kₘ and V_max values (Table I). Thus, at the very least, the 311/312 mutations affect neither the weak binding interactions between actin and S1 nor the activation of S1 MgATPase by actin. Moreover, the in vitro motilities of the mutant and wt actins in the absence of regulatory proteins were the same, as was also the binding of S1-ADP to actin-Tm complexes containing either wt or mutant actin (Table I).

The strong binding interactions (i.e. in the absence of nucleotides) between each of the actins and S1 were examined using cosedimentation assays. Here too, the results did not reveal any significant difference between the two actins in their binding of S1 (Table I).

Finally, we compared the pCa dependence of regulated acto-S1 Mg-ATPase of both wt and 311/312 mutant actin to determine whether the mutation alters the calcium affinity of TnC in the regulatory complex (Fig. 5). We found no difference between the ATPases measured with wt or 311/312 mutant actin. Both had the same degree of activation versus pCa and the same maximal ATPase rates in experiments using bovine Ca²⁺ was impacted by these mutations in actin, or (iii) the structure was not disturbed, but the amino acid substitutions changed the interaction between actin and the regulatory proteins. We performed a series of experiments to test, and ultimately exclude, the first two possibilities.

**Solution Interactions**—The rate at which actin polymerizes from monomeric G-actin to polymeric F-actin is sensitive to its substructure and conformational state (35–37). We compared the rates of MgCl₂-induced polymerization of both the 311/312 mutant and wt actins by measuring the increase in light scattering of 5.0 μM G-actin solutions after the addition of 3.0 mM MgCl₂. We found no significant differences between the two actins (Table I).
cardiac Tn and either bovine cardiac Tm (Fig. 5) or skeletal Tm (data not shown). We thus conclude that there is no difference in Ca\(^{2+}\) affinity between thin filaments reconstituted with either of the actins.

All in all, the above assays suggest that there are no significant structural differences between wt and the 311/312 mutant actin. The results of the polymerization, acto-S1 MgATPase, regulated acto-S1 MgATPase, in vitro motility, S1 and S1-ADP binding, CD, and tryptophan fluorescence experiments strongly indicate that neither the structure nor the function of the mutant actin has been modified. Thus, the differences in the in vitro motility regulation that we report in this study probably stem from the differences in the interactions between the actins and the regulatory proteins.

DISCUSSION

The goal of this study was to test predictions of the Lorenz et al. (1) model of the actin-Tm complex in the closed state. According to this model, actin residue 311 contributes significantly to the closed-state interaction of actin and Tm. Our initial hypothesis, based on the above model, was that a mutation at this residue should reduce the affinity of Tm for actin by about 4-fold and thus destabilize the closed-state binding of Tm to the mutant actin. This, in turn, would be reflected in an altered Ca\(^{2+}\) sensitivity of the regulated mutant actin filaments in the in vitro motility assays.

As a first step, we established that wt yeast actin is fully regulated by Tm-Tn (38). Thus, one important result of this work is that regulation can be conveniently studied using yeast actin in the in vitro motility assays. Korman and Tobacman (39) have also shown yeast actin to be fully regulated in acto-S1 ATPase studies. These results pave the way for regulation experiments using mutated yeast actins.

The main result of this work is that in the in vitro motility assays, the regulated 311/312 mutant actin filaments move faster at high pCa values than do regulated wt actin filaments. Because structural and functional comparisons of the two actins did not reveal any significant differences, these motility results indicate an increased Ca\(^{2+}\) sensitivity of the regulated 311/312 actin filaments.

At first glance, these results appear consistent with the prediction of Lorenz et al. (1) regarding the role of actin residue 311 in actin-Tm binding. According to the McKillop and Geeves (4) three-state model, the binding of Ca\(^{2+}\) to Tn induces conformational changes and steric transitions, which expose myosin weak binding sites on F-actin. At the same time the Tm-Tn complex moves into the closed position. If, in the mutant actin thin filaments, the Tm-Tn complex is not as firmly stabilized in the closed (+Ca\(^{2+}\)) position, then a smaller number of myosin heads will be sufficient to induce a shift of the Tm-Tn complex into the open position. This should be especially apparent at low Ca\(^{2+}\) concentrations, where few cross-bridges bind to the thin filaments. Our findings seemed to indicate that this is the case. In addition, the fact that larger fractions of mutant than of wt filaments moved at high pCa values lent support to this premise.

The explanation that the mutant actin regulation results are due, at least to some extent, to the destabilization of the actin-Tm complex in the closed state is not indicated by direct binding measurements. Tm binds to wt and 311/312 mutant actins with similar affinities under both closed-state (Tm alone) and open-state (Tm in the presence of S1 at a saturating concentration) conditions. Similar binding of S1-ADP to wt and mutant actin-Tm complexes rules out another possible cause for unequal stability of these actin-Tm complexes in the closed state. The unchanged binding properties of the 311/312 mutant suggest that explanations other than changes in equilibrium binding must be considered to account for our observations.

These possibilities include:

The three-state equilibrium could be shifted toward the open state if the mutation actually increases the 311/312 actin open-state affinity for Tm. However, our results suggest that Tm binding to actin under open-state conditions is unchanged by the mutation. This implies that the improved sliding of regulated 311/312 actin filaments at high pCa is not caused by a stabilization of the open-state complex.

The increased Ca\(^{2+}\) sensitivity of the regulated 311/312 mutant actin may be because of a destabilization of the actin-Tm-Tn complex in the blocked state (+Ca\(^{2+}\)−Tm), shifting the equilibrium toward the closed state. Such an effect could be the result of a reduced blocked-state affinity for Tm or an increase in the affinity of Tn for Ca\(^{2+}\) in the mutant actin-Tm-Tn complex. Using regulated actin-activated S1 MgATPase assays, we showed that the 311/312 mutation does not change the affinity of Tn for calcium. It may appear surprising that the pCa profiles for regulated wt and mutant actin filaments are the same in ATPase activity measurements but different in the in vitro motility assays. However, the rate-limiting steps in the two types of experiments are different. Unlike the ATPase reaction, filament sliding is an analog of unloaded muscle fiber shortening and is rate-limited by ADP release from actomyosin-ADP (40). Consequently, ATPase values are not necessarily predictive of filament sliding speeds in the motility assays (41).

Regarding changes in blocked state stability, although we cannot exclude this possibility, circumstantial evidence argues against it. The modulation of actin motility by Tm alone shows that 311/312 mutation-induced changes also occur in the absence of the blocked state. Moreover, because actin residues 311 and 312 are not within the Tm binding site in the blocked state, any change in such binding to 311/312 mutant actin would be allosteric in nature.

Interpreting our data in terms of the Lorenz et al. (1) model of the actin-Tm complex and the three-state model of regulation (4) may not be the only way to approach this issue. Squire and Morris (42) speculate that Tm alone could occupy a range of positions on the F-actin filament and that the modeled closed...
state of Tm on actin (1) could very well be an average of these Tm positions. If so, this would affect the identification of amino acid residues involved in the actin-Tm interaction and the estimation of their ΔG contributions. An example of this would be the recent study of the actin mutation E93K in the Drosophila flight muscle (43). This residue was not implicated in the Lorenz et al. (1) study but, nevertheless, strongly affects the function of Actin-Tm filaments in the in vitro motility assay.

Finally, the 311/312 mutation may modify the regulation via allosteric shifts in the regulated actin system. One possible scenario is that the binding of S1 to the actin-Tm-Tn complex in the presence of ATP and at low calcium concentrations is enhanced by this mutation. Results of motility experiments with the actin-Tm (no Tn) complexes are also consistent with allosteric explanations. Inhibition of actin sliding by Tm at low densities of HMM on the motility assay surface could be described in purely steric terms, i.e., an insufficient density of myosin heads binding to the closed-state actin-Tm complex to tilt the equilibrium toward the open state (active form) of this complex. (This argument may also explain the prior observations that acto-S1 MgATPase is inhibited by Tm at low S1 concentrations (5–7).) However, the potentiation of the 311/312 mutant actin-Tm sliding at higher concentrations of HMM together with the reported potentiation of acto-S1 MgATPase by Tm as S1 concentrations are increased (5–7) (albeit not at conditions close to V_max) cannot be explained using a steric-block model of regulation or stronger S1 contributions. An example of this would be S1 binding step and the subsequently more rapid release of the mutant actin-Tm complex leading to kinetic changes in the mutant actin-Tm complex.

It should be noted that a missense mutation in actin (R312H) in skeletal actin in the in vivo function of Actin-Tm filaments in the motility assay uncovers notable differences in their binding of Tm, S1, and S1-ADP and small, if any differences in other nonregulated functions. Our results underscore the importance of allosteric factors in the regulation of actomyosin interactions.

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