Cooperative Binding of Tropomyosin to Muscle and Acanthamoeba Actin*

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The association of tropomyosin with F-actin presents an interesting problem in protein-protein interaction. The 40-nm-long tropomyosin molecules appear to lie end-to-end along the two grooves of the F-actin filament (1) and to span the entire length of the actin filament with each tropomyosin molecule interacting with seven consecutive actin monomers (2, 3). In a complex system such as this, it is of interest to examine if the tropomyosin molecules bind independently or cooperatively along the F-actin filament, especially in view of the regulatory role of this protein. Although Drabikowski et al. (4) reported independent binding of tropomyosin to low and high affinity binding sites on F-actin, recent evidence suggests that a cooperative interaction of tropomyosin molecules along the F-actin filament may be of importance in determining the behavior of the regulated F-actin filaments. A. Weber and her colleagues (5–7) have demonstrated that F-actin in a complex with tropomyosin or troponin-tropomyosin shows a cooperative interaction with HMM1 or S-1 in the presence of ATP. This cooperativity could involve the interaction of adjacent tropomyosin molecules along the F-actin filament. It has also been shown (8, 9) that the removal of a few amino acid residues from the COOH terminus of tropomyosin results in a molecule that is no longer capable of end-to-end association. This nonpolymerizable tropomyosin still combines with F-actin and troponin. However, the resulting complex exhibits a more gradual response to changing Ca2+ concentration in the superprecipitation reaction than does normal regulated F-actin (8). Finally, Poo and Hartshorne (10) recently found that cross-linking F-actin with glutaraldehyde completely prevents the bound troponin-tropomyosin complex from regulating the interaction of F-actin with HMM, in the presence of ATP. These data suggest that a cooperative interaction in the F-actin filament might be involved in the action of the troponin-tropomyosin complex.

In the present study, we investigated the binding of tropomyosin to F-actin under varied conditions. We found that, under all conditions tested, the binding of tropomyosin to F-actin shows positive cooperativity. Increasing concentrations of Mg2+, KCl, HMM, or S-1 increased the binding affinity of tropomyosin to muscle or Acanthamoeba F-actin but, in all cases, the binding remained cooperative. In an attempt to determine whether the structure of F-actin itself affected this cooperativity, we studied the binding of tropomyosin to F-actin cross-linked with glutaraldehyde. However, in contrast to the results of Poo and Hartshorne (10), we were unable to find any condition where tropomyosin was able to bind to F-actin cross-linked with glutaraldehyde.

MATERIALS AND METHODS

Preparation of Proteins—Actin, myosin, and heavy meromyosin were prepared from rabbit skeletal muscle as previously described (11). Myosin subfragment-1 was prepared by the method of Weeds and Taylor (12). Tropomyosin, which is free from troponin contamination, was isolated and purified according to the procedures described in our last report (11). Acanthamoeba actin was prepared by the procedures of Gordon et al. (13).

Binding of Tropomyosin to Actin—The binding of 125I-labeled tropomyosin to actin was measured by the amount of radioactivity sedimentable with F-actin according to previous procedures (11). Except when the effect of HMM or S-1 was investigated, all reactions were carried out at 25°C in the presence of 1 mM EGTA, 2 mM ATP, and 2 mM imidazole, pH 7.0. ATP was omitted when HMM or S-1 was added. In the computation of the binding data, corrections were made for small fractions of actin, and of tropomyosin, that presumably did not participate in binding. The correction for actin, about 5%, was determined by measuring the amount of actin that did not sediment in the absence of tropomyosin. For tropomyosin, the correction was less than 5%; it represented the fraction that sedimented in the absence of actin.

Determinations of Protein Concentration and ATPase Assays—The concentration of protein was determined spectrophotometrically using the following extinction coefficients: 638 cm-1/g at 290 nm for F-actin, 647 cm-1/g at 280 nm for HMM (14), 700 cm-1/g at 280 nm for S-1 (15), and 290 cm-1/g at 278 nm for tropomyosin (16). The molecular weights used for the calculation of the molarity of these proteins were 42,000 for actin, 350,000 for HMM, 120,000 for S-1, and 68,000 for tropomyosin. The concentration of the cross-linked actin, as well as the unmodified actin that served as its control, was determined by the method of Lowry et al. (17). Actin-activated HMM ATPase activity was determined by the pH-stat method (11). The activity contributed by HMM alone was subtracted from each assay.

Cross-linking of F-actin with Glutaraldehyde—Muscle actin so-

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1 The abbreviations used are: HMM, heavy meromyosin; S-1, myosin subfragment-1; EGTA, ethylene glycol bis(β-aminoethyl ether) N,N,N’,N’-tetraacetic acid; 1M, tropomyosin; SDS, sodium dodecyl sulfate.
RESULTS

Cooperative Binding of Tropomyosin to Muscle Actin in the Presence of $Mg^{2+}$—As we previously showed (19), the binding of tropomyosin to actin is very dependent on $Mg^{2+}$ concentration. At a given tropomyosin concentration, no binding occurs unless the $Mg^{2+}$ concentration exceeds a threshold level, and then a small increment in $Mg^{2+}$ concentration results in a sharp increase in binding until, at an optimal level of $Mg^{2+}$, the binding is complete. An example of this effect is shown by the solid circles in Fig. 4. The threshold and optimal $Mg^{2+}$ concentrations are greater for Acanthamoeba actin than for muscle actin but, at saturation, the stoichiometry of binding for both actins is the same: seven actin monomers per tropomyosin (19).

When 2 mM ATP and 4.6 mM MgCl$_2$ are present, 5 $\mu$M F-actin is completely saturated with tropomyosin when the added tropomyosin concentration is 1.25 $\mu$M. Accurate measurement of the free tropomyosin concentration requires somewhat weaker binding of tropomyosin to F-actin. Therefore, in the binding experiment shown in Fig. 1, the $Mg^{2+}$ concentration was maintained at 4.4 mM. Our results show that, under this condition, when the actin concentration is held constant at 5 $\mu$M and the tropomyosin concentration is varied, a sigmoidal binding curve is obtained. Therefore, under this condition, the binding of tropomyosin to F-actin appears to show positive cooperativity. This is confirmed by the Scatchard plot shown in the inset of Fig. 1. Schwarz (20) has shown that when positive cooperativity is present the Scatchard plot will be a convex curve which passes through a maximum. The apparent or mean binding constant, $K_{app}$, is then calculated as 1/(free tropomyosin concentration) at the point where the (bound tropomyosin)/(added actin concentration, $v$) is half its maximal value. The Scatchard plot shown in the inset of Fig. 1 is very convex, confirming that under this condition the binding of tropomyosin to F-actin is highly cooperative. The value of $K_{app}$ under this condition is about 1.5 x 10$^4$ M$^{-1}$.

Effect of KCl on the Binding of Tropomyosin to Muscle and Acanthamoeba Actin—Although almost no tropomyosin binds to Acanthamoeba actin at 5 mM $Mg^{2+}$, the addition of 80 mM KCl allows binding to occur to an appreciable extent, approaching that of muscle actin. The open symbols in Fig. 2a show that the addition of 80 mM KCl increases $K_{app}$ to 3.8 x 10$^4$ M$^{-1}$. Despite the stronger binding constant, however, the highly curved Scatchard plot demonstrates that the binding of tropomyosin to muscle actin is still highly cooperative.

Induction of the Binding of Tropomyosin to Acanthamoeba Actin by Myosin Subfragments—We showed (11) previously that Acanthamoeba actin, which did not bind tropomyosin at 5 mM $Mg^{2+}$, became saturated with tropomyosin when HMM was added at a ratio of one myosin head per actin monomer. Under these conditions, since ATP is absent, all of the actin monomers would be associated with HMM as acto-HMM, or rigor complexes.

In the present study, we examined the binding of tropomyosin to the rigor complexes at 2.5 $\mu$M HMM and 5 $\mu$M actin (Fig. 3). The presence of HMM bound to the Acanthamoeba F-actin strengthened the tropomyosin binding even more than the addition of 80 mM KCl; the apparent binding constant had a value of about 9.0 x 10$^4$ M$^{-1}$. Myosin heads, therefore, induce the binding of tropomyosin to Acanthamoeba actin by strongly shifting the equilibrium in favor of association. The tropomyosin binding still appears to be cooperative. However, the Scatchard plot is not as curved as under the other conditions where we studied the binding. Thus, the strong binding of tropomyosin induced by the bound HMM seems to be accompanied by a reduced cooperative interaction between the tropomyosin molecules as they bind along the F-actin filament.

Cross-linked Muscle Actin and Its Interaction with Tropomyosin and Myosin Subfragments—One possible mechanism for the cooperative interaction of tropomyosin with F-actin is a cooperative conformational change occurring along the F-actin filament. Ando and Asai (21) have presented evidence suggesting that the binding of HMM to actin induces a cooperative conformational change in the actin filament. If such a cooperative conformational change were involved in the binding of tropomyosin, immobilization of actin filaments by cross-linking the actin monomers might abolish the cooperative nature of the tropomyosin binding. We used glutaraldehyde as the cross-linker since it has been reported (10) that muscle actin cross-linked with glutaraldehyde retains its ability to activate the myosin or S-1 ATPase and also to bind tropomyosin or the tropo-actin-tropomyosin complex, although the bound troponin-tropomyosin has no effect on the actin-activated ATPase.

Table I confirms the earlier observation of Poo and Hartshorne (10) that glutaraldehyde-cross-linked actin is fully competent to activate the HMM ATPase and that this activation is not affected by tropomyosin. However, in contrast to the results of Poo and Hartshorne (10), we found that the reason tropomyosin does not affect the actin-activated ATP-
Cooperative Binding of Tropomyosin to Actin

FIG. 2. Effect of KCl on the binding of tropomyosin to muscle and Acanthamoeba actin. Reactions were carried out in the presence of 5 mM MgCl₂, 80 mM KCl, 2 mM ATP, 1 mM EGTA, and 2 mM imidazole, pH 7.0. In a, the concentrations of muscle actin (solid symbols) and Acanthamoeba actin (open symbols) were 25 μM. In b, 5 μM of Acanthamoeba actin was used.

FIG. 3. Effect of HMM on the binding of tropomyosin to Acanthamoeba F-actin. The reaction mixture contained 5 mM MgCl₂, 1 mM EGTA, 2 mM imidazole, pH 7.0, and no ATP. It also contained 5 μM actin, 2.5 μM HMM, and varied concentrations of tropomyosin.

TABLE I
Effect of tropomyosin on the ability of cross-linked actin to activate the HMM ATPase

<table>
<thead>
<tr>
<th>Conditions: 5 mM MgCl₂, 2 mM ATP, 2.5 mM KCl, 1 mM EGTA, 2 mM imidazole, pH 7.0, 0.2 mg/ml of HMM, and 2.5 μM tropomyosin.</th>
<th>ATPase activity (μmol P₃/mg HMM/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control actin</td>
<td>0.84</td>
</tr>
<tr>
<td>Cross-linked actin**</td>
<td>1.06</td>
</tr>
</tbody>
</table>

* By SDS-electrophoretic gel analysis, about 60% of the actin subunits were cross-linked and 40% were unmodified. The high specific activity relative to that of normal actin may be an artifact derived from the estimation of protein concentration by the method of Lowry et al. (17); the cross-linked actin may interact differently than unmodified actin with the Folin reagent.

FIG. 4 (left). Binding of tropomyosin to glutaraldehyde-cross-linked muscle F-actin. The reaction was carried out in 0.125 mM ATP, 1 mM EGTA, 2 mM imidazole, 2.5 mM KCl, and varied concentrations of Mg²⁺. Each reaction mixture was carefully adjusted to pH 7.0. The reaction mixture also contained 1.2 μM tropomyosin, and either 5 μM unmodified actin (○) or 5 μM glutaraldehyde-cross-linked actin (◊). In experiments to test the effect of S-1, the reaction mixture contained 5 μM unmodified actin (○) or 5 μM glutaraldehyde-cross-linked actin (◊) and 1.2 μM tropomyosin with no ATP present. Fig. 5 (right). Binding of tropomyosin to cross-linked muscle actin at 0.1 M KCl. The reaction contained 0.1 M KCl, 0.5 mM ATP, 1 mM EGTA, 2 mM imidazole, pH 7.0, 1.2 μM tropomyosin, and either 5 μM unmodified actin (○) or 5 μM glutaraldehyde-cross-linked actin (◊).

However, our binding studies were performed in the absence of KCl whereas Poo and Hartshorne (10) performed their experiments at 0.1 M KCl. We, therefore, repeated our experiment at 0.1 M KCl (Fig. 5). Our results show that even at 0.1 M KCl, the binding is very poor. When the ratio of tropomyosin to actin was 0.5, the binding of tropomyosin to the cross-linked actin was only 17% of the control value. A similar experiment carried out at 3 mM Mg²⁺ with cross-linked F-actin resulted in a binding curve identical with the curve shown in Fig. 5. Therefore, neither Mg²⁺, KCl, or S-1 were able to induce tropomyosin to bind to the glutaraldehyde-cross-linked F-actin.

DISCUSSION

The results of our Scatchard plot analysis, showing highly cooperative binding of tropomyosin to F-actin, differ from the results of Drabikowski et al. (4), who used a similar analysis but concluded that F-actin contained low and high affinity tropomyosin binding sites. However, whereas most of their binding data were collected with tropomyosin concentrations...
no more than 3.5-fold in excess of what was required to saturate actin, the bulk of the binding data of Drabikowski et al. (4) were obtained at much higher concentrations where large errors in the measurement of binding are to be expected. Therefore, we think that our finding of positive cooperativity is valid although the mechanism of the positive cooperativity remains unclear.

The occurrence of this strong positive cooperativity means that the binding of the first few tropomyosin molecules along the F-actin filament is much weaker than the binding of the subsequent molecules. This phenomenon may be related to the ability of the tropomyosin molecules to associate end-to-end with each other along the F-actin filament. Such end-to-end association could have the effect of greatly increasing the binding strength of tropomyosin molecules which bind not only to F-actin but also to adjacent tropomyosin molecules. The major effect of Mg^{2+}, KCl, and S-1 would then be to increase the affinity of the first few tropomyosin molecules which bind to F-actin.

If the positive cooperativity we observe were due to end-to-end association of tropomyosin along F-actin, it might be independent of conformational changes occurring in the F-actin filament, itself. We attempted to test whether this was the case by investigating the binding of tropomyosin to F-actin cross-linked with glutaraldehyde. However, in contrast to the results of Poo and Hartshorne (10), we could not find any conditions where tropomyosin was able to bind to the cross-linked F-actin. It is not clear why our results differ from those of Poo and Hartshorne (10). Since it is now possible to prepare tropomyosin which shows no end-to-end association (8, 9), it should be possible to test directly whether end-to-end association is involved in the cooperative binding of tropomyosin. Such studies may also clarify whether cooperative effects of tropomyosin on the acto-HMM ATPase are related to the cooperative binding which we observed in the present study.

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