Binding of Tropomyosin to Copolymers of Acanthamoeba Actin and Muscle Actin*

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The binding of tropomyosin to F-actin is strongly dependent on Mg\(^{2+}\) concentration. With muscle actin, in the presence of 2 mM ATP, binding begins at 4 mM Mg\(^{2+}\) and is complete at about 4.75 mM Mg\(^{2+}\) while, with Acanthamoeba actin, binding is initiated at 6 mM Mg\(^{2+}\) and reaches saturation at 8.5 mM Mg\(^{2+}\). Copolymers of muscle and Acanthamoeba actin, however, behave as unique species of actin, each with a characteristic Mg\(^{2+}\) dependence and not as a mixture of homopolymers. In all cases, the stoichiometry of binding is one tropomyosin molecule to seven actin monomers. These results suggest that the actin monomers are randomly distributed in the copolymers so that one tropomyosin molecule binds to seven actin monomers of mixed origin and, therefore, neither the muscle nor Acanthamoeba actin can express its individual properties with respect to tropomyosin binding.

In 9 mM Mg\(^{2+}\) and 2 mM ATP, bound tropomyosin inhibits the muscle actin-activated heavy meromyosin ATPase but has no effect on the Acanthamoeba actin-activated heavy meromyosin ATPase. The effect of tropomyosin on the ATPase activation by copolymers of muscle and Acanthamoeba actin was proportional to the ratio of the two kinds of actin just as in mixtures of homopolymers. Similarly, in 5 mM Mg\(^{2+}\), 80 mM KCl, and 2 mM ATP, bound tropomyosin inhibits the muscle actin-activated heavy meromyosin ATPase while it increases the Acanthamoeba actin-activated heavy meromyosin ATPase both with copolymers and mixtures of homopolymers. These results cannot be attributed to an inability of tropomyosin to inhibit the activation of muscle heavy meromyosin ATPase by Acanthamoeba actin since, in the absence of Ca\(^{2+}\), the addition of tropoinin together with tropomyosin strongly inhibits the activation of the heavy meromyosin ATPase by Acanthamoeba actin both with homopolymers and copolymers. These data suggest that adjacent actin monomers can interact independently with heavy meromyosin in the presence and absence of tropomyosin and that one tropomyosin molecule can have different effects on adjacent actin monomers in random copolymers.

Actins isolated from different organisms can copolymerize. Hatano et al. (1) demonstrated that Physarum actin and muscle actin form hybrid actin polymers and more recently Gordon et al. (2) showed by viscometry that Acanthamoeba actin and muscle actin copolymerize. These observations suggest that the isoactins recently shown to co-exist in some vertebrate cells (3-6) might form copolymers in situ unless there are specific mechanisms that prevent their copolymerization. Since Acanthamoeba and muscle actin are each single isolectric species (7) with significantly different properties (2, 8, 9), their copolymers should serve as a useful model system to determine whether the different characteristics of each type of actin in a copolymer can be expressed independently.

We previously showed (9) that Acanthamoeba actin binds muscle tropomyosin at a molar ratio of seven actin monomers per tropomyosin molecule just as does muscle actin. However, this bound tropomyosin, which inhibits the muscle actin-activated HMM\(^{2+}\) ATPase, stimulates the Acanthamoeba actin-activated muscle HMM ATPase. We now find that the binding between actin and tropomyosin is strongly affected by Mg\(^{2+}\) with homopolymers of Acanthamoeba actin requiring a higher Mg\(^{2+}\) level than homopolymers of muscle actin for binding tropomyosin. Copolymers of muscle and Acanthamoeba actin are intermediate in their Mg\(^{2+}\) requirement for tropomyosin binding, which suggests that the muscle and Acanthamoeba actin monomers are randomly distributed in the copolymers. These data also show that neighboring actin monomers affect each other in their interaction with tropomyosin. In contrast, in their activation of HMM ATPase, the copolymers, both with and without tropomyosin, act like a mixture of homopolymers, showing that adjacent actin monomers in the copolymers act independently in their activation of HMM ATPase. This is an unexpected result in terms of the steric blocking model of tropomyosin action (10-12) which, in its simplest form, might predict a more cooperative effect of tropomyosin on the behavior of copolymers.

**MATERIALS AND METHODS**

Isolation and Puriﬁcation of Acanthamoeba Actin—Acanthamoeba actin was prepared by DEAE-chromatography and gel filtration as before (8) with the following modiﬁcations: the DEAE-cellulose column chromatography was carried out by directly applying the crude extract to the column, omitting elutions with low ionic strength buffer prior to and after sample application. Subsequently, the column was eluted with a buffer containing 0.1 M KCl, 0.1 mM CaCl\(_2\), 0.5 mM ATP, 0.7 mM β-mercaptoethanol, 0.01% NaN\(_3\), and 10 mM imidazole, pH 7.5. Usually 2 bed volumes or more of this buffer were passed through the column before the gradient elution was started. A linear gradient between 0.1 and 0.4 M KCl was used instead of 0.1 to 0.5 M KCl. The final purification step was by chromatography on Sephadex G-200 as described below.

Preparation of Muscle Proteins—Myosin, heavy meromyosin, and tropomyosin were prepared as described previously (9). Tropomysin was isolated and purified according to the procedures of Eisenberg and Kielley (13). Actin was isolated and purified as before (9), then depolymerized and chromatographed on a Sephadex G-200 column.

*The abbreviations used are: HMM, heavy meromyosin; EGTA, ethylene glycol bis(β-aminoethyl ether)N,N′-tetraacetic acid.
**Tropomyosin Binding to Actin Copolymers**

**Sephadex G-200 Chromatography of Acanthamoeba Actin and Muscle Actin**—To prepare random copolymers, it is important that each kind of G-actin be devoid of nuclei (dimer, trimer, or oligomers) since the presence of nuclei can give rise to copolymers with relatively long segments containing only muscle actin or Acanthamoeba actin. Therefore, Acanthamoeba and muscle G-actins were chromatographed in parallel on separate Sephadex G-200 columns under carefully controlled conditions.

Both columns (Pharmacia; gel beds, 26 × 850 mm) were pre-equilibrated with the same stock buffer containing 0.5 mM CaCl₂, 0.7 mM β-mercaptoethanol, 0.01% NaN₃, and 5 mM imidazole, pH 7.5. Identical flow rates were maintained. The two actin preparations were dialyzed against the same buffer used to equilibrate the columns, centrifuged at 100,000 × g for 90 min to remove aggregates, and adjusted to concentrations of 6 to 8 mg/ml before applying to the columns. As much as 250 mg of actin was applied to the columns, which were eluted with the same buffer. It was assumed that the two chromatograms would be comparable when flow rates, sample concentrations, and sample loads were identical. Fractions from the leading edge of the actin peak, comprising about 10% of the total peak, were discarded to avoid possible presence of dimers and trimers. Fractions with protein concentrations below 0.5 mg/ml from the trailing edge were also discarded. The remaining fractions, which presumably contained only actin monomers, were pooled. The monomeric Acanthamoeba actin and muscle actin thus obtained were then dialyzed in the same buffer against fresh buffer of the same composition as above. The dialysate was used as the reference buffer in the spectrophotometric determination of actin concentrations and for subsequent adjustment of protein concentrations. Dialed actin stocks, in concentrations of 1.0 to 2.7 mg/ml, were diluted to working stocks of precisely 1.0 mg/ml for the preparation of copolymers.

**Copolymerization**—Copolymers of various compositions were prepared by mixing appropriate amounts (determined by weight rather than volume) of the two working stocks of Acanthamoeba and muscle actin monomers and subsequently polymerizing the mixtures. Each actin mixture was incubated in a water bath at 25°C for 20 min and polymerization then was initiated by the addition of MgCl₂ to 1 mM with moderate stirring. After 2 h, the copolymers were collected by sedimentation at 100,000 × g for 90 min. Homopolymer of Acanthamoeba actin and muscle actin were prepared in identical manner. Pelleted polymers were resuspended by gentle homogenization in a buffer containing 0.5 mM ATP, 2 mM MgCl₂, 0.01% NaN₃, and 5 mM imidazole, pH 7.0, and then dialyzed against two changes of the same buffer. Following dialysis, each F-actin solution was examined under a polarizer; when aggregates were present, gentle mechanical stirring was employed to achieve even dispersion. The concentration of each F-actin solution was determined in duplicate and diluted to 4 to 5 mg/ml by addition of the dialysate.

**Radioiodination of Muscle Tropomyosin**—¹²⁵I-labeled tropomyosin was described as before (9) except that the removal of unreacted I⁻⁻ was accomplished by dialysis against fresh buffer of the same composition as above. The dialysate was used as the reference buffer in the spectrophotometric determination of actin concentrations and for subsequent adjustment of protein concentrations. Dialed actin stocks, in concentrations of 1.0 to 2.7 mg/ml, were diluted to working stocks of precisely 1.0 mg/ml for the preparation of copolymers.
Mg$^{2+}$. After polymerization, each reaction mixture was centrifuged and the actin concentration in the supernatant was measured. The amount of nonsedimentable actin was remarkably constant for all experiments even though the ratio of muscle to Acanthamoeba actin varied from 4:1 to 0.5 (Table I). Presumably, the amount of nonsedimentable actin would have varied if there had been preferential incorporation of one type of actin into the copolymers. Therefore, it is very likely that the compositions of the copolymers were identical to the compositions of the actin mixtures from which they were formed.

**Binding of Tropomyosin to Actin Copolymers**—The effect of Mg$^{2+}$ on tropomyosin binding to two actin copolymers as well as to mixtures of homopolymers is shown in Fig. 1. The copolymers behaved as if they were single new actin species with threshold Mg$^{2+}$ concentrations and transition midpoints between the extremes set by the two homopolymers. This is strong evidence that random copolymerization has, in fact, occurred since, if copolymerization were not random, the binding curves would have resembled the binding curves for mixtures of homopolymers. It is possible, however, that non-random sequences might not have been detected if the binding of one tropomyosin molecule affects the binding of other tropomyosin molecules. But, in view of the care taken to ensure random copolymerization, we think it is much more likely that the copolymers were indeed random. Therefore, since the copolymers bound tropomyosin with the same stoichiometry as did homopolymers, tropomyosin probably binds simultaneously to seven adjacent actin monomers of mixed origins in the copolymers at high Mg$^{2+}$ concentrations (>8 mM), where all copolymers can be saturated with tropomyosin. These experiments established, therefore, that, at 9 mM Mg$^{2+}$ we could study the effect of bound tropomyosin on the activation of HMM ATPase by random copolymers of muscle and Acanthamoeba actin.

**Stimulation of HMM ATPase by Actin Copolymers and Homopolymers**—We first studied the copolymer actin-activated HMM ATPase in the absence of tropomyosin. All actin-activated HMM ATPase activities were carried out at 10 mM ATP and 9 mM MgCl$_2$. Reaction mixtures contained 10 $\mu$m actin, 0.46 mg/ml of HMM, 2 mM ATP, 9 mM MgCl$_2$, 1 mM EGTA, and 2 mM imidazole, pH 7.0, at 25°C. Each point represents the average of two determinations.

![Fig. 2. Activation of HMM ATPase by copolymers and mixed homopolymers of muscle and Acanthamoeba actin. Reaction mixtures contained 10 $\mu$m actin, 0.46 mg/ml of HMM, 2 mM ATP, 9 mM MgCl$_2$, 1 mM EGTA, and 2 mM imidazole, pH 7.0, at 25°C. Each point represents the average of two determinations.](http://www.jbc.org/)

**TABLE I**

**Incorporation of muscle and Acanthamoeba actin into copolymers**

Varying compositions of copolymers were prepared as described under "Materials and Methods." In all combinations, the reaction mixture contained 1 mg/ml of total actin monomers, 0.5 mM ATP, 0.1 mM CaCl$_2$, 0.7 mM $\beta$-mercaptoethanol, 0.01% NaN$_3$, and 5 mM imidazole, pH 7.0.

<table>
<thead>
<tr>
<th>Amount of G-actins</th>
<th>Actin remaining in supernatant (%</th>
<th>%</th>
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<tbody>
<tr>
<td>Muscle</td>
<td>Acanthamoeba</td>
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</tr>
<tr>
<td>24</td>
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<td>0</td>
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*Percentage of muscle actin and Acanthamoeba actin not sedimentable following polymerization.*

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The effects of bound tropomyosin on the abilities of muscle and Acanthamoeba actin to activate the HMM ATPase were determined in the presence of 2 mM ATP and 9 mM Mg$^{2+}$, a condition in which tropomyosin binds maximally to both actins (Fig. 1). In agreement with our previous finding (9, 14), tropomyosin inhibited the muscle actin-activated HMM ATPase in proportion to the amount of tropomyosin bound (Fig. 3). A maximum of 85% inhibition was reached when the muscle actin was saturated with tropomyosin. The effect of tropomyosin was different with Acanthamoeba actin. Previously (9), we had observed that, in 5 mM MgCl$_2$ and 80 mM KCl bound tropomyosin stimulated the Acanthamoeba actin-activated HMM ATPase. In 9 mM Mg$^{2+}$, however, tropomyosin had no effect at all on the Acanthamoeba actin-activated HMM ATPase even when tropomyosin was present in excess (Fig. 3).

We next tested whether the differential effects of tropomyosin on the activation of HMM ATPase by muscle and Acanthamoeba actin would also occur with the copolymers. The theoretical curves (dashed) in Fig. 4 illustrate the possible consequences of copolymer actin-HMM interactions, as a function of copolymer composition, if tropomyosin were to exert a uniform effect on all of the actin monomers in the copolymers, i.e. if one type of actin dominated completely.
reaction mixture before the addition of HMM.  

For example, if all of the actin in the copolymers behaved as Acanthamoeba actin does in its homopolymer, tropomyosin would not inhibit the copolymer actin-activated HMM ATPase until the composition of the actin copolymers approached 100% muscle actin (upper theoretical curve). On the other hand, if tropomyosin had the same inhibitory effect on all of the actin monomers in the copolymers as it has on the muscle actin homopolymer, then the lower theoretical curve of Fig. 4 would be expected. A third, and perhaps more likely possibility, would be a sharp transition from the lower curve to the upper curve at some particular mole fraction of Acanthamoeba actin, e.g. 0.5. In contrast to these possibilities, the experimental data showed (Fig. 4, solid curve) that the copolymer actin-activated HMM ATPases were inhibited by tropomyosin to the same extent as were the HMM ATPases that were activated by mixed homopolymers of identical composition. Therefore, in the copolymer actin-activated HMM ATPase, tropomyosin inhibited the muscle actin-activated HMM ATPase but did not affect the Acanthamoeba actin-activated HMM ATPase even though, presumably, the muscle and Acanthamoeba actin monomers are adjacent to each other in the copolymers. Thus, the same tropomyosin molecule can affect adjacent actin monomers differently. This conclusion is supported by experiments carried out under different conditions. In 5 mM MgCl₂ and 80 mM KCl, both Acanthamoeba actin and muscle actin are saturated with tropomyosin but the tropomyosin of the Acanthamoeba actin-HMM ATPase while still inhibits the muscle actin-activated HMM ATPase (10). Under these ionic conditions, in the presence of tropomyosin, the actin-activated HMM ATPase activities were almost identical for the 1:1 copolymer and the equimolar mixture of the two actin homopolymers (Table II). These data also indicate that tropomyosin can affect adjacent actin monomers in the copolymer differently. The lack of inhibition by tropomyosin of the Acanthamoeba actin-activated HMM ATPase cannot be attributed to an inherent inability of Acanthamoeba actin to be inhibited because, as shown in Fig. 5, the tropomyosin-troponin complex caused 93% inhibition of both Acanthamoeba actin and muscle actin-activated HMM ATPase when added to either homopolymers or copolymers of actin.

The interpretation of our experiments depends strongly on whether or not the muscle and Acanthamoeba actin monomers were randomly distributed along the copolymer filament. The best evidence for a random distribution, in addition to the procedure used to synthesize the copolymers, comes from the studies on the Mg²⁺ dependence of the tropomyosin binding to the actins. The copolymers did not act at all like mixtures of homopolymers of muscle and Acanthamoeba actin. Rather, the copolymers behaved like new species of F-actin, each of which shows a Mg²⁺ dependency for tropomyosin binding distinctly different from, and intermediate between, those of pure muscle F-actin and pure Acanthamoeba F-actin.

On the other hand, in terms of the effect of tropomyosin on the acto-HMM ATPase activity, the copolymers were experimentally indistinguishable from mixtures of homopolymers. If the copolymers were, as we believe, truly random, then a tropomyosin molecule, in binding to seven adjacent actin monomers in a copolymer filament, can have a different effect on two neighboring actin monomers. Specifically, the tropomyosin can inhibit the activation of the HMM ATPase by a muscle actin monomer while simultaneously either enhancing, or having no effect, on the activation of the HMM ATPase by an adjacent Acanthamoeba actin monomer in the same copolymer filament.
This interpretation of our data does not support the "steric-blocking" model proposed by Huxley and co-workers (10-12) to explain the action of tropomyosin in vivo. According to their model, in resting muscle, tropomyosin physically blocks the myosin-attachment sites on the actin filaments, thus preventing the actin and myosin filaments from interacting while, during contraction, tropomyosin moves to a position where it no longer blocks the myosin-attachment sites so the actin and myosin filaments can interact. If the effect of tropomyosin on the interaction of actin and myosin is related to its position on the actin filament, then our data for the homopolymers would require that tropomyosin be located at a different position when bound to Acanthamoeba F-actin (position A) than when bound to muscle F-actin (position M). The position of tropomyosin when bound to a copolymer of Acanthamoeba and muscle actin would, then, be expected to be determined by the composition of the copolymer, i.e. the properties of one of the actin monomers should dominate. When a copolymer is rich in muscle actin, the tropomyosin should occupy position M and inhibit the copolymer actin-activated HMM ATPase. Conversely, when the copolymer is rich in Acanthamoeba actin, the tropomyosin should occupy position A and either stimulate (in 80 mM KCl) or have no effect (in 9 mM Mg²⁺) on the copolymer actin-activated HMM ATPase. As the ratio of Acanthamoeba actin to muscle actin in the copolymer is increased, and, assuming that all of the actin monomers influence the binding of tropomyosin, there should be an abrupt switch of the tropomyosin from position M to position A with a concomitant shift in the effect of tropomyosin from inhibition to stimulation (or no effect). These predictions follow from the fact that tropomyosin, a 40-nm long rigid molecule, would be expected to move as a unit from a blocking to a nonblocking position and, therefore, exert a uniform effect on the seven consecutive actin monomers to which it is bound. This conclusion assumes, of course, that the myosin attachment site is in an equivalent position on both types of F-actin.

Contrary to the predictions based on the steric-blocking model, our results show that tropomyosin has an independent effect on adjacent muscle and Acanthamoeba monomers in the copolymers. The effect of tropomyosin on the copolymers is always indistinguishable from its effect on mixed homopolymers of the same composition. Of course, our results in no way suggest that a shift in the binding position of tropomyosin does not occur but they do indicate that such a shift may not be causally related to the effect of tropomyosin on the actin-activated myosin ATPase.

Finally, our data on the formation and properties of copolymers of muscle and Acanthamoeba actin suggest that the isoactins that co-exist in many vertebrate cells will form random copolymers unless there are specific cellular mechanisms to prevent their formation. Moreover, the isoactin monomers in the copolymers will act independently in their activation of the myosin ATPase and in their regulation by the tropomyosin-troponin system.

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