constant (Marston et al., 1979). When a muscle fiber is shortening, any rigor heads must experience a negative tension, and the similarity of the inhibition constants describing shortening velocity (Pate & Cooke, 1984) with those describing acto-S1 and myofibrillar ATPase is again consistent with the small size of any chemomechanical effect.

ACKNOWLEDGMENTS

We thank Professor R. M. Simmons and Dr. M. Irving for their helpful comments on the manuscript.

Registry No. ADP, 58-64-0; PP_i, 14000-31-8; AMPPNP, 25612-73-1; ATPase, 9000-83-3; ATP, 56-65-5.

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Effect of Muscle Tropomyosin on the Kinetics of Polymerization of Muscle Actin†

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Received August 6, 1985

ABSTRACT: At saturating concentrations, tropomyosin inhibited the rate of spontaneous polymerization of ATP-actin and also inhibited by 40% the rates of association and dissociation of actin monomers to and from filaments. However, tropomyosin had no effect on the critical concentrations of ATP-actin or ADP-actin. The tropomyosin–troponin complex, with or without Ca^{2+}, had a similar effect as tropomyosin alone on the rate of polymerization of ATP-actin. Although tropomyosin binds to F-actin and not to G-actin, the absence of an effect on the actin critical concentration is probably explicable in terms of the highly cooperative nature of the binding of tropomyosin to F-actin and its very low affinity for a single F-actin subunit relative to the affinity of one actin subunit for another in F-actin.

Polymerization of actin has two major phases: the relatively slow formation of nuclei, most probably trimers, and the more rapid elongation of nuclei to long filaments by addition of monomers to each end (Korn, 1982). The details of the nucleation process are still rather obscure, but considerable information is now available about the elongation phase. Most simply, the kinetics of elongation can be described by the equation

$$dF/dt = k_+Nc_1 - k_-N = k_+N(c_1 - c_c) \quad (1)$$

where k_+ and k_- are the sums of the association and dissociation rate constants at the barbed and pointed ends[1] of the filaments, N is the filament number concentration, c_1 is the actin monomer concentration, and c_c is the critical concentration = k_-/k_+.

† Supported in part by a grant from the Muscular Dystrophy Association.

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[1] The ends of actin filaments are designated "barbed" and "pointed" from the appearance in the electron microscope of filaments decorated with muscle heavy meromyosin.

The hydrolysis of ATP[2] that accompanies the polymerization of ATP–G-actin to ADP–F-actin occurs on the filament subsequent to the elongation step. At high actin concentrations, the rate of polymerization is faster than the rate of ATP hydrolysis so that a long stretch of ATP–F-actin subunits transiently forms (Pardee & Spudich, 1982; Pollard & Weeds, 1984; Carlier et al., 1984). At steady state, i.e., when c_1 is reduced to c_c, a small number (perhaps 2) of ATP–actin subunits cap and stabilize the barbed end of the filament[2] (Carlier et al., 1984, 1985; Pantaloni et al., 1985a,b) while the rest of the filament, including the pointed end (Carlier et al., 1986), consists of ADP–actin subunits.

There are, therefore, at least four sets of interactions at the ends of actin filaments: (1) the addition of ATP–actin to and loss of ATP–actin from filaments with long stretches of ATP–actin subunits, (2) the addition of ATP–actin to and loss of ATP–actin from ATP-capped filaments, (3) the addition of ATP–actin to and loss of ADP–actin from an ADP–actin end, and (4) the addition of ADP–actin to and loss of ADP–actin from an ADP–actin end. The first set of reactions occurs at both ends of the filament during the early phases of polymerization at high actin concentrations relative to the critical concentration; the second predominates at the barbed end at steady state; the third predominates at the pointed end at steady state; and the fourth is the major set of events occurring at both ends of the filament when actin at steady state is diluted appreciably below its critical concentration. When ADP–actin is polymerized, only the fourth set of interactions is possible. Because both the association and dissociation rate constants are very much larger at the barbed end than at the pointed end, the association and dissociation rates and critical concentration measured in solution, as in the experiments to be described in this paper, are essentially those of the barbed end. To characterize the pointed end, the barbed end must be completely blocked (for example, by a capping protein such as gelsolin), which introduces appreciable additional complications (Coué & Korn, 1985).

Muscle and nonmuscle cells contain many proteins that bind to monomeric and polymeric actin (Korn, 1982; Weeds, 1982; Craig & Pollard, 1982). These interactions are expected to affect the concentration of actin monomers, filament ends, and/or the association and dissociation rate constants that enter into eq 1. Through the study of the effects of the actin-binding proteins on actin polymerization, it should be possible to learn something about their probable functions in cells and to obtain more information about the process of actin polymerization.

Muscle tropomyosin is a 40 nm long coiled-coil rod composed of two highly helical 33000-dalton polypeptides (Smillie, 1979). At sufficiently high Mg^{2+} concentration, tropomyosin forms head-to-tail polymers that lie in the grooves of the F-actin double helix with a ratio of tropomyosin:actin subunits of 1:7 at saturation. Not surprisingly, muscle tropomyosin has been reported to stabilize actin filaments against the filament-severing actions of villin (Bonder & Mooseker, 1983) and an actin-depolymerizing protein from brain (Bernstein & Bamburg, 1982). Tropomyosins from nonmuscle cells (Smillie, 1979; Côté, 1983) are very similar to muscle tropomyosin but are shorter by 37 amino acids because of deletions near the amino terminus and contain significant sequence differences at the carboxyl-terminal end (Lewis et al., 1983). Amost certainly as a consequence of these differences, nonmuscle tropomyosins self-associate and bind to F-actin much more weakly than does muscle tropomyosin. Both muscle and nonmuscle tropomyosins associate with the troponin I, troponin C, troponin T complex and exert Ca^{2+}-dependent regulation of muscle actomyosin ATPase activity (Smillie, 1979; Côté, 1983).

Because tropomyosin binds to actin filaments and not to actin monomers, it might be expected to reduce the actin critical concentration and, perhaps, also to affect the rate of polymerization or depolymerization. Indeed, Pragay and Gergely (1968) reported that muscle tropomyosin accelerates the rate of polymerization of ATP–actin and, even more, of ADP–actin, as measured by the increase in viscosity that accompanies polymerization. Earlier work by Maruyama (1964), however, showed an inhibition by tropomyosin of polymerization of ATP–actin, as measured by the increase in flow birefringence. More recently, Walsh and Wegner (1980) reported that tropomyosin had no effect on the critical concentration of ATP–actin, as measured by the increase in fluorescence of NBD[2]-labeled actin, and Wegner (1982) concluded that the inhibition of the rate of actin polymerization by tropomyosin could be explained entirely by its ability to inhibit spontaneous fragmentation of actin filaments, thus reducing the concentration of filament ends (N in eq 1) available for elongation. In a preliminary paper, Hitchcock-DeGregori and Maris (1983) also found no effect of tropomyosin on the critical concentration but suggested that tropomyosin may inhibit the rates of both nucleation and elongation.

Because of the possible importance of tropomyosin as a regulatory protein in muscle and nonmuscle cells and the discrepancies among the previous studies, we decided to reinvestigate its effects on actin polymerization by using the sensitive assay of the increase in fluorescence of pyrenyl-labeled actin to measure the formation of F-actin. We have measured the rates of spontaneous polymerization (under conditions in which filament fragmentation does not have a significant effect), the rates of elongation of seeded solutions, the rates of depolymerization of filaments, and the critical concentrations of muscle ATP–actin and ADP–actin in the absence and presence of muscle tropomyosin and the tropomyosin–troponin complex. Muscle tropomyosin was used because it binds more tightly to actin than does nonmuscle tropomyosin and, therefore, is expected to have greater effects.

Materials and Methods

Muscle G-actin was prepared from rabbit muscle according to the procedure of Spudich and Watt (1971), as modified by Eisenberg and Kielley (1974), and gel-filtered through Sephadex G-200. Monomeric actin was stored on ice in buffer consisting of 5 mM Tris·HCl, pH 7.5, 0.1 mM ATP, 0.1 mM $CaCl_2$, 0.5 mM DTT, and 0.01% NaN_3 (buffer G). The concentration of monomeric actin was determined from its absorbance at 290 nm by using an extinction coefficient of 0.617 mg^{-1} mL cm^{-1} (Gordon et al., 1976). Pyrenyl-labeled actin was prepared by reacting F-actin with N-pyrenyliodoacetamide by using the procedure of Kouyama and Mihashi (1981) with minor modifications (Brenner & Korn, 1983). ADP–actin was prepared according to the procedure of Lal et al. (1984) and used within 6–8 h. Tropomyosin was purified according to the procedure of Eisenberg and Kielley (1974) and was a gift of Dr. David L. Williams, Jr., of the National

[2] Abbreviations: DTT, dithiothreitol; pyrenyl-labeled actin, actin labeled on Cys-374 by reaction with N-pyrenyliodoacetamide; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; SDS, sodium dodecyl sulfate; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride; ATP, adenosine 5′-triphosphate; ATPase, adenosinetriphosphatase; ADP, adenosine 5′-diphosphate; TN, troponin; TM, tropomyosin; NBD, 4-nitro-2,1,3-benzoxadiazole.

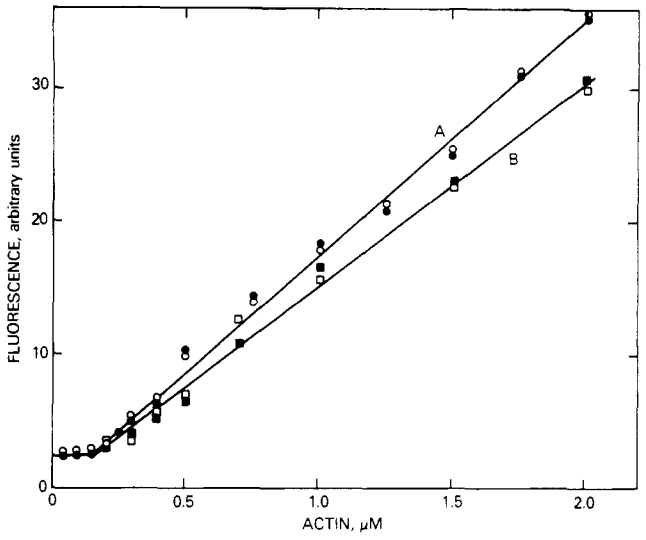

FIGURE 1: Critical concentration of ATP–actin in the presence of saturating concentrations of tropomyosin. Lower curve: 30 μM actin (5% pyrenyl labeled) was polymerized for 1 h in buffer F (buffer G + 2 mM $MgCl_2$, 100 mM KCl, and 1 mM EGTA). Polymerized actin was diluted to 20 μM with 6.66 μM tropomyosin (■) and without (□) tropomyosin in buffer F. The two samples were kept at 25 °C before diluting to various actin concentrations. Fluorescence intensity was measured for each sample at steady state. Upper curve: 20 μM F-actin (5% pyrenyl labeled) was diluted to various concentrations in the presence of tropomyosin (●) or in the absence of tropomyosins (○). The final ratios of tropomyosin:actin were the same as in the lower curve.

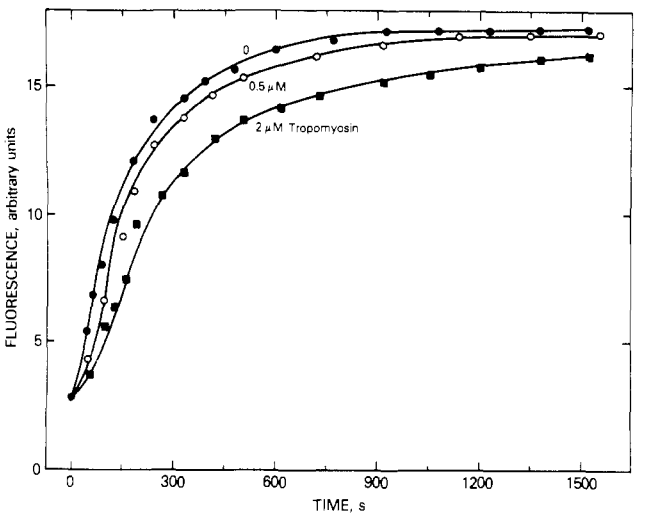

FIGURE 2: Spontaneous polymerization of actin in the absence and presence of tropomyosin. G-actin (5% pyrenyl labeled) was preincubated with 1 mM EGTA for 1 min in buffer G. Polymerization was started by addition of 2 mM $MgCl_2$ and 100 mM KCl. 6 μM actin alone (●); 6 μM actin with 0.5 μM tropomyosin (○); 6 μM actin with 2 μM tropomyosin (■).

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Measurements of actin critical concentrations were made as previously described (Brenner & Korn, 1983). Time courses of actin polymerization and depolymerization were followed by measuring the fluorescence changes of the pyrenyl probe covalently attached to actin, using a Spex Fluorolog 212 spectrofluorometer with a sample chamber thermostated at 25 °C. The excitation and emission wavelengths were 366 and 386 nm, respectively. To avoid bleaching of the fluorophore, pyrenyl-labeled actin was prepared and stored in the dark and was exposed to the light source only intermittently during the fluorescence measurement. Unless otherwise stated, polymerization was started by the addition of 2 mM $MgCl_2$, 100 mM KCl, and 1 mm EGTA to G-actin solution in buffer G.

ATP hydrolysis during actin polymerization was monitored by extraction of $^{32}P_i$ liberated from [γ-^{32}P]ATP (Pollard & Korn, 1973). Binding of tropomyosin to F-actin was quantified by polymerizing 6 μM actin in the presence of 2 μM tropomyosin followed by centrifuging aliquots at various time intervals in a Beckman airfuge at 20 psi for 20 min. The portion of the tubes containing the actin pellet was cut off and boiled directly in sample buffer and loaded onto 7% SDS gels. Electrophoresis was performed according to the procedure of Laemmli (1970). Nonspecific binding of tropomyosin to actin was corrected for by centrifuging tropomyosin alone and centrifuging F-actin with ^3H_2O and bovine serum albumin. Known quantities of actin and tropomyosin were simultaneously analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis for the purpose of quantification. Gels were stained according to the procedure of Fairbanks et al. (1971) and were scanned at 595 nm with a Beckman DU-8 spectrophotometer.

RESULTS

In the first set of experiments, we measured the effect on the critical concentration of ATP–actin at steady state of the addition of tropomyosin to F-actin. No effect was found (Figure 1). Samples at low, intermediate, and high actin concentrations were sedimented, and the ratio of bound tropomyosin to actin was found to be 1:7 in all cases (range 1:6.4 to 1:7.7); i.e., the actin filaments were saturated with tropomyosin at all points along the critical concentration curves.

Although it had no effect on the amount of polymerized actin at steady state, tropomyosin did inhibit the rate of actin polymerization (Figure 2). The maximal effect was observed with 2 μM tropomyosin, which was sufficient to saturate the actin filaments (6 μM total actin). The measured ratios of tropomyosin:F-actin subunits were 1:7.8, 1:7.7, and 1:7.4 for samples taken 300, 600, and 1200 s after initiation of polymerization.

We reported previously (Carlier et al., 1984) that the fluorescence intensity of pyrenyl-labeled F-actin is only 50% as high when ATP is bound to the F-actin as when ADP is bound. Therefore, it seemed possible that the slower rate of increase of fluorescence in Figure 2 in the presence of tropomyosin might not have been due to an inhibition of the rate of polymerization but to an inhibition of the rate of ATP hydrolysis on the F-actin. To test this possibility, we measured the rate of polymerization by the increase in fluorescence and the rate of hydrolysis of ATP in the same samples (Figure 3). In both the presence and absence of tropomyosin, ATP hydrolysis lagged slightly behind polymerization, as reported previously, but the relative rates of ATP hydrolysis and actin polymerization were the same in both samples. Tropomyosin, then, did not appear to inhibit the rate of ATP hydrolysis either during polymerization or at steady state. Thus, the reduced rate of fluorescence increase in the presence of tropomyosin could not have been due to an accumulation of ATP–F-actin subunits.

We next measured the effect of tropomyosin on the rate of elongation of F-actin seeds added to ATP–G-actin and on the rate of depolymerization of F-actin when solutions at steady state were diluted. These assays are measures of the effects of tropomyosin on the association and dissociation rate constants, respectively. The results (Figure 4) indicate that both k_+ and k_- are inhibited about 40% by tropomyosin at saturating levels. These results can explain the inhibition of the rate of spontaneous polymerization by tropomyosin (Figure 2) with no effect on the critical concentration (Figure 1)

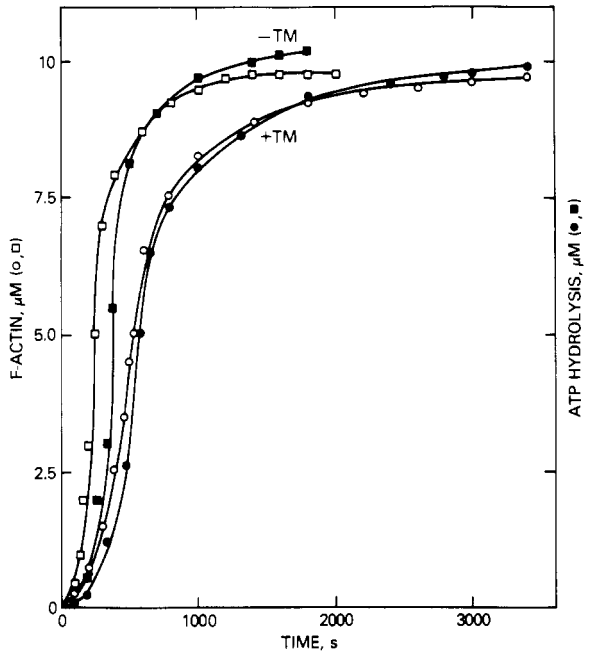

FIGURE 3: Time course of ATP hydrolysis and polymerization of actin with and without tropomyosin. G-actin (5% pyrenyl labeled) at a concentration of 20 μM was incubated for 3 h on ice in the presence of 110 μM [γ-^{32}P]ATP to exchange actin-bound ATP. Actin at a concentration of 6 μM was then polymerized with 2 μM tropomyosin (O) and without tropomyosin (□) as in Figure 2. ATP hydrolysis was followed during polymerization with tropomyosin (●) and without tropomyosin (■).

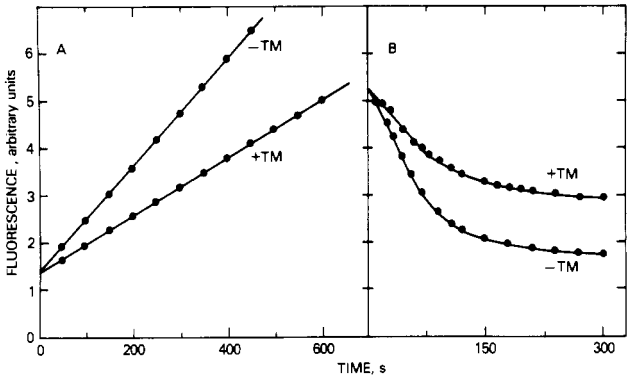

FIGURE 4: Effect of tropomyosin on the association and dissociation rates of the actin polymerization process. (A) F-actin filaments saturated with tropomyosin (40 μL of 6 μM actin and 2 μM tropomyosin) were used to nucleate the assembly of 0.8 μM actin with 0.26 μM tropomyosin (TM) or without tropomyosin in buffer F. (B) F-actin (20 μM, 5% pyrenyl labeled) was incubated with 6.66 μM tropomyosin or without tropomyosin for 3 h in buffer F. The samples were then diluted 20-fold in buffer F, and the decrease in fluorescence intensity was monitored.

because the ratio $k_-:k_+$ would be unchanged by tropomyosin. It is possible that the increase in the lag phase of spontaneous polymerization caused by tropomyosin in Figure 2 was due to an independent effect on nucleation, but it could have resulted simply from the decrease in k_+ (Tobacman & Korn, 1983).

In muscle, tropomyosin exists as a complex with troponins I, C, and T, and the native complex is responsible for Ca^{2+}-dependent activation of actomyosin ATPase activity. The data in Figure 5 show that Ca^{2+} is not required for the inhibition of the rate of actin polymerization by the tropomyosin–troponin complex, which was very similar to the effect of tropomyosin alone. In experiments similar to those described in Figures 1 and 4, we also found no difference between

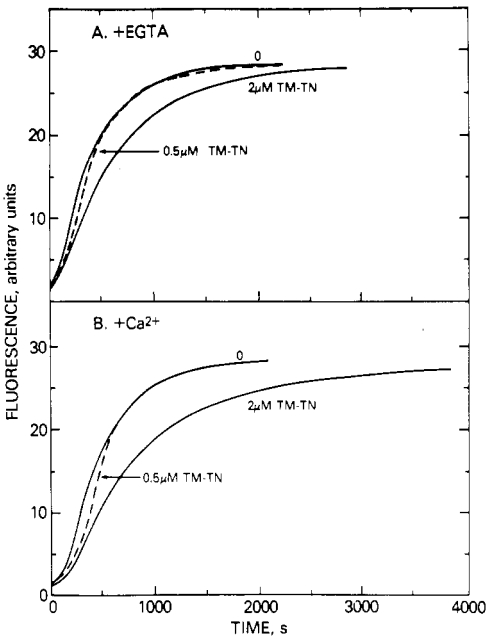

FIGURE 5: Effect of tropomyosin–troponin complex on actin polymerization with and without added Ca^{2+}. (A) Actin (6 μM, 5% pyrenyl labeled) was polymerized either in the presence of 0.5 μM and 2 μM tropomyosin–troponin (TM–TN) or without TM–TN in buffer F containing 1 mM EGTA as in Figure 2. (B) EGTA was not added, but the rest of the conditions were identical with those in A.

the effects of tropomyosin–troponin, with or without Ca^{2+}, and tropomyosin alone on the critical concentration or the rate constants for actin polymerization.

The polymerization of ATP–actin is a steady-state process in which ATP is irreversibly hydrolyzed while the polymerization of ADP–actin is a true equilibrium that we thought might be more significantly affected by the binding of tropomyosin to F-actin. However, in experiments identical with those of Figure 1, except for the replacement of ATP by ADP, we also found no effect of tropomyosin on the critical concentration of ADP–actin.

Discussion

Our finding that tropomyosin inhibits the rates of polymerization of ATP–actin and ADP–actin (data not shown) with no effect on the critical concentration of either disagrees with the observations of Pragay and Gergely (1968) and confirms the findings of Walsh and Wegner (1980) and Hitchcock-DeGregori and Maris (1983). In contrast to Wegner (1982), however, we find that tropomyosin does inhibit both the association and dissociation rate constants, although not substantially. We have no doubt that tropomyosin also inhibits the rate of spontaneous filament fragmentation, as reported by Wegner (1982), but our experiments were performed under conditions where the contribution of fragmentation to the polymerization kinetics is too low to detect.

Intuitively, one might have expected that tropomyosin would lower the actin critical concentration because it binds to F-actin and not to G-actin. That it does not is probably explained by the highly cooperative nature of the binding of tropomyosin to actin filaments (Yang et al., 1979; Wegner, 1979). In fact, the affinity of tropomyosin for F-actin is very low, with a K_D of approximately 1 mM (Wegner, 1979) when less than saturating concentrations of tropomyosin are used, so that there is no overlap of tropomyosin molecules on the actin filament. At saturating concentrations of tropomyosin, the K_D is reduced to about 1 μM (Wegner, 1979) because of the cooperativity introduced by the polymerization of tropomyosin itself within

the groove of the actin helix. The critical concentration, which is a measure of the affinity of actin monomers for filament ends, was about 0.2 μM for ATP–actin and 0.8 μM for ADP–actin in the experiments reported in this paper, i.e., about the same as the K_D for the interaction of tropomyosin with seven F-actin subunits. Therefore, the affinity of tropomyosin for one actin subunit at the end of the filament must be substantially less than the affinity of the actin–actin interaction. This would explain the insensitivity of the critical concentration of actin to the presence of tropomyosin while steric or other effects of tropomyosin reduce slightly the absolute magnitudes, but not the ratio, of the association and dissociation rate constants for actin elongation.

We conclude that muscle tropomyosin and tropomyosin–troponin have only trivial effects on the polymerization of actin. We would expect even smaller effects from nonmuscle tropomyosins because they bind to only six actin subunits in the filament, lack the carboxyl-terminal residues necessary for head-to-tail polymerization, and, as a result, have less affinity for F-actin. The main structural consequence of the interaction of tropomyosin with F-actin, then, would seem to be the inhibition of spontaneous fragmentation or active shearing of actin filaments by severing proteins. Nonmuscle tropomyosins share this property (Fattoum et al., 1983).

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