Fragments of Rabbit Striated Muscle \( \alpha \)-Tropomyosin

II. BINDING TO TROPOVIN-T*

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The interactions of a variety of large fragments of rabbit skeletal muscle \( \alpha \)-tropomyosin, prepared as previously described, with troponin-T and a soluble tropomyosin-binding fragment of troponin-T (CB1) have been investigated by affinity chromatography and gel filtration. No specific interactions between NH\(_2\)-terminal fragments encompassing residues 1-189 with troponin, troponin-T, or CB1 immobilized on a Sephadex 4B column could be demonstrated. Similarly, there was no interaction between these fragments and CB1 on a gel filtration column operated in 0.1 M KCl, 10 mM imidazole pH 7.0 buffer. On the other hand, all fragments encompassing residues 190-284 showed interaction with troponin-T on the affinity column and with CB1 by gel filtration. When mixtures of two fragments, one of which had the intact NH\(_2\)-terminal sequence of the original tropomyosin structure and the other the intact COOH-terminal sequence, were applied to the gel filtration column, there was no indication of interaction between them. However, when CB1 was included in the mixture, a ternary complex of the three components was demonstrable. Fragments in which 10 or 12 residues at the NH\(_2\)-terminal end of the \( \alpha \)-tropomyosin sequence were absent showed no evidence of forming a ternary complex with CB1 and the COOH-terminal fragments. We conclude that the binding of the troponin-T fragment, CB1, to the COOH-terminal third of the \( \alpha \)-tropomyosin molecule enhances head-to-tail aggregation of tropomyosin molecules either indirectly by the transmission of conformational changes to the head-to-tail overlap region or more directly by binding close to or at this region.

Numerous studies have been directed toward delineating the nature of the interaction between tropomyosin and troponin in attempts to more fully understand the molecular mechanisms by which these two protein complexes participate in the actin-linked calcium regulatory systems of skeletal and cardiac muscle. Interaction between these proteins has been demonstrated by the dramatic increase in viscosity that occurs upon the addition of the Tn' complex to TM solutions (1, 2) and the co-sedimentation of the two as a hypersharp peak in the analytical ultracentrifuge (3). The binding of Tn to TM through the troponin-T component of the complex (4, 5) is independent of the presence of calcium (6-8) and magnesium ions (9) but is affected by the ionic strength (5, 10).

Studies directed toward the identification of the interaction sites for TM and the relatively insoluble Tn-T molecule have been facilitated by the preparation of soluble cyanogen bromide fragments of Tn-T (10-13). Thus, Tn-T fragments CB1 (residues 1-151) and CB2 (residues 71-151) have been shown to bind to TM immobilized on Sepharose 4B (13) and to increase the viscosity of TM solutions (10). The high \( \alpha \)-helical content (80%) of fragment CB2 implies that the interaction between the two proteins involves the formation of a multi-\( \alpha \)-helical complex (13).

Evidence for the binding site on TM for Tn is derived largely from electron microscopic examination of crystals and the fibrous magnesium tectoids of TM in the presence and absence of Tn and its components (14-21). Interpretation of the detailed uranyl acetate staining pattern of the tectoids in terms of the periodicity of the acidic residues in the amino acid sequence has led to the conclusion that the site of Tn-binding is about one-third of the distance from the COOH-terminal end (22). Based on certain irregularities in the sequence, McLachlan and Stewart (23) have suggested that residues 197-217 may represent the Tn-T binding site on TM. However, their arguments are now less compelling since equine platelet TM, which binds only poorly to Tn (24), has been shown to have a very similar amino acid sequence to muscle TM in the region of these residues. These observations, coupled with the fact that this nonmuscle TM has markedly different NH\(_2\)- and COOH-terminal sequences and polymerizes weakly in a head-to-tail manner (25), has prompted us to investigate the Tn-T-binding properties of a number of muscle TM fragments derived from various regions of its structure. The preparation, identification, and stability properties of these fragments have been described in the preceding paper (26). In the present work, we demonstrate by both affinity chromatographic and gel filtration techniques that while fragments derived from the NH\(_2\)-terminal two-thirds of the TM molecule show no evidence of binding to the soluble TM-binding fragment (CB1) of Tn-T, those derived from the COOH-terminal third do. It is also shown that when fragments with intact NH\(_2\)-terminal and COOH-terminal sequences are combined, a ternary complex with CB1 is formed under conditions in which the two TM fragments alone show no interaction. We interpret these observations as indicating that the binding of the Tn-T fragment, CB1, to the COOH-terminal third of the \( \alpha \)-TM molecule enhances head-to-tail aggregation of TM molecules either indirectly by the transmission of conformational changes to the head-to-tail overlap region or more directly by binding close to or at this region.

MATERIALS AND METHODS
The preparation of \( \alpha \)-TM and its fragments has been described in

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The abbreviations used are: Tn, troponin; TM, tropomyosin; Tn-T, troponin-T; Tn-TC, a complex of troponin-T and troponine-C; SDS, sodium dodecyl sulfate.
Binding of Tropomyosin Fragments to Troponin-T

RESULTS

Interaction Studies by Affinity Chromatography—In these experiments, α-TM and its individual fragments were applied to the Tn-T affinity column and the KCl concentration at which they were eluted was taken as a measure of their strength of binding to the ligand. Typical elution profiles for bovine serum albumin, α-TM, and fragments CnIA and CnIB are shown in Fig. 1. An elution profile for a mixture of fragments T3 and CnIB from the column is shown in Fig. 2. Of those, α-TM eluted the most strongly bound, being eluted at a KCl concentration of 0.2 M. Fragments CnIA and CnIB were eluted at 0.05 M and 0.10 M KCl, respectively, while bovine serum albumin was eluted in the void volume of the column. The data for all the proteins and fragments tested are assembled in Table I which also includes a less complete set of data for the affinity columns prepared with either whole Tn or the TM-binding fragment CB1 of Tn-T. There appeared to be no significant differences in the concentrations of KCl required for the elution of any one protein or fragment from the three types of affinity columns. Of the control proteins tested, bovine serum albumin, ovalbumin, and myoglobin were not retained by the columns, whereas cytochrome c and some of the fragments of a cyanogen bromide digest of light meromyosin were retained and eluted at a concentration of 0.04 to 0.06 M KCl. The elution of the latter two preparations at this KCl concentration was taken as an indication of a degree of nonspecific adsorption to the column. Whereas α-TM was eluted at a KCl concentration of 0.2 M, all the fragments derived from the NH₂-terminal half of the molecule were...
eluted at 0.05 M KCl or less and thus could not be distinguished from nonspecific adsorption. On the other hand, all fragments derived from the COOH-terminal half were eluted at 0.10 M KCl. The only exception to this was T5, the smallest of the fragments tested, which was not retained by the column. In the previous paper, it was shown to have little or no coiled-coil structure because of its small size (26). These results are therefore consistent with the view that the binding site for Tn-T is located in the COOH-terminal third of the molecule between residues 190 and 284.

Interaction Studies by Gel Filtration—Interaction studies between α-TM and its fragments and Tn-T are complicated by the insolubility of Tn-T at normal physiological ionic strengths. However, the availability of a fragment of Tn-T, namely the cyanogen bromide fragment CB1 (residues 1-151), which has been shown to interact with α-TM (10, 13) and which is soluble in water and low salt concentrations, offered the possibility of studying its interaction with various α-TM fragments at normal physiological salt concentrations. Because of the asymmetric rodlike nature of α-TM and its fragments, it was anticipated that adequate separation of the α-TM fragments and CB1 would be possible on gel filtration columns for the purpose of demonstrating interaction between them. Such a system would also permit the unambiguous identification of individual components under any particular elution profile peak by the analysis of appropriate fractions by SDS-polyacrylamide gel electrophoresis.

That this approach had potential was demonstrated by the elution profiles of CB1 alone, of α-TM alone, and of CB1 plus α-TM on a column (1.2 x 100 cm) of Bio-Gel A-0.5m equilibrated with 0.1 M KCl, 10 mM imidazole, 1 mM dithiothreitol, 0.01% sodium azide, pH 7.0, at room temperature (see Fig. 3, A to C). It can be seen that CB1 alone (Fig. 3A) was eluted as a single peak centered at Fraction 39. The shape and position of this peak was reproducible for more than five control runs. α-TM alone was eluted as a single peak at Fraction 25 (Fig. 3B). However, a mixture of α-TM and CB1 appeared as a single peak at Fraction 23 (Fig. 3C). The presence of both α-TM and CB1 in this peak was confirmed by SDS-polyacrylamide gel electrophoresis. Different molar ratios of CB1 to α-TM have been used in these binding studies. Quantitative binding between CB1 and α-TM was observed when CB1:α-TM was less than 1. This is illustrated in Fig. 3C where CB1:α-TM was 1:2.5. The absence of CB1 in Fractions 37 to 43, where free CB1 would be expected to be eluted, indicated that it was completely bound as a complex with α-TM. However, when CB1:α-TM was greater than 1, the excess CB1 not involved in binding to α-TM was detected at Fractions 37 to 43 (not shown).

Binding of α-TM Fragments and CB1—These studies are divided into three groups. The first group involves the possible interactions between fragments derived from the NH₂-terminal half of α-TM and CB1 and includes fragments Cy1 (residues 1-189), CT1 (residues 1-169), T1 (residues 1-133), and Cn1A (residues 11-127). The second group deals with binding between COOH-terminal fragments and CB1 and includes Cy2 (residues 190-284), CT2 (residues 170-284), T2 (residues 134-284), and Cn1B (residues 142-281 (284)). The third group of experiments was designed to detect formation of ternary complexes in mixtures of NH₂-terminal α-TM fragments, COOH-terminal α-TM fragments, and CB1. The results of these three groups of experiments are illustrated in this paper by the results obtained with the fragments Cy1, Cy2, and CB1 (see Fig. 4, A to G).

Lack of Interaction between NH₂-terminal Fragments and CB1—A typical set of results is illustrated by the studies on Cy1 and CB1 (Fig. 4, A, C, and D). Fragment Cy1 was eluted as a single peak at Fraction 32 (Fig. 4A) and a mixture of Cy1 and CB1 appeared as two distinct peaks centered at Fractions 32 and 39 (Fig. 4D). The first peak contained only Cy1 and the second peak consisted of pure CB1. This indicated the lack of complex formation between Cy1 and CB1. Similar results were obtained when other NH₂-terminal fragments were studied; they included T1, CT1, and Cn1A. These results therefore agree with those obtained by affinity chromatography which showed that none of the NH₂-terminal fragments formed a complex with CB1 in 0.1 M KCl.

Interaction between COOH-terminal Fragments and CB1—The COOH-terminal fragment Cy2 appeared as a single peak at Fraction 39 (Fig. 4B) in the same position as free CB1 (Fig. 4C). Evidence for interaction between Cy2 and CB1 was obtained by the appearance of a new peak centered at Fraction 34 partially resolved from the second peak at Fraction 37 (Fig. 4E). Analyses of the fractions from the two peaks by SDS-gel electrophoresis showed the presence of both Cy2 and CB1 in the first peak and only Cy2 in the second peak. Similar complex formation was also observed when other COOH-terminal fragments were applied to the column as mixtures with CB1. These COOH-terminal fragments included T2, CT2, and Cn1B. Since Cy2 (residues 190-284) is the shortest of the COOH-terminal fragments tested, these results indicate that the binding site(s) on TM for Tn-T Fragment CB1 is located between residues 190 and 284.

Detection of Ternary Complexes by Gel Filtration—The head-to-tail polymerization of α-TM molecules, observable as
a large increase in relative viscosity, is markedly dependent on salt concentration (10, 25, 29, 30). At low salt concentrations, the viscosity increases sharply as the ionic-strength is reduced below 0.1 M. The viscosity is also markedly increased by the addition of troponin or Tn-T fragment CB1 to a TM solution (1, 10). This sharp increase in viscosity as the result of complex formation between TM and Tn or CB1 may result from an enhancement of the head-to-tail polymerization of TM molecules. To investigate this phenomenon further, we have tested the abilities of NH2-terminal and COOH-terminal fragments to engage in head-to-tail interactions in the absence and presence of CB1. A typical experiment is illustrated here using fragments Cy1, Cy2, and CB1 (Fig. 4, F and G). When a mixture of Cy1 and Cy2 was eluted from the gel filtration column, two distinct peaks were observed at Fractions 32 and 39, respectively (Fig. 4F). Analysis of these peaks by SDS-polyacrylamide gel electrophoresis demonstrated that Peak 1 consisted only of Cy1 while Peak 2 was pure Cy2. Thus, no head-to-tail complex formation was detectable between these two fragments in 0.1 M KCl. Similar results (not shown) were obtained for other combinations of NH2-terminal and COOH-terminal fragments including CT1 plus CT2, T1 plus CT2, CT1 plus CT2, and Cy1 plus CT2.

The elution profile of a mixture of Cy1, Cy2, and CB1 (molar ratio of 2:2:1) is shown in Fig. 4G. Three partially separated peaks centered on Fractions 27, 32, and 37 were observed. The first peak at Fraction 27 contained all three components as shown by gel electrophoresis and is taken as evidence for the formation of a ternary complex of the three. The second peak at Fraction 32 contained only Cy1 which likely represented excess Cy1 not involved in the ternary complex. The first portion of the third peak showed the presence of Cy2 and CB1 while the later part of this peak contained only Cy2. Apparently, essentially all of the CB1 was engaged in the formation of a ternary complex with Cy1 and Cy2 or as a binary complex with Cy2.

We have also observed that other combinations of NH2-terminal and COOH-terminal fragments form ternary complexes with CB1 under these conditions. These include CT1 plus CT2, T1 plus CT2, CT1 plus Cy2, and Cy1 plus CT2. It must be emphasized that all of these fragments have intact NH2-terminal and COOH-terminal sequences involved in the head-to-tail polymerization of intact α-TM molecules. On the other hand, when a mixture of CnIA (residues 11-127), which lacks the NH2-terminal sequence for head-to-tail interaction, Cy2 (residues 190-284) and CB1 were applied to the gel filtration column, no evidence for the formation of a ternary complex was observed (Fig. 5A to C). Such a complex (66,000 daltons) would be expected to appear in the region of Fractions 27 to 30. However, no such complex was detected in the elution profile of Fig. 5C. Instead, a binary complex of Cy2 and CB1 was eluted at Fraction 35 (see Fig. 4E) where it coincided with the peak of CnIA alone. Excess Cy2 was found at Fraction 39.

These results strongly suggest that the formation of a ternary complex in 0.1 M KCl is the result of head-to-tail...
association of NH$_2$-terminal and COOH-terminal ends of the a-TM fragments and that this interaction is induced by the binding of CB1 to the COOH-terminal fragment.

**DISCUSSION**

In the present work, we have employed two experimental approaches for investigating the interaction properties of a variety of fragments of a-TM with Tn-T and its soluble TM-binding fragment CB1. The first of these involves the measurement of the ionic strength at which a-TM and its fragments are eluted from affinity columns in which Tn-T, CB1, or troponin are immobilized on Sepharose 4B. Under the buffer conditions employed, a-TM was eluted as a peak centered about 0.2 M KCl. This concentration of salt is lower than that previously reported for the complete dissociation of troponin and a-TM (3-6, 10) and may be due to steric hindrance of the interaction site(s) on troponin and Tn-T for TM by virtue of their immobilization on the Sepharose 4B support. Alternatively, the chemical procedure for the coupling of the ligand may have chemically modified the binding site on Tn-T for a-TM. However, the demonstration that several control proteins or protein fragments were eluted at salt concentrations 0.05 M or lower indicated that the binding of a-TM was the result of specific interaction between it and the ligand. This experimental approach for testing the binding of a-TM fragments to Tn-T had the additional advantage that the identity of proteins and fragments in the eluted fractions could be unambiguously identified by SDS-polyacrylamide gel electrophoresis. The identification of the components of putative complexes observed by other techniques such as polyacrylamide gel electrophoresis or sedimentation velocity ultracentrifugation is often much more experimentally difficult and subject to misinterpretation.

The feasibility of employing gel filtration for investigating the interaction properties of a-TM and its fragments with the soluble a-TM-binding fragment of Tn-T, namely CB1, was demonstrated on Bio-Gel A-0.5m. The differing elution positions observed for a-TM, its fragments, and CB1 and the complexes formed among them are clearly dependent on differences in the size and shape of the components. The fact that a-TM and its fragments are highly asymmetric coiled coils undoubtedly is largely responsible for the satisfactory separations observed. This experimental approach also had the advantage that the interactions could be studied under salt and buffer conditions approximating physiological conditions. This is not true of polyacrylamide gel electrophoresis where the buffer conditions are dictated by those appropriate for the resolution of the components under investigation. Under such conditions, the observed interactions may in some cases be artifactual.

In general, the separation between the complexes and individual components was adequate enough to permit their unambiguous identification by SDS-polyacrylamide gel electrophoresis of appropriate fractions. However, in most cases the resolution was not sufficient to allow accurate calculation of stoichiometry of the individual components in a particular complex. Thus, in the present work, we have not attempted to establish the stoichiometry of the complexes in any quantitative sense. In all the studies reported in Figs. 3 and 4, the amount of CB1 was always less than that of the a-TM fragments on a molar basis. This was done to avoid the appearance of free CB1 which might have unnecessarily complicated the interpretation of some of the elution profiles.

Employing both affinity chromatography and gel filtration, we have obtained an entirely consistent set of results which are interpretable in terms of a binding site(s) for Tn-T on a-TM between residues 190 and 284. No evidence for binding of any of the NH$_2$-terminal fragments encompassing residues 1-189 was obtained. However, evidence from the gel filtration experiments indicates that, under conditions in which NH$_2$-terminal and COOH-terminal fragments show no head-to-tail interaction, formation of a ternary complex is induced by the addition of CB1 to the mixture. Since NH$_2$-terminal fragments which are truncated by 10 or 12 residues at the NH$_2$-terminal end do not show this phenomenon, we conclude that the binding of the Tn-T fragment to the COOH-terminal third of the molecule enhances the head-to-tail aggregation between the NH$_2$-terminal and COOH-terminal portions of TM molecules.

Our results and interpretations are in disagreement with those of Ueno and Ooi (31, 32) and Ueno (33) who observed cooperative binding of a mixture of NH$_2$-terminal and COOH-terminal fragments (e.g. S-fragment (a mixture of fragments tentatively identified as residues 13-149 and 13-128) and C-chain (residues 190-284) (their nomenclature)) to Tn or Tn-TC but observed little or no binding of individual fragments to Tn or Tn-TC using gel electrophoresis analysis in a running buffer of 10 mM Tris, 100 mM glycine, pH 8.1. The authors suggested the presence of two Tn-T binding sites on a-TM such that one of the sites is located near Cys-190 and extends
to some unknown region in the COOH-terminal half of the molecule while the other site is located around residue 150. We consider the presence of a Tn-T binding site near residue 150 as highly unlikely based on our findings that: 1) the two NH2-terminal fragments CT1 (residues 1-169) and Cy1 (1-189) do not bind to Tn, Tn-T, or CB1; 2) ternary complex formation was demonstrated between T1 (1-133), CT2 (170-284), and CB1 (or Tn-T) in which the central region of the original TM structure (residues 134-169) is absent.

The observations in the present work from both affinity chromatographic and gel filtration experiments suggest strongly that Tn-T binding occurs at one or more sites between residues 190 and 284 of the α-TM molecule. Furthermore, head-to-tail aggregation appears to be induced by binding CB1 to α-TM. If the site of binding of Tn-T is in the region of residues 197-217 as suggested by previous studies (14-23), it would suggest that a conformational change on the α-TM molecule caused by the binding of Tn-T would be transmitted through a distance of some 10 nm to the COOH-terminal end of the thin filament structure from close to the NH2 terminus. The hypothesis that there is a Tn-T binding site close to the COOH terminus of α-TM now also seems more attractive in light of recent studies of the nonmuscle TM from equine platelets which binds only poorly to muscle Tn and Tn-T (24). Amino acid sequence analyses of this protein have demonstrated that its primary structure in the region of residues 197-217 is very similar to that of muscle α-TM while both its NH2- and COOH-terminal sequences are markedly different (24, 25). Clearly, further biochemical and structural studies on the interactions between the muscle TM molecule and Tn-T and troponin are required before their structural and functional relationships can be more fully understood.

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