Interaction between Acanthamoeba Actin and Rabbit Skeletal Muscle Tropomyosin

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The binding of 15S-labeled muscle tropomyosin to Acanthamoeba and muscle actin was studied by ultracentrifugation and by the effect of tropomyosin on the actin-activated muscle heavy meromyosin ATPase activity. Binding of muscle tropomyosin to Acanthamoeba actin was much weaker than its binding to muscle actin. For example, at 5 mM MgCl2, 2 mM ATP, and 5 μM actin, tropomyosin bound strongly to muscle actin but not detectably to Acanthamoeba actin. When the concentration of actin was raised strongly to muscle actin but not detectably to Acanthamoeba muscle heavy meromyosin ATPase activity. Binding of muscle tropomyosin to Acanthamoeba actin approached its binding to muscle actin. As with muscle actin, the addition of muscle heavy meromyosin in the absence of ATP induced binding of tropomyosin to Acanthamoeba actin under conditions where binding would otherwise not have occurred. The most striking difference between the interactions of muscle tropomyosin with the two actins, however, was that under conditions where tropomyosin was bound to both actins, it stimulated the Acanthamoeba actin-activated heavy meromyosin ATPase but inhibited the muscle actin-activated heavy meromyosin ATPase.

In the past few years, it has become evident that eukaryotic cell motility, one of the most striking characteristics of living organisms, depends on actin and myosin (1, 2). To understand the molecular mechanisms of this very complicated cellular process, we have chosen to study a simple primitive organism, Acanthamoeba castellanii, because it is very motile and because of the relative ease of obtaining large quantities of cells for biochemical studies. Several cytoplasmic motility proteins have now been identified in Acanthamoeba: actin (3), myosin (4), myosin cofactor (5), and more recently several gelation factors and a Ca2+-ATPase that interact with actin (6). Of these proteins, actin is the best characterized.

Acanthamoeba actin is similar to muscle actin in its amino acid composition (3, 7) and sequence (8), stoichiometry of bound nucleotide (7), stoichiometry of binding to HMM (1) and to myosin subfragment 1 (7), and in the qualitative aspects of the kinetics and thermodynamics of its polymerization (9). Acanthamoeba actin differs from muscle actin in the presence of N- methyllysines (3, 7, 10) and in its critical concentrations for polymerization (9). Finally, Acanthamoeba actin activates muscle HMM ATPase only about one-third as well as does muscle actin (1).

Tropomyosin is a rod-like protein that lies along the grooves of the actin helix (11) in skeletal muscle. With troponin, tropomyosin regulates muscle contraction by controlling the interaction of actin and myosin filaments. Tropomyosin has also been demonstrated in, and isolated from, vertebrate non-muscle cells (12-16) where its function is not yet clear. Although tropomyosin has not been detected in Acanthamoeba, it has been demonstrated (17) that the tropomyosin-troponin complex from skeletal muscle confers Ca2+ sensitivity on the Acanthamoeba actin-activated HMM ATPase, as it does also on the actin-activated HMM ATPase.

In the present study, we compared the interactions of tropomyosin with Acanthamoeba actin and muscle actin to characterize further the Acanthamoeba actin molecule and to gain additional insight into the nature of the interactions between tropomyosin and muscle actin, of which Acanthamoeba actin can be considered a natural variant. Our results show that muscle tropomyosin binds to Acanthamoeba actin much more weakly than to muscle actin. Furthermore, under conditions in which it binds to both actins, tropomyosin stimulates the Acanthamoeba actin-activated HMM ATPase whereas, on the contrary, as shown previously by Eaton et al. (18), tropomyosin inhibits the muscle actin-activated HMM ATPase.

EXPERIMENTAL PROCEDURES

Preparation of Acanthamoeba Actin—Routinely, 150 g (wet weight) of cells were obtained from two 15-liter suspension cultures (3). Acanthamoeba actin was purified as previously reported (7). The yield was approximately 100 to 150 mg.

Preparation of Actin, Heavy Meromyosin, and Tropomyosin from Rabbit Skeletal Muscle—White muscle from the back and hind legs was used for the extraction of all muscle proteins. Actin was prepared according to the procedure of Spudich and Watt (19) and treated twice with 0.8 M KCl to ensure the complete removal of tropomyosin. Myosin and heavy meromyosin were obtained using the methods described previously (18). The tropomyosin-troponin complex was extracted from an ethanol/ether powder of muscle (20) and subsequently fractionated by the procedure of Greaser and Gergely (21) to obtain tropomyosin. One single cycle of isoelectric precipitation, at pH 4.6, followed by ammonium sulfate fractionation between 40 and 60% saturation resulted in a tropomyosin preparation with 7 to 10% troponin contamination. This troponin-poor tropomyosin was chromatographed on a hydroxyapatite column to remove troponin and nucleic acids (22). The homogeneity of tropomyosin thus obtained was established by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, according to the procedure of Laemmli (23). It was important for the tropomyosin to be homogeneous since slight contamination with component(s) still undent-
fied resulted in various degrees of activation of Acanthamoeba actin-activated HMM ATPase activity under conditions where pure tropomyosin had no effect. A preparation was judged acceptable if no contaminant could be detected when 50 μg of tropomyosin were loaded onto the gel. Repurification of tropomyosin, when necessary, was achieved by repeating the isoelectric precipitation and by ammonium sulfate fractionation between 53 and 60% saturation (21). The purified protein was exhaustively dialyzed against deionized water, lyophilized and stored at -20°C. In a given series of experiments, tropomyosin from the same preparation was used for radioiodination, binding analyses, and ATPase assays.

Radioiodination of Tropomyosin — Lyophilized tropomyosin was dissolved in 1 M KCl, 10 mM Tris/HCl, pH 8.0, and dialyzed against a buffer containing 0.37 M KCl and 0.063 M potassium phosphate buffer, pH 7.0. After centrifugation at 100,000 × g to remove trace amounts of denatured protein and aggregates, the tropomyosin was labeled with 125I by the lactoperoxidase procedure as modified by Eaton et al. (18) except that in some batches, the concentration of Na125I (New England Nuclear) was doubled to increase the specific activity of the labeled tropomyosin. The labeled tropomyosin was stored frozen in 2 mM imidazole, pH 7.0, at -20°C and was used within 3 months.

Determination of Tropomyosin Binding to F-Actin by Ultracentrifugation — The binding of 125I-tropomyosin to Acanthamoeba actin or muscle actin was determined by centrifuging a mixture of F-actin and 125I-tropomyosin to separate the bound (pellet) from the free (supernatant) tropomyosin. The general procedure of Eaton et al. (18) was used for the present investigation with the following modifications.

Just before the experiment, the stock 125I-tropomyosin (~200 μM) was diluted to a concentration twice that of the desired final working solution, 10 and 50 μM in the experiments at low and high tropomyosin concentration. In the experiments at high concentrations of tropomyosin, the 125I-tropomyosin was mixed with unlabeled tropomyosin in the ratio of approximately 1:4 to obtain the desired specific activity. This concentrated solution was centrifuged at 100,000 × g for 1 h to remove small amounts of aggregates and then diluted to either 5 or 25 μM tropomyosin. F-actin was diluted to 10 or 50 μM just prior to the experiment. The concentration of each diluted protein was redetermined by ultraviolet absorption and readjusted when necessary. Binding assays were carried out in centrifuge tubes containing 2 ml of reaction mixtures which were incubated with continual agitation for 20 min in a constant temperature water bath at 25°C. At the end of the incubation, 0.5-ml aliquots from each tube were set aside to determine the total radioactivity. The remaining solution was centrifuged at 40,000 rpm for 30 min in a 40 rotor (Beckman) at 25°C. Aliquots of 0.5 ml were removed from the supernatant to determine the radioactivity of unbound tropomyosin. Each 0.5-ml aliquot was mixed with 0.25 ml of 8% sodium dodecyl sulfate, 9% mercaptoethanol and the mixture was heated at 100°C for 15 min. Duplicate 0.15-ml aliquots from this mixture were added to 90 ml of Aquasol (New England Nuclear) and the radioactivity was determined in a Beckman LS-250 liquid scintillation counter. When 125I-tropomyosin was not treated with sodium dodecyl sulfate and mercaptoethanol, the observed radioactivity was significantly lower than the true radioactivity, possibly because of aggregation of the tropomyosin in the Aquasol.

For each sample, a control was carried out simultaneously to determine the amount of 125I-tropomyosin sedimentable in the absence of actin; it was never more than 5% of the total tropomyosin. Bound tropomyosin was calculated by subtracting the free tropomyosin and that which sedimented in the absence of actin from the total tropomyosin. Nonspecific association of tropomyosin with F-actin filaments or HMM was insignificant.

Measurement of Protein Concentration — The concentration of protein was determined by ultraviolet absorption using the following extinction coefficients: 1149 cm⁻¹/g at 280 nm for F-actin (24), 647 cm⁻¹/g at 260 nm for HMM (25), and 290 cm⁻¹/g at 278 nm for tropomyosin (26). The molecular weights used for calculating the molarity of these proteins were: 44,000 for actin, 350,000 for HMM (or 175,000 for each head), and 70,000 for tropomyosin.

ATPase Assays — HMM and actin-activated HMM ATPase activities were determined by the pH-stat method of Eisenberg and Moos (24). The reaction was carried out at 25°C and pH 7.0. HMM was always added to the reaction mixture last to initiate the reaction; its concentration was adjusted for each reaction to give rates that could be determined accurately. Rates for actin-activated HMM ATPase were measured, and when less than 25% of the ATP had been hydrolyzed, tropomyosin was added to determine its effect on the ATPase activities.

RESULTS

Binding of Tropomyosin to Acanthamoeba Actin — In 5 mM MgCl2, 2 mM ATP and with 5 μM actin, tropomyosin bound to muscle F-actin reaching saturation at a ratio of one tropomyosin per seven actin monomers (Fig. 1 and Ref. 18). In contrast, under identical conditions, tropomyosin did not bind detectably to Acanthamoeba F-actin (Fig. 1). Muscle tropomyosin and Acanthamoeba actin could be induced to bind, however, by the addition of HMM in the absence of ATP (Fig. 2). Omission of ATP had no effect on the binding of tropomyosin to actin in the absence of HMM. When tropomyosin and Acanthamoeba actin were mixed in a molar ratio of one tropomyosin to seven actin monomers, complete saturation of the actin filaments with tropomyosin occurred when the HMM heads were present in excess of one head per actin monomer. No binding was detected between tropomyosin and HMM in the absence of actin, confirming the observation of Kominz and Maruyama (27), suggesting that HMM does not serve as a bridge between tropomyosin and Acanthamoeba actin. A similar observation was made for muscle actin by Eaton (28), who showed that HMM induced stoichiometric binding of tropomyosin to muscle actin under conditions in which binding would otherwise not have occurred.

Since, in the presence of HMM, tropomyosin bound to Acanthamoeba actin with the same stoichiometry as to muscle actin, its failure to bind to Acanthamoeba actin in 5 mM MgCl2 (Fig. 1) must reflect a low binding affinity between the two proteins and not an absence of binding sites. Interaction between tropomyosin and Acanthamoeba actin could be en-
hanced by increasing the concentrations of both proteins or by raising the ionic strength of the incubation medium (Fig. 3). In the absence of KCl, a 5-fold increase in the concentrations of both Acanthamoeba actin (from 5 to 25 μM) and tropomyosin (the molar ratio of tropomyosin to actin was varied over the same range at the two actin concentrations) significantly elevated the binding between the two proteins so that at a molar ratio of total tropomyosin to Acanthamoeba actin of 0.5, the F-actin was about 64% saturated with tropomyosin. Alternatively, when the actin concentration was maintained at 5 μM, the addition of 80 mM KCl increased the binding to 80% saturation at the same molar ratio (0.5) of total tropomyosin to actin. When both the protein concentrations and the ionic strength were increased (Fig. 3), the binding between tropomyosin and Acanthamoeba actin approached the levels attained with tropomyosin and muscle actin. Such conditions, however, had no observable effect on the binding between tropomyosin and muscle actin (Fig. 3), which was already maximal at low ionic strength and low protein concentrations.

Effect of Tropomyosin on Acanthamoeba Actin-activated HMM ATPase—Not unexpectedly, at 5 mM MgCl₂ and 2 mM ATP, conditions in which tropomyosin does not bind to Acanthamoeba actin (Fig. 1), tropomyosin had no effect on the Acanthamoeba actin-activated HMM ATPase (Fig. 4), whereas, in agreement with the observation of Eaton et al. (18), tropomyosin inhibited muscle actin-activated HMM ATPase by more than 60% (Fig. 4). It was quite unexpected, however, that, under conditions where binding did occur, tropomyosin had the opposite effect on the Acanthamoeba actin-activated HMM ATPase. Thus, at 25 μM actin and 80 mM KCl, conditions that are favorable for binding between tropomyosin and Acanthamoeba actin, tropomyosin stimulated the actin-activated HMM ATPase more than 2-fold at maximum activation (Fig. 5). In contrast, under identical conditions, tropomyo-
activated HMM ATPase activity. This is somewhat greater than the 33% previously reported (7), probably because of the higher concentration of Mg\(^{2+}\) in the present experiments. As expected, both the Acanthamoeba and muscle actin-activated HMM ATPase were strongly inhibited by the presence of 80 mM KCl despite the 5-fold increase in actin concentrations in these experiments.

**DISCUSSION**

It is clear from the present study that the binding of muscle tropomyosin to Acanthamoeba actin is much weaker than its binding to muscle actin. The most striking difference, however, is that when tropomyosin does bind to Acanthamoeba actin it stimulates the Acanthamoeba actin-activated HMM ATPase, whereas, under the same conditions, tropomyosin inhibits the muscle actin-activated HMM ATPase. Previously, tropomyosin had been shown to stimulate the muscle actin HMM or actomyosin ATPases only under very different conditions than the experiments with 80 mM KCl, 5 mM MgCl\(_2\), 2 mM ATP, 1 mM EGTA, and 2 mM imidazole, pH 7.0.

**TABLE I**

Comparison of activities of muscle and Acanthamoeba actin-activated heavy meromyosin ATPases in presence and absence of tropomyosin and KCl.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Muscle actin</th>
<th>Acanthamoeba actin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-1M</td>
<td>+1M</td>
</tr>
<tr>
<td>0 mM KCl, 5 (\mu)M actin</td>
<td>0.79</td>
<td>0.92</td>
</tr>
<tr>
<td>80 mM KCl, 25 (\mu)M actin</td>
<td>0.22</td>
<td>0.08</td>
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</tbody>
</table>

The mechanisms of the inhibitory and the stimulatory effects of tropomyosin on the muscle actomyosin ATPase are not currently understood. Huxley and his colleagues (33–35) have proposed that tropomyosin inhibits in situ by physically blocking the interaction of actin and myosin in relaxed muscle and moves away from the blocking position in actively contracting muscle. Thus, tropomyosin would occupy two positions on the actin filament: the "on" position, when the myosin ATPase is activated by actin, and the "off" position, when no activation occurs. If this model were applicable to the situation in vitro, our observation that tropomyosin inhibits the muscle actin-activated HMM ATPase by 60% could only be explained by postulating that tropomyosin can occupy an intermediate position on the actin. Another difficulty with the steric blocking model is that it does not adequately explain either the stimulation of the actin-activated HMM ATPase or the related finding that the binding of HMM to actin strengthens the binding of tropomyosin to actin, an effect that has been observed with both muscle actin (28) and Acanthamoeba actin (Fig. 2). In addition, the finding (22) that, in the absence of tropomyosin, the complex of troponin-I and tropomin-T, at relatively low ratios to actin, inhibits the actin-activated HMM ATPase activity also suggests that the mechanism of action of the tropomyosin-troponin complex may be more complex than a simple shift of the position of the tropomyosin on the actin.

An alternative possibility is that tropomyosin regulates the actin-myosin interaction by inducing conformational changes in the F-actin filaments. The various effects of tropomyosin could then be explained by postulating that actin monomers can be induced to assume different conformational states depending on the source of the actin and the conditions of the interaction. In support of this notion are the observations of Yanagida et al. (36) who found changes in the linear ultraviolet dichromism of muscle thin filaments in the presence and absence of Ca\(^{2+}\) and the indirect evidence from Poo and Hartshorne (37) that showed that, when the actin conformation is "fixed" with glutaraldehyde, the actin filament is no longer regulated by the tropomyosin-troponin complex, even though it binds the complex normally.

Whatever the mechanism of action of tropomyosin, it is clear from our data that it interacts differently with muscle and Acanthamoeba actin. This difference in tropomyosin binding must be related to a structural difference between the two actins. Thus far, the amino acid sequence analyses have revealed only a few conservative differences between Acanthamoeba and muscle actin (8). When these studies are complete, they may provide interesting insights into the mechanism of interaction of tropomyosin and actin.

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