Proteolytic Fragments of Troponin C

INTERACTIONS WITH THE OTHER TROPONIN SUBUNITS AND BIOLOGICAL ACTIVITY*

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Fragments of rabbit skeletal muscle Ca²⁺-binding subunit of troponin (TnC), obtained by cleavage with trypsin, thrombin, and CNBr, were tested for their ability to form binary and ternary complexes with ATPase inhibitory subunit (TnI) and tropomyosin-binding subunit (TnT) and their ability to replace TnC in reversing Tn inhibition of actomyosin ATPase activity. Three regions of TnC were found to be involved in interaction with TnI. Regions near Ca²⁺-binding sites I and II require Ca²⁺ for the interaction, while a third region near Ca²⁺-binding site IV binds TnI whether or not Ca²⁺ is present. The TnT binding site has been localized in the NH₂-terminal half of TnC. Several of the TnC fragments form soluble ternary complexes with TnI and TnT. Fragments that contain amino acid residues 89-100 and at least one pair of Ca²⁺-binding sites are able to reverse the TnI inhibition of actomyosin ATPase activity, which exhibits the same [Ca²⁺]₁/₂ regardless of which of the Ca²⁺-binding sites are present in the fragment.

An important step in the initiation of skeletal muscle contraction is the binding of Ca²⁺ to troponin C. The effects of the structural rearrangements induced in the latter by Ca²⁺ ions are transmitted to other components of the regulatory system, e.g., TnI, TnT, and tropomyosin, and in an as yet not fully understood way lead to the activation of myosin ATPase and tension development. There are four homologous domains within the amino acid sequence of skeletal muscle TnC, numbered I-IV from the NH₂ terminus, corresponding to four Ca²⁺-binding sites (1, 2). Each domain consists of a Ca²⁺-binding loop rich in Asp and Glu residues, flanked by two α-helical segments which in this paper will be designated with subscripts N and C, depending on whether their position in the sequence is closer to the NH₂ or COOH terminus of the molecule, respectively. Two of the sites, the so-called Ca²⁺-specific sites (I and II) have high affinity for Ca²⁺ (Kₐpp = 2 × 10⁶ M⁻¹) and also competitively bind Mg²⁺. The two others are the Ca²⁺-specific or low affinity sites (Kₐpp = 5 × 10⁷ M⁻¹) (3). A variety of studies including those using tryptic, thrombin, and cyanogen bromide peptides show that sites I and II are the low affinity sites and sites III and IV are the high affinity Ca²⁺-Mg²⁺ sites (4-6).

Binding of Ca²⁺ to the high affinity sites (III and IV) causes extensive conformational changes in that region, indicated by various physicochemical techniques (4, 6-11). Binding of Ca²⁺ to sites I and II is accompanied by minor structural changes, but various pieces of evidence point to the importance of these sites in the regulatory process itself (3, 6, 12). Although it is well established that TnC can form binary complexes with TnI and TnT and a ternary complex containing one molecule of each subunit (13, 14; also, for a dissenting view, cf. Ref. 15), information concerning precise sites of interaction is lacking. Previous studies with proteolytic fragments of TnC (4) have suggested at least two sites of interaction with TnI, a view supported by recent work on reactivity of Lys residues of TnC (16). Studies on TnI fragments (17) show that two regions of TnI interact with TnC. Little is known about the sites of interaction between TnC and TnT. Preliminary results suggested that TnT is bound to the COOH-terminal domain of TnC (4).

In this report, we present a systematic study on the interaction of TnC with other troponin subunits, using proteolytic fragments of TnC described in a previous paper (4) as well as a new set of peptides produced by tryptic digestion in the absence of Ca²⁺. The current data indicate that TnT binds to the NH₂-terminal half of TnC rather than to the region of the Ca²⁺-binding domain IV as concluded from earlier preliminary studies (4). The data suggest that there are three sites of interaction between TnC and TnI located in helical fragments N,N',N⁵-tetraacetic acid; SDS, sodium dodecyl sulfate; PIPES, 1,4-piperazine diethanesulfonic acid; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid).

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II_N, III_N, and IV_N. Interactions involving the first two sites appear to require Ca^{2+}, whereas involving site IV_N is Ca^{2+}-independent. A number of fragments form ternary complexes with one molecule each of TnI and TnT. Incorporation of complexes comprising fragments that contain residues 89-100 of the TnC sequence into the thin filament confer Ca^{2+}-sensitivity upon actomyosin ATPase.

**MATERIALS AND METHODS**

**Troponin Components**—TnC, TnI, and TnT were prepared from rabbit back and leg muscles using methods previously described (13, 18). Myosin was obtained from rabbit skeletal muscles according to Ferry (19).

Actin was extracted at 0 °C from the acetone-dried powder prepared from rabbit skeletal muscles according to Drabikowski and Gergely (20).

Tropomyosin was prepared according to Bailey (21).

**Tryptic Peptides**—TnC tryptic peptides were obtained by digestion in the presence of Ca^{2+} (TnC) or EDTA (TnC). TR(C) (amino acid residues 9-84) and TR(C) (amino acid residues 89-159) were obtained by digestion of TnC (1 mg/ml) with trypsin (10 μg/ml) at 25 °C for 90 min in 0.1 M NH_4HCO_3, pH 8.2, in the presence of 1 mM CaCl_2. Digestion was stopped by addition of soybean trypsin inhibitor (2-fold excess (w/w) over the amount of trypsin). Resulting peptides were separated by preparative urea polyacrylamide gel electrophoresis as previously described (4, 22).

To obtain TnE peptides, TnC (1 mg/ml) was digested with trypsin (2 μg/ml) for 10 min at 25 °C in a solution containing 0.1 M NH_4HCO_3, pH 8.2, 1 mM EDTA. Under these conditions, initially two TnC fragments are formed: TR(E) (amino acid residues 1-100) and TR(E) (amino acid residues 101-159) (23). These peptides were separated by preparative electrophoresis as previously described for the TnC peptides (4, 22). TR(E) was obtained in homogenous form. TR(E), however, although showing a single band in urea gel electrophoresis, proved to be a mixture of two peptides: TR(E) (amino acid residues 101-159) and TR(E) (amino acid residues 101-159) as derived from amino acid composition (23). These peptides are separable in polyacrylamide gel electrophoresis in the absence of urea and also exhibited different sensitivity upon actomyosin ATPase.

**Preparation of Immobilized TnI**—In order to couple TnI to CNBr Sepharose, we used a TnI-TnC complex to protect the TnC-binding site of TnI. To prevent coupling of TnC to Sepharose, its NH_2 groups were blocked with acetic anhydride prior to the formation of the complex with TnI. This was accomplished by mixing 30 mg of TnC (3 mg/ml) in half-saturated sodium acetate (0.37 g of CH_3COONa/ml of H_2O) with 60 μl of acetic anhydride, added in 6 portions over a period of 1 h, the pH being maintained at 9 with 1 N NaOH (cf. Ref. 30). After overnight dialysis against 1 mM NaHCO_3, TR(C) was checked for its ability to bind to CNBr activated Sepharose. To the solution of TR(C) (2 mg in 2 ml of 0.1 M NaHCO_3), 0.3 g of freshly washed CNBr-activated Sepharose 4B (Sigma) was added and the mixture was stirred for 30 min. Bound protein, estimated from the difference in A_280, was found to be only 8% of the initial amount of TR(C). Acetylated TnC (25 mg) was combined with TnI in a 1:1 molar ratio in a solution containing 6 mM urea, 1 mM dithiothreitol, 1 mM CaCl_2, 0.1 M NaHCO_3, and 0.1 M NaHCO_3. After overnight dialysis against 1 mM NaHCO_3, TnC was checked by coupling to CNBr-activated Sepharose (4.5 g of powder) which had been swollen for 10 min in 100 ml of 1 M HCl and washed with the same solution (400 ml) on a fritted glass funnel, followed by washing with 200 ml of 0.1 M NaHCO_3. The washed Sepharose was suspended in a small volume of 0.1 M NaHCO_3 and 50 mg of TR(C)-TnC complex (in 0.1 M NaHCO_3, 1 mM CaCl_2) were added, giving a final volume of 40 ml. The suspension was mixed gently for 2 h at room temperature. Following coupling, the remaining groups on the Sepharose were blocked with 1 M ethanolamine. Unbound material was washed out with 0.1 M NaHCO_3. The gel was loaded on a column, and acetylated TnC was washed by washing with solution containing 1 mM EDTA, 6 mM urea, 0.1 M KCl, 25 mM Hepes, pH 7.5.

**Affinity Chromatography**—A sample of peptides (2-4 mg) was placed on the Sepharose-TnI column (1 × 10 cm) equilibrated with 25 mM Hepes, pH 7.5, 0.1 M KCl, containing 1 mM CaCl_2. Bound material was eluted stepwise with the same solution containing either 1 mM EDTA or 1 mM EDTA plus 6 mM urea instead of CaCl_2. Although immobilization of TnI on Sepharose partially decreased its ability to bind TnC, owing to random chemical modification of its amino groups, in Table I.

<table>
<thead>
<tr>
<th>Peptides used in this study</th>
<th>Method of digestion and peptide</th>
<th>Amino acid residues</th>
<th>Ca^{2+}-binding sites</th>
<th>p(Ca^{2+})</th>
<th>Synonyms used in other papers (ref.)</th>
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<tr>
<td>Trypsin +</td>
<td>TR(C)</td>
<td>9-84</td>
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<td>7.8</td>
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<td>TR(E)</td>
<td>1-100</td>
<td>I, II</td>
<td>E_1, (26)</td>
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<td>E_2</td>
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<td></td>
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<td>101-153</td>
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<td>1-120</td>
<td>I, II, III</td>
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<tr>
<td></td>
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this would not influence our results, since the amount of peptides applied to the column did not exceed 20% of the total capacity of the bound TnI.

Complex Formation—Unless it is otherwise specified, the complexes of TnC fragments with TnI or TnL and TnT were formed by mixing stoichiometric amounts of proteins in 25 mM PIPES, pH 7.0, containing 6 M urea, 0.1 M KCl, 5 mM dithiothreitol, 1 mM CaCl₂, followed by dialysis overnight against 25 mM PIPES, pH 7.0, 0.1 M KCl, 0.1 mM CaCl₂. Any precipitate present was removed by centrifugation for 10 min at 27,000 rpm. In the case of ternary complexes, the supernatant solution was applied to a Sephadex G-150 column equilibrated with the above buffer. The first peak contained the ternary complex.

ATPase Activity—Two methods were used. In one, the steady state hydrolysis of ATP in each sample was determined by measuring the release of inorganic phosphate according to Fiske and SubbaRow (31). In the second, the pH-stat technique was used (32).

RESULTS

Interaction of TnC Fragments with Troponin Subunits

TnI—It should be noted that native TnC forms a binary complex with TnI both in the absence and presence of Ca²⁺ but, in solutions containing 6 M urea, only when Ca²⁺ is present (9, 33). All of the TnC fragments studied, with the exception of TH₁, form complexes with TnI in the presence of Ca²⁺ (Figs. 1-3). Of these, however, only two (TR₂C and TR₂E) remain complexed when Ca²⁺ is removed, behaving in this respect as intact TnC does. These observations suggest that at least one site not requiring Ca²⁺ for interaction with TnI is located within the COOH-terminal part of TnC, residues 101-159, and that sites exist elsewhere in the molecule that require Ca²⁺ for complex formation (cf. Table II, CB₁, TR₂C, CB₂).

Two of the fragments studied, TH₁ and TR₂E, although homogeneous on polyacrylamide gel electrophoresis, were eluted as two peaks from the Sepharose-TnI column (Fig. 1), one emerging upon addition of EDTA to the eluent, and the other on switching to an eluent containing 6 M urea. In the case of TH₁, the first peak contained the monomeric form of the peptide, while the second contained the dimer; the latter peptide was not observed if dithiothreitol was present. Analysis of the two peaks of TR₂E led us to discover a heretofore unsuspected heterogeneity, viz. the first peak consisted of a peptide containing residues 101-153, referred to in this report as TR₂E, the second containing TR₂E, residues 101-159 (see "Materials and Methods", Table I).

TABLE II

<table>
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<tr>
<th>Peptide</th>
<th>structure</th>
<th>Ca²⁺</th>
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<td>VIII</td>
<td>102</td>
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</tr>
</tbody>
</table>

* indicates that formation of the complex was observed by chromatography on a TnI-Sepharose column but not by polyacrylamide gel electrophoresis.
* Taken from Leavis et al. (4).

In the presence of 6 M urea and Ca²⁺, three of the fragments, TH₁, TR₂E, and TR₃C, bind to TnL (Fig. 3 and Ref. 4) as does TnC. Although the fragments differ with respect to the Ca²⁺-binding domains, both contain the amino acid sequence comprising residues 89-100, which implicates this region as an interaction site in urea.

TnT—gel electrophoresis with or without urea or Ca²⁺ failed to provide evidence of complex formation between TnT and fragments of TnC. Although some fragments did bind to a TnT affinity column, they were gradually eluted upon

![Fig. 1. Chromatography of TnC fragments on a TnI column.](image)

Protein samples (2-4 mg) in 25 mM Hepes, pH 7.5, 0.1 M KCl, 1 mM CaCl₂ (buffer A) were loaded on a TnI-Sepharose column (1 x 15 cm) and eluted stepwise with three different eluents (A, B, C). About 2 column volumes of the effluent were collected in each step. Eluents contained 25 mM Hepes, pH 7.5, 0.1 M KCl, and either 1 mM CaCl₂ (A), 1 mM EDTA (B), or 1 mM EDTA plus 9 M urea (C). I, TnC; II, TH₁; III, TR₂E; IV, CB₁; V, TR₁C; VI, TR₂E + TR₂E; VII, TH₂. Protein was detected in the effluent by monitoring A₂₈₀.

![Fig. 2. Separation of Ca⁺⁺ from TR₂C attached to a TnI column.](image)

Two mg of TR₂C in a solution containing 0.1 M KCl, 25 mM Hepes, pH 7.5, 0.1 mM CaCl₂, 0.05 µCi of ⁴⁰Ca/ml were applied to a TnI-Sepharose column equilibrated with the same solution. The eluent contained 0.1 M KCl, 25 mM Hepes, pH 7.5, and A, 0.1 mM CaCl₂, 0.05 µCi of ⁴⁰Ca/ml; B, 1 mM EDTA, 0.05 µCi of ⁴⁰Ca/ml; C, 1 mM EDTA; or D, 1 mM EDTA, 6 M urea. Protein was detected by measuring A₂₈₀. Radioactivity (---) was measured in 0.1-ml aliquots taken from each fraction by scintillation counting. The separate elution of the ⁴⁰Ca at B and protein at D verifies that the latter remains bound to the column even when Ca²⁺ is entirely removed.
continued washing with the loading solution, suggesting that the interactions were weak. Complex formation, however, could be deduced from the effects of the fragments on the solubility of TnT at low ionic strength. While TnT alone is virtually insoluble at \( \mu < 0.2 \) M, it is soluble up to millimolar concentrations, even at low ionic strength, when complexed to TnC. TnT is completely solubilized by two fragments, THi and TRiE, in the presence of Ca\(^{2+}\) (Fig. 4). Partial solubilization is indicated by the weak tryptophan fluorescence in the supernatant solutions in the case of TRiC and CBi, suggesting the formation of weak complexes with TnT (Fig. 4). Fragment-TnT but not TnC-TnT complexes are dissociated if EDTA is present, resulting in the precipitation of TnT.

**Ternary Complexes**—Ternary complexes containing equimolar amounts of TnC, TnI, and TnT can be reconstituted from the purified components by combining them in 6 M urea followed by dialysis (see "Materials and Methods"). Such a complex is fully soluble at low ionic strength, binds Ca\(^{2+}\), and confers Ca\(^{2+}\) sensitivity upon actin-activated myosin ATPase activity; it is thus indistinguishable in this respect from "native" whole troponin (13). Most of the TnC fragments, when substituted for TnC in a system containing TnT + TnI, form complexes that are soluble at low ionic strength. The complexes are eluted as a single peak in the void volume from a Sephadex G-150 column (not shown). SDS-polyacrylamide gel electrophoresis shows that the peaks contain all three components. Two of the peptides, THiE and THi, that form ternary complexes are also able to form binary complexes with TnT or TnI. In the case of TRiC, TRiE, and CBi, which do not form ternary complexes, no binary complex formation with TnT can be demonstrated, suggesting that in these ternary complexes, TnT might be bound directly to the TnI subunit (see "Discussion"). With TRiC, a precipitate is formed upon removal of urea by dialysis. Gel electrophoresis of the precipitate shows that it contains all three troponin components, suggesting formation of a ternary complex of limited solubility at low ionic strength. No evidence of ternary complex formation was found with fragments TRiE and THi.

**Regulation of Actomyosin ATPase Activity by Complexes of TnC Fragments**

The ability of the TnC fragments to confer Ca\(^{2+}\) control on myosin ATPase activated by regulated actin was checked by adding fragments to an actin/myosin solution whose ATPase activity was inhibited by the presence of tropomyosin, TnI, and TnT. Addition of intact TnC in the presence of Ca\(^{2+}\) completely restores the ATPase activity at a TnC/TnI molar ratio of about 1 (Fig. 5). Three TnC fragments, THi, TRiE, and TRiC, also produce Ca\(^{2+}\)-dependent reversal of the inhibition (Fig. 5), although there is some variability among them with respect to the level of the ATPase activity regained. Particularly interesting is the fact that both TRiE and TRiC peptides show similar activity, although TRiE contains only low affinity Ca\(^{2+}\)-specific sites and TRiC contains only the high affinity Ca\(^{2+}\)-Mg\(^{2+}\) sites; the only sequence they share are residues 89–100. Slight activity, less than 10% of that restored by TnC, was also observed for TRiC and CBi. None of the other peptides showed any capacity to reverse ATPase inhibition except at an extremely high molar ratio of peptide to TnI (>30), which most likely represents nonspecific effects. Finally, it should be noted that mixtures of complementary peptides, e.g. THi + THi, TRiC + TRiC, or TRiE + TRiE, did not enhance or reduce the effects seen in Fig. 5, suggesting

**Fig. 3. Polyacrylamide gel electrophoresis of complexes of TnC fragments with TnI.** Electrophoresis was performed on 10% polyacrylamide gel slabs in 60 mM Tris/glycine buffer, pH 8.6, containing 0.1 mM CaCl\(_2\) and, in gels 1-4, 5 M urea. Samples contained 0.01 mg of TnI, 0.01 mg of TnT, and various amounts of TnC. Samples (1 mg/ml) in a 1:1 molar ratio in the presence of 5 M urea (see "Materials and Methods") followed by dialysis and centrifugation to remove insoluble material. Samples were diluted 10 times with a solution containing 25 mM Pipes, pH 7.0, 0.1 M KCl, and 0.1 mM CaCl\(_2\) (open bars) or 1 mM EDTA (stippled bars). Samples were centrifuged and fluorescence emission of the supernatants was measured. Note that only peptides TRiE and THi do not form complexes. The diffuse band of CBi peptide when TnI is present (gel 13) in comparison to CBi alone (gel 14) indicates weak interaction.

**Fig. 4. Complex formation between TnT and TnC fragments.** TnC fragments were mixed with TnT (1 mg/ml) in a 1:1 molar ratio in the presence of 6 M urea (see "Materials and Methods") followed by dialysis and centrifugation to remove insoluble material. Samples were diluted 10 times with a solution containing 25 mM Pipes, pH 7.0, 0.1 M KCl, and 1 mM EDTA (open bars) or 1 mM EDTA (stippled bars) or 1 mM CaCl\(_2\) (open bars) or 1 mM CaCl\(_2\) (solid symbols). The reaction was started by addition of ATP, stopped by addition of 2 ml of 2% SDS and the liberated phosphate determined by the method of Fiske and SubbaRow (31). The activity of actomyosin (usually 0.38–0.43 \( \mu \)mol of P\(_i\)/mg/min) was taken as 100%, and that of actomyosin in the presence of tropomyosin, TnI, and TnT (usually 0.08–0.12 \( \mu \)mol of P\(_i\)/mg/min) was taken as 0%.

**Fig. 5. Neutralization of TnI inhibition of actomyosin ATPase activity by TnC and its fragments.** One-ml samples containing 0.2 mg of myosin, 0.04 mg of F-actin, 0.02 mg of tropomyosin, 0.006 mg of TnI, 0.01 mg of TnT, and various amounts of TnC, TRiC, THi, TRiE, or TRiE, were incubated for 5 min at 25 \( ^\circ \)C in a solution containing 10 mM Tris-HCl, pH 7.5, 1 mM ATP, 1 mM MgCl\(_2\), 30 mM KCl, and 1 mM EDTA (open symbols) or 0.1 mM CaCl\(_2\) (solid symbols). The reaction was started by addition of ATP, stopped by addition of 2 ml of 2% SDS and the liberated phosphate determined by the method of Fiske and SubbaRow (31). The activity of actomyosin (usually 0.38–0.43 \( \mu \)mol of P\(_i\)/mg/min) was taken as 100%, and that of actomyosin in the presence of tropomyosin, TnI, and TnT (usually 0.08–0.12 \( \mu \)mol of P\(_i\)/mg/min) was taken as 0%.
FIG. 6. Calcium dependence of neutralization of TnI inhibition of actomyosin ATPase. ATPase activity was determined from the rate of H+ liberation using a pH-stat (32). Three-m1l samples contained 0.6 mg of myosin, 0.12 mg of actin, 0.03 mg of COOH-terminal half, viz. helices IIIc (residues 115-122) and IVN (residues 127-138), that exhibit helical structure whether or not Ca2+ is present (8, 37). Furthermore, helix IVN contains several closely spaced acidic amino acid residues (Asp128, 137; Glu129, 130, 132) that may form electrostatic contacts with basic residues on TnI (cf. Ref. 17), leading us to propose that region IVN is a site of interaction with TnI, independent of Ca2+. This view is supported by recent studies showing a reduction in the reactivity of lysines 136 and 140 of TnC when it is complexed to TnI (16). If helix IVN is indeed a site of binding to TnI, the failure of either TR2E or TH2 to form a Ca2+-independent complex must result from loss of the six COOH-terminal residues (154-159) in the former case and the absence of domain III in the latter, both of which may result in loss of structure in the region IVN, necessary for binding of TnI. TR2E and TH2 indeed have decreased structure as deduced from CD spectra and decreased affinity to Ca2+ in comparison to TR2C or TnC (Ref. 4 and Footnote 2).

TnC-like behavior in 6 M urea, viz. the complex is stable if Ca2+ is present, is exhibited by fragments TH2, TR2E, and TR2C. Although these fragments differ with respect to the Ca2+-binding sites, they possess a single overlapping region comprising residues 89-100, possibly another TnI-binding site. This region, like helix IVN, contains a cluster of acidic residues (Glu92, 93, 94, and 97). It is attractive to speculate that the Ca2+ requirement for complex formation is tied to the cation-induced folding of residues 91-100 into an a-helix resulting in the alignment of the glutamate side chains along one side of the helix. Because this occurs both under native conditions and in 6 M urea (8), it seems reasonable to consider residues 89-100 as a site of interaction under both conditions. We have previously advanced the view that residues 89-100 constitute an important region in the TnC-TnI interaction (38-40), a view that is supported by the observation of CB3 binding to TnI (41) and the recent results of Wall et al. (42).

Two fragments, TR,C (residues 9-84) and CB3 (residues 46-78), that do not contain either residues 89-100 or 127-138, nevertheless bind to TnI, but only in the presence of Ca2+. 1H-NMR studies (43) as well as the observation that reactivity of Lys52 decreases upon complex formation with TnI (16), suggest a binding site in the region of helix IIN (residues 51-62).

Our studies, then, suggest that interaction with TnI involves three different regions of the TnC molecule (Fig. 7), two of which (IIN and IIIb) are Ca2+-dependent and one (IVN) Ca2+-independent under native conditions. Studies by Syska et al. (17) yielded two TnI peptides able to form complexes with TnC, containing a large number of basic amino acid residues. If three regions of TnC are involved in binding to TnI, it is possible that two of them may be close together and bind to a single area in TnI. The putative sites of interaction that we propose share several features. First, they are all on a-helices on the NH2-terminal side of binding domains II-IV. Second, they all contain a remarkably similar cluster of acidic residues along one surface of the helix (see Fig. 7). This is not the case for any of the five other a-helical segments of the molecule. Third, all the lysines located in these sites (viz. Lys52, 84, 88, 90, 136, and 140) exhibit reduced reactivities in the complex with TnI relative to TnI alone (16). The other three lysine residues in the molecule are unaffected by complex formation.

It seems that the stabilizing effect of Ca2+ on complex formation involving residues 89-100 may require binding to the high affinity sites (III, IV), whereas those involving residues 51-62 require binding of Ca2+ to low affinity sites (I, II). Complex stabilization by the high affinity sites is supported

that these peptides do not interact to modify their individual activities. Also, the ability of a digest of TnC to reverse inhibition by TnI was proportional to the amount of undigested TnC remaining. At the point where electrophoresis showed only TRC and TR2C peptides in the digest, the ATPase activity was similar to that for TR2C peptide alone.

We have examined the Ca2+ dependence of the effect of fragments on the regulated actomyosin ATPase. The ATPase activity of the reconstituted system containing TnC, TH2, TR2E, or TR2C in addition to tropomyosin, TnI, and TnT increased with increasing [Ca2+] all systems having a transition midpoint of Ca2+ (Fig. 6). In this experiment, peptide complexes with TnI and TnT were preformed in 6 M urea (see "Materials and Methods") and appeared to interact to modify their individual activities. Noteworthy is the steepness of the curves (Hill coefficient = 4), indicating cooperativity with increasing Ca2+-binding in the activation of ATPase.

**DISCUSSION**

The TnC-TnI complex is known to be stable under native conditions both in the presence and absence of Ca2+ (9, 33, 34). Ca2+, however, has been implicated in the "tightening" of the existing bonds between the two proteins (35, 36) and possibly in the formation of additional points of contact (4). The fact that virtually all of the TnC fragments studied required Ca2+ to form complexes with TnI suggests that a specific, Ca2+-dependent, spatial arrangement of residues involved in the binding is essential for the interaction to occur. This idea is further supported by our previous observations that Ca2+ induces structure in the peptides (4). Such residues would exist in both halves of the TnC molecule, since fragments from both the NH2-terminal (TRC, TR2E, CB3) and COOH-terminal halves (TR2C, TR2E, CB3) bind to TnI when Ca2+ is present. It is interesting that, although the two peptides TR2C and TR2E, which share amino acid residues 101-159, undergo large structural changes upon binding of Ca2+, they bind TnI in the presence of Ca2+ as well as in its absence. This suggests that readily observable Ca2+-induced structural changes in the COOH-terminal half of TnC are not essential for TnI binding per se. There are, however, regions within the

2 P. C. Leavis and Z. Grabarek, unpublished observation.
specific interaction between TnT and TnC. If both TnT and TnI can bind to the same sites in the NH2-terminal fragments of TnC in binary complexes, the binding of the former appears to be weaker as judged by the failure of electrophoresis or affinity chromatography to demonstrate complexes of TnT with TnC fragments. Weak, if any, interactions are also indicated by cross-linking studies showing reduced linking of TnC to TnT relative to either TnC to TnI or TnI to TnT (44). We have previously reported that TnT binds to the COOH-terminal fragment (TH1) (4). In view of the fact that none of the other fragments comprising the COOH-terminal half of TnC bind to TnT and in light the present demonstration of binding to the NH2-terminal half, we conclude that TnT-TH1 interaction is probably nonspecific.

Although only two of the TnC fragments were able to form binary complexes with TnT, virtually all fragments were able to form ternary complexes with 1 mol each of TnI and TnT. This suggests that the integrity of ternary complexes of TR1E, TR2C, and CB9 involves bonds between TnT and TnI, a link long known to exist in early tropinin B preparations (45, 46), and more recently investigated by Horwitz et al. (47). All of the fragments able to form ternary complexes were tested for their ability to confer Ca2+ sensitivity on ATP hydrolysis; however, only the three shown in Fig. 5, viz. TnIC, TnC, and TR2C, showed significant TnC-like behavior. All three peptides restored the Ca2+ sensitivity of ATPase activity and all required approximately 1 mol of fragment/mol of TnI. It is, however, puzzling that the degree of neutralization of the inhibition was less than that in the case of TnC, ranging from about 60% for TH1 to only 20% for TR2E. Of particular interest in these experiments is the fact that both TR1E and TR2C exhibit similar neutralizing activity even though they contain only the Ca2+-specific or Ca2+-Mg2+-binding sites, respectively. A slight (~10%) Ca2+-dependent neutralization was also shown by CB9 and TR1C. The former has been shown to neutralize ATPase inhibition in a system similar to ours but lacking TnT (41). In those studies, the neutralization exhibited very little Ca2+ dependence either for CB9 or intact TnC, reaffirming the observation by Greaser and Gergely (13) that all three subunits are required for Ca2+ sensitivity of the ATPase activity of reconstituted actomyosin.

Three features are evident from the Ca2+ titration curves in Fig. 6. First, the ATPase curves are steep (Hill coefficient = 4), indicating a high degree of cooperativity in Ca2+ binding, possibly due to interaction between adjacent tropomyosin molecules (48). Second, the values of [Ca2+]1/2 are identical for TnC, TH1, TR2E, and TR1C (i.e. [Ca2+]1/2 = 1.5 x 10^{-7} M). Third, all peptides restored the ATPase activity to the same extent as TnC. The failure of the fragments to completely restore actomyosin ATPase activity in the first experiment (Fig. 5), in which peptides are directly added to the actomyosin solution, compared to the second experiment, using preformed complexes (Fig. 6), may reflect differences in the fragment-TnI-TnT complex formed under the two experimental conditions. It is possible that in the former case, conditions for the proper alignment of binding sites between proteins are not met, resulting in only a partially active complex. This is consistent with the earlier observations of Greaser and Gergely (13) that an active tropinin complex could be reconstituted from a solution containing actin. It is surprising that TR1C containing only the Ca2+-Mg2+-binding sites, which are not considered to be involved in regulation, can replace TnC in conferring Ca2+ sensitivity of the actomyosin ATPase activity. One can speculate that the symmetry between the NH2- and COOH-terminal halves of the TnC molecule may permit TR1C to occupy the position in the complex usually occupied by the region of TnC involved in regulation, presumably sites I and by recent fluorescence quenching studies with a probe attached to Cys 98, showing that Ca2+ or Mg2+ binding to the high affinity sites in the TnC-TnI complex resulted in a large decrease in the accessibility of the probe to quencher molecules (36). This indicates that the probe was covered by a tightening of the bond between TnI and TnC in the area of Cys 98. Evidence for low affinity Ca2+ binding in modulating the region containing residues 51-62 comes from 'H-NMR studies showing changes in resonances assigned to threonine residues 49 and 51 and Glu side chains when TnI is added to CB9 (43).

Two peptides bind to TnT in the presence of Ca2+, viz. TH1 and TR2E, suggesting a site or sites in the NH2-terminal half of the protein. The Ca2+ dependence is reminiscent of the behavior of these peptides with TnI, raising the possibility that TnT might form contacts with regions 51–62 or 89–100 or both. This possibility is further supported by fluorescence quenching studies that show TnT-induced changes in the “accessibility” of extrinsic fluorophores on TnC that are similar to those induced by TnI (36). Furthermore, Hitchcock (16) showed that the reactivities of Lys 52, 84, 88, and 90 are reduced by both the TnT and TnI. However, the reactivity of Lys 37 changes only for TnT-TnC complex formation but is insensitive to TnI binding, thus leaving open the possibility of

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**Fig. 7.** Representation of the relative positions of amino acids in α-helical segments II_N, III_N, and IV_N. For the purpose of comparison, all three helices are shown in the same spatial orientation. Note the positions of Glu (E) and Asp (D) residues in each helix (drawn from coordinates given in Ref. 2) A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; I, isoleucine; K, lysine; L, leucine; N, glutamine.

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[Diagram of α-helical segments with amino acid positions indicated.]
II. Alternatively, it must be considered that although TR,E and TR,C contain different classes of Ca$^{2+}$-binding sites, they are both likely to interact with TnI via their common amino acid region, residues 89-100. It is possible that Ca$^{2+}$ binding to either class of sites may produce similar changes in the structure of this sequence which can subsequently be transmitted to TnI.

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