Cooperative Interactions between Adjacent Troponin-Tropomyosin Complexes May Be Transmitted through the Actin Filament*

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We have recently demonstrated that troponin does not strengthen interactions between adjacent, actin-bound tropomyosin molecules (8). In addition, lack of tropomyosin amino-terminal acetylation, which weakens tropomyosin binding to actin (9–11), has little or no effect on the cooperative interactions involved in thin filament assembly (12). These results suggest that the primary source of these interactions may not be direct contacts between neighboring tropo- 

nyosin-tropomyosin molecules; indirect interactions involving the actin lattice may be critical. To continue this line of investigation, we now describe the properties of carboxy-peptidase A-digested tropomyosin, previously termed non-polymerizable tropomyosin (13), which lacks 11 carboxyl-terminal amino acids. The term non-polymerizable tropomyosin will not be used in the present report, because both unacetylated tropomyosin and carboxypeptidase A-digested tropomyosin polymerize poorly. Similar to the results testing the effects of troponin and the effects of absent tropomyosin amino-terminal acetylation, altering the tropomyosin carboxyl terminus has only modest effects on the cooperativity of thin filament assembly.

This report also describes the effect on thin filament assembly of combined amino- and carboxyl-terminal alterations; unacetylated tropomyosin was digested with carboxypeptidase A. Surprisingly, the effects of the two alterations were not additive. Lack of amino-terminal acetylation had no further effect on the binding of carboxyl-terminal-digested tropomyosin (in the presence of troponin) to an isolated site on the actin filament, despite a 10–40-fold effect of acetylation on full-length tropomyosin (12). Furthermore, binding of the doubly modified tropomyosin to actin (in the presence of troponin) remained highly cooperative.

EXPERIMENTAL PROCEDURES

Protein Purification—Rabbit fast skeletal muscle actin (14) and tropomyosin (15) and bovine cardiac troponin and tropomyosin (16) were obtained as previously described. Unacetylated tropomyosin was purified as bacterially expressed rat skeletal muscle α-tropomyosin (12), using the pET3d vector developed by Studier (17). Skeletal muscle and cardiac muscle α-tropomyosins have identical amino acid sequences (18, 19).

Carboxypeptidase A (Calbiochem) digestion of tropomyosin, monitored by SDS-polyacrylamide gel electrophoresis (20) and by amino acid analysis, was carried out to completion (removal of 11 amino acids) (13). Digestion of bovine cardiac tropomyosin was conducted after prior treatment with alkaline phosphatase, followed by isoelec- 

tric point precipitation (21). The carboxypeptidase A was added in a 1:50 ratio to tropomyosin, and the two proteins were then incubated in 10 mM KPO4 (pH 7.5), 100 mM KCl at 23 °C for 16 h. The digest was chromatographed over a Sephacryl S200 column. To digest 11 amino acids from unacetylated tropomyosin in a similar manner, carboxypeptidase A was added in a 1:100 ratio to tropomyosin under the same conditions as above, and after 4 h an equal amount of fresh enzyme was added. The incubation was then continued for another 12 h. Amino acid analysis on a Beckman model 6300 high performance amino acid analyzer confirmed stoichiometric removal of 11 residues,

Recent analyses of the assembly of thin filaments containing altered forms of troponin (or no troponin) suggested that the strongly cooperative nature of troponin-tropomyosin binding to actin might be primarily caused by indirect interactions involving the actin lattice, rather than by direct contacts between neighboring troponin-tropomyosin molecules. To test this hypothesis, thin filament assembly was examined using either cardiac tropomyosin digested with carboxy-peptidase A (cbpTm) or a tropomyosin with defective function at both amino and carboxyl termini (unacetylated cbpTm). Compared to intact troponin-tropomyosin, both tropo- 

myosin-cbpTm and tropomin-unacetylated cbpTm had much weaker binding to actin; however, cooperative interactions were only slightly reduced. These data support the implication that the primary source of the cooperativity involves tropinin-tropo- 

myosin-promoted conformational changes within the actin polymer.

Surprisingly, the effects of tropomyosin amino- and carboxyl-terminal structural defects on tropo- 

nyosin binding to actin were not additive. In the presence of troponin, tropomyosin molecules with either defect had the same diminution in actin affinity as molecules with both defects. Finally, the Ca++ sensitivity of tropinin-tropomyosin binding to actin was increased by alteration of either end of tropomyosin.

The regulatory proteins troponin and tropomyosin bind to the actin filament in a 1:1:7 troponin:tropomyosin:actin ratio (1–5). By positioning the troponin-tropomyosin complexes precisely seven actin molecules apart, there will be no gaps of unregulated actin between tropomyosins. Therefore, thin filament assembly must include energetic effects that strongly promote tropomyosin’s proper positioning along the thin filament. Experimental evidence for this idea includes the pattern of equilibrium binding of tropomyosin or troponin-tro- 

pomyosin to actin; the shape of such binding isotherms is dictated by the strength of interactions between adjacent ligands along the thin filament. Indeed, binding data indicate that adjacent tropomyosins or tropomin-tropomyosins strongly interact (6–8). To understand how these interactions are mediated, this report characterizes the assembly of thin filaments containing altered forms of tropomyosin.

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stopping at a Glu-Glu carboxyl-terminal pair. The digestion was terminated by precipitation of the tropomyosin at pH 4.6, and the resuspended pellet was purified over Sephacryl S200.

Assays—To measure binding of tropomyosin to actin, each of the various tropomyosins was stoichiometrically labeled on Cys-190 with [3H]iodoacetic acid (Du Pont-New England Nuclear), as previously described (8). The specific activity of the labeled tropomyosins ranged between 1.4 \times 10^4 and 4.8 \times 10^4 cpm/mmol. Binding experiments were performed at room temperature in the presence of 10 mM imidazole (pH 7.5), 0.2 mM dithiothreitol, 0.3 ng/ml bovine serum albumin, and between 60 and 300 mM KCl. 180-μl samples were incubated for 30 min, transferred to microcentrifuge tubes, duplicate 15-μl aliquots removed for liquid scintillation counting, and centrifuged for 20 min at 30 p.s.i. in a TL.100 Beckman Airfuge rotor, after which supernatant aliquots were counted.

Binding curves were analyzed as a linear lattice problem (22, 23) with the ligand (troponin-tropomyosin) spanning seven binding sites (actin monomers) on the lattice. Tropomyosin was added in stoichiometric excess over tropomyosin, enough to saturate the tropinin-induced promotion of tropomyosin-actin binding as determined by data in Fig. 1 (see "Results"). Based upon this evidence, the modeling employs the approximation that all of free tropomyosin, as well as the actin-tropomyosin complex, is associated with troponin. Nonlinear least squares curve fitting was performed using the program MATLAB (Mathworks, Inc.). The data were fit to the linear lattice binding equation (12, 24), which relates the bound ligand concentration to the free ligand concentration, depending upon four parameters: the number of lattice sites (seven in this case) covered by each ligand, the lattice (F-actin) concentration, the affinity (K_i) of troponin-tropomyosin for an isolated site on the actin filament, and the -fold increase in affinity (\gamma) when binding occurs adjacent to one already bound ligand.

RESULTS

Effect of Ionic Strength on the Ability of Tropinin to Promote Actin Binding of Carboxypeptidase A-treated Tropomyosin—To quantitatively compare properties of intact tropomyosin and carboxypeptidase A-digested tropomyosin (cbpTm), it would be preferable to study both molecules under identical conditions. Unfortunately, this could not be accomplished with respect to thin filament assembly. High ionic strength (>300 mM KCl) is required when measuring the binding of tropinin-tropomyosin to actin using intact tropomyosin (8). This is because tropinin-tropomyosin tends to polymerize in the presence of lower ionic strength, even in the absence of actin. On the other hand, as shown below, troponin-cbpTm binding to actin can only be studied in the presence of 300 mM KCl.

Fig. 1 shows tropinin-induced binding of cbpTm to actin. Data were obtained in the presence of KC1 concentrations ranging between 60 and 300 mM, and in the presence of <10^{-8} M free Ca^{2+}. For all of these ionic strengths, there was negligible binding of cbpTm to actin in the absence of tropinin. The ratio of troponin to tropomyosin was high enough to saturate tropinin-tropomyosin to actin using intact tropomyosin (8). The proportion of unbound tropomyosin to actin in the presence of ionic strength that varied the proportion of free tropomyosin, as well as the actin-tropomyosin complex, is associated with troponin. Nonlinear least squares curve fitting was performed using the program MATLAB (Mathworks, Inc.). The data were fit to the linear lattice binding equation (12, 24), which relates the bound ligand concentration to the free ligand concentration, depending upon four parameters: the number of lattice sites (seven in this case) covered by each ligand, the lattice (F-actin) concentration, the affinity (K_i) of troponin-tropomyosin for an isolated site on the actin filament, and the -fold increase in affinity (\gamma) when binding occurs adjacent to one already bound ligand.

Fig. 1 indicates that the higher the ionic strength, the more tropinin did not saturate in the presence of 300 mM KCl. Conditions were as described as under "Experimental Procedures" with the addition of 0.5 mM Br_2BAPTA.

does not indicate saturation of all the sites on the actin filament, but rather saturation of the effect of tropinin.) These data imply relatively weak affinity of troponin for cardiac tropinin. In agreement with previous reports (25, 26), similar data were also obtained in the presence of 30^{-4} M Ca^{2+}. Ca^{2+} lowered the plateau levels of tropomyosin binding but did not change the tropinin concentrations required to reach saturation (data not shown).

Fig. 1 indicates that the higher the ionic strength, the more tropinin was required to induce cbpTm-actin binding, particularly for cardiac tropinin. In agreement with previous reports (27, 28), this suggests that tropinin binding to tropo-
myosin is inhibited by increasing ionic strength. It also suggests that it will be difficult to study the affinity of the troponin-cbpTm complex for actin at KCl concentrations much higher than 120 mM for cardiac troponin and 180 mM for skeletal muscle troponin. At higher KCl concentrations one cannot be certain that troponin binds to cbpTm; failure of troponin to cause cbpTm-actin binding could be due to weak affinity of troponin for cbpTm, rather than weak affinity of troponin-cbpTm for actin.

**Linear Lattice Analysis of Skeletal Muscle Troponin-cbpTm**

**Binding to Actin—** Binding of the troponin-cbpTm complex to actin in the presence of 60 mM KCl is shown in the representative experiment in Fig. 2. The solid lines are best-fit theoretical curves, based upon the linear lattice equation (12, 24). Binding was much weaker in the presence of 10^{-4} M Ca^{2+} (circles) than in the absence of Ca^{2+} (squares). Both binding curves are highly cooperative, although the scale of the abscissa permits easy visualization of the S-shaped pattern only in the presence of Ca^{2+}. Despite removal of 11 carboxyl-terminal residues from tropomyosin, binding of the troponin-cbpTm complex to actin remained a very cooperative process; binding adjacent to an already bound molecule was 35-fold more likely than binding to an isolated site (Table I; average value for $y = 35$). This agrees with the results of Heeley et al. (26), who also found that troponin-cbpTm bound to actin cooperatively. The quantitative agreement between Table I and this previous report is reasonably good; applying the above, direct comparison of cbpTm to these other tropomyosins under one set of experimental conditions was not possible. Nevertheless, carboxypeptidase digestion of tropomyosin greatly weakens troponin-tropomyosin binding to an isolated site on the actin filament. This conclusion applies both in the presence of tropinin (above) and in its absence; cbpTm binds very poorly to actin in the absence of tropinin (Table I).

**Amino-terminal Acetylation Has No Further Effect on Binding of Troponin-cbpTm to Actin—** To further assess the roles of the amino- and carboxyl-terminal ends of tropomyosin on thin filament assembly, dually defective tropomyosin (ua-

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**Table 1: Assembly of thin filaments containing altered tropomyosin**

<table>
<thead>
<tr>
<th>$K_e$</th>
<th>$pCa_{Ca^{2+}}$</th>
<th>$y$</th>
<th>$y_{K_e}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$&gt; 8$</td>
<td>$= 4$</td>
<td>$&gt; 8$</td>
</tr>
<tr>
<td>60 mM KCl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>uaTm + S Tn</td>
<td>340</td>
<td>125</td>
<td>97</td>
</tr>
<tr>
<td>cbpTm + S Tn</td>
<td>420</td>
<td>48</td>
<td>32</td>
</tr>
<tr>
<td>ua-cbpTm + S Tn</td>
<td>240</td>
<td>83</td>
<td>46</td>
</tr>
<tr>
<td>cbpTm + C Tn</td>
<td>240</td>
<td>100</td>
<td>42</td>
</tr>
<tr>
<td>uaTm</td>
<td>$&lt; 0.2$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cbpTm</td>
<td>$&lt; 0.3$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tropomyosin</td>
<td>17</td>
<td>90</td>
<td>1.5</td>
</tr>
<tr>
<td>120 mM KCl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cbpTm + S Tn</td>
<td>470</td>
<td>20</td>
<td>15</td>
</tr>
<tr>
<td>ua-cbpTm + S Tn</td>
<td>170</td>
<td>28</td>
<td>28</td>
</tr>
<tr>
<td>cbpTm + C Tn</td>
<td>67</td>
<td>55</td>
<td>30</td>
</tr>
<tr>
<td>180 mM KCl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cbpTm + S Tn</td>
<td>32</td>
<td>(10)</td>
<td>43</td>
</tr>
<tr>
<td>ua-cbpTm + S Tn</td>
<td>74</td>
<td>23</td>
<td>1.7</td>
</tr>
</tbody>
</table>

| $^{6,4}$ Ref. 12. |       |       |       |       |       |
| $^{6}$ Estimated from experiments with high actin concentrations, as in Ref. 12. |       |       |       |       |       |
| $^{6}$ Assumus $y = 30$, the mean of the values at lower ionic strength. |       |       |       |       |       |
cbpTm) was prepared by digesting unacetylated tropomyosin with carboxypeptidase A. Binding of troponin-ua-cbpTm to actin is illustrated by the representative experiment in Fig. 3. Remarkably, the affinity of this complex for actin, both in the presence (circles) and in the absence (squares) of Ca\(^{2+}\), was indistinguishable from the actin-affinity of troponin-cbpTm. The 40-fold effect of tropomyosin amino-terminal acetylation on actin affinity (12) was completely abolished by removal of 11 residues at the opposite end of this extended coiled coil molecule (11, 29). For example, Table I shows that the overall affinity constant \(y_K\) which can be determined with greater precision than either \(y\) or \(K_o\) was (in the presence of Ca\(^{2+}\)) either \(1.9 \times 10^8\) M\(^{-1}\) using cbpTm or \(1.8 \times 10^8\) M\(^{-1}\) using ua-cbpTm. Similarly, acetylation made no difference in the absence of Ca\(^{2+}\), with values of \(y_K\) equaling 1.3 or 1.1 \(\times 10^8\) M\(^{-1}\), depending upon whether the tropomyosin was acetylated.

One possible explanation for these observations was that tropomyosin acetylation had no effect on troponin-tropomyosin binding to actin in the presence of 60 mM KCl but had a strong effect in the previous investigation (12) because it was conducted in the presence of higher ionic strength (300 mM KCl). If this were true, the identical results for cbpTm and ua-cbpTm might be a peculiarity of the ionic conditions of Figs. 2 and 3. This possibility is excluded, however, by data included in Fig. 4. Comparison of the filled circles and filled triangles indicates that troponin-cbpTm and troponin-ua-cbpTm have indistinguishable overall binding constants for actin, regardless of ionic strength. Therefore, the observation is generalizable; the strong effect of tropomyosin amino-terminal acetylation on actin binding is eliminated by removal of amino acids at the tropomyosin carboxyl terminus.

**Effect of Ca\(^{2+}\) on the Affinity of Troponin-cbpTm for Actin—** Comparison of the filled and open symbols in Fig. 4A shows the substantial effect of Ca\(^{2+}\) on the actin affinity of troponin-tropomyosin after carboxypeptidase A digestion of the tropomyosin. Removal of Ca\(^{2+}\) from troponin increases \(y_K\) by an order of magnitude. This was not an artifact due to weak binding of troponin to cbpTm in the presence of Ca\(^{2+}\); higher troponin concentrations did not influence the result (data not shown). An effect of Ca\(^{2+}\) on troponin-cbpTm binding to actin has been recently reported (26, 30) and is consistent with models for thin filament regulation in which Ca\(^{2+}\) binding to TnC alters several binary interactions between thin filament proteins, including TnC-TnI, TnI-actin, and TnT-tropomyosin (4, 5, 25, 26, 31-34). Notably, the magnitude of the effect in Fig. 4A (8-fold) is greater than seen for unacetylated tropomyosin (4-fold; Table I) and much larger than we recently reported for acetylated tropomyosin with normal COOH and NH\(_2\) termini (only 2-fold) (12).

To determine the significance of this relatively large effect of Ca\(^{2+}\) we considered two issues. The greater effect seen in the presence of cbpTm cannot be explained by the higher
Cooperative Interactions within the Actin Filament

FIG. 5. Schematic illustrations of the mechanism for cooperative thin filament assembly and of the implications of non-additive NH₂- and COOH-terminal defects in tropomyosin. The cooperativity parameter, \( y \), is a quasi-equilibrium constant describing the repositioning of an isolated tropinin-tropomyosin to a position adjacent to another tropinin-tropomyosin. \( y \) remains large despite combined defects in both the NH₂ and COOH termini of tropomyosin (present work) and is not decreased by removal of either tropinin or the portion of TnT spanning the tropinin-tropomyosin overlap joint (8, 12). Therefore, it is now proposed that tropinin-tropomyosin causes a conformational change (schematically represented by filled versus open circles) in actin and that differences in actin-actin contacts (arrows) are the primary source for cooperative tropinin-tropomyosin binding to actin.

日内 scientists have considered the specificity of the observation for skeletal muscle tropinin. Cardiac and skeletal muscle tropinins have significant sequence differences in all three subunits (35-40), and Ca²⁺ has only a slight effect on cardiac tropinin binding to actin-tropomyosin (41). Fig. 4B shows that Ca²⁺ had much less effect on the binding of cardiac tropinin-tropomyosin to actin than it did on skeletal muscle tropinin-tropomyosin to actin. There was, in fact, no effect of Ca²⁺ on yKₑ when unaltered cardiac tropomyosin was used (filled and open diamonds). However, the figure also indicates a pattern in the cardiac tropinin data that resembles the skeletal muscle results; alterations in the amino or carboxyl termini of tropomyosin increased the effect of Ca²⁺ on tropinin-tropomyosin binding to actin.

DISCUSSION

The cooperativity parameter, \( y \), is a quasi-equilibrium constant for the process illustrated in Fig. 5: movement of a tropinin-tropomyosin complex from an isolated position to a position adjacent to another such complex. The intermolecular interactions responsible for this cooperativity are now proposed to be between actin molecules, as illustrated in the figure. This proposal arises from accumulating evidence against the alternative source of this cooperativity, interactions between neighboring tropinin-tropomyosin complexes. The high value of the cooperativity parameter, \( y \), persists after removal of the tropomyosin carboxyl terminus; Table I suggests it decreases only by a factor of about 0.5. Recent work also indicates that the nearest neighbor interactions are not increased by tropinin (8) and are independent of a properly functional tropomyosin amino terminus (12). These results imply that direct contacts between adjacent tropinin-tropomyosin complexes are not primarily responsible for cooperative thin filament assembly. The remaining explanation is that tropomyosin-actin binding produces conformational changes within the actin lattice itself, as illustrated in Fig. 5, and that these conformational changes in the actin monomers are primarily responsible for cooperative thin filament assembly. This idea has been raised previously (e.g. Refs. 10 and 26). We now conclude it to be the mechanism most consistent with current data. (Because actin-binding is too weak for \( y \) to be measured for either cbpTm or unacetylated tropomyosin in the presence of tropinin, we cannot determine whether this actin-mediated cooperativity is also present for tropomyosin-actin binding in the absence of tropinin.)

It should be emphasized that the analysis and conclusions in this paper depend upon the assumption that the tropinin concentration is near saturation and is not limiting. This is based upon numerous experiments such as those shown in Fig. 1. However, more comprehensive analysis of this multiprotein system would be facilitated by direct measurement of tropinin binding to tropomyosin and also by simultaneous determination of both tropinin and tropomyosin binding to actin. Methods are being developed to perform these measurements.

A surprising finding in this paper is that altering both the amino and carboxyl termini of tropomyosin did not have an additive effect on thin filament assembly, whereas the effect of either alteration separately was substantial. If tropomyosin carboxyl-terminal digestion and lack of amino-terminal acetylation had each inhibited tropinin-tropomyosin binding to actin by abolishing cooperativity, one could have easily rationalized the observation that simultaneous alteration of both ends had no additional effect. However, neither alteration had a major effect on cooperativity; the pattern of tropinin-tropomyosin binding to actin implied the existence of strong nearest neighbor interactions despite either (or both) of the tropinin alterations. Therefore, barring an undetected systematic error in the data, the explanation for the dually altered tropomyosin results is unlikely to be the absence of nearest neighbor cooperativity. Rather, either alteration seems to weaken binding of an individual tropinin-tropomyosin complex to actin fully as much as do both alterations. The mechanism for this observation is unclear.

The magnitude of the effect of Ca²⁺ in Fig. 4A has a parallel in a previous study of cooperative binding of myosin S-1 to thin filaments containing cbpTm (42). Pan et al. found that Ca²⁺ has a larger influence on myosin binding to the thin filament when tropomyosin lacks its carboxyl terminus. Normal tropomyosin and cbpTm are indistinguishable in the presence of Ca²⁺, but Ca²⁺ removal causes more inhibition of myosin binding when cbpTm was present. In other words, the absence of the tropomyosin carboxyl terminus makes the thin filament more difficult to activate and results in a greater effect of Ca²⁺. This is explained, in part, by an equilibrium shift toward a more inactive thin filament. Similarly, Walsh et al. (21) found that removal of the tropomyosin carboxyl terminus results in a thin filament requiring higher Ca²⁺ concentrations for myosin S-1 MgATPase activation and lower MgATPase rates in the presence of saturating Ca²⁺; thin filament activation is energetically less favorable.

These previous observations are supported by analogous results in the present investigation. The assembly of Ca²⁺-activated thin filaments containing cbpTm was energetically unfavorable (weak \( yKₑ \)). Therefore, removal of the tropomyosin carboxyl terminus seems to stabilize the inhibited state of the thin filament with respect to myosin binding, Ca²⁺ binding, and thin filament assembly. This suggests that the ends of tropomyosin promote muscle activation; they are anti-hibitory. This could be a direct effect; the tropomyosin ends, particularly the carboxyl terminus, may bind more strongly to actin when Ca²⁺ is bound to tropinin than when Ca²⁺ is absent. This proposed effect of Ca²⁺ would strengthen tropinin-tropomyosin binding to actin and oppose the Ca²⁺-induced diminution of the TnI-actin interaction.

Finally, it is worth noting that tropomyosin-tropomyosin binding to actin...
interactions have a significance beyond their role in thin filament assembly. Both physiological and biochemical experiments suggest that the very cooperative response of muscle contraction to the intracellular Ca²⁺ concentration involves longitudinal cooperativity along the thin filament (41-53); the response of the muscle fiber depends in part upon interactions between neighboring proteins within the thin filament. Significantly, many of these interactions critical for regulation are (in theoretical models) formally the same as the nearest neighbor interactions that govern thin filament assembly (41, 43, 44, 50). It is hoped that further structure-function studies of thin filament assembly will provide a better understanding of the regulatory properties of the assembled filament.

REFERENCES