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Troponin–Tropomyosin Interactions. Fluorescence Studies of the Binding of Troponin, Troponin T, and Chymotryptic Troponin T Fragments to Specifically Labeled Tropomyosin†

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ABSTRACT: We have studied the interaction between troponin and tropomyosin by means of a fluorescent probe, *N*-(1-anilinonaphth-4-yl)maleimide (ANM), attached to the cysteine-190 residues of tropomyosin. The binding of troponin and troponin T to ANM–tropomyosin produces substantial increases in the label fluorescence. Analysis of the binding profiles indicates that both troponin and troponin T bind with a 1:1 stoichiometry. We have obtained and characterized several chymotryptic fragments of troponin T by digestion of isolated troponin T or whole troponin. An N-terminal fragment from troponin T which is slightly less than two-thirds of the whole molecule binds to tropomyosin without affecting the label fluorescence; a C-terminal fragment composed of the rest of the troponin T molecule causes a substantial enhancement of the label fluorescence. We have also isolated a complex containing the C-terminal troponin T fragment together with troponin I and troponin C from whole troponin, which also enhanced the label fluorescence. These observations indicate an elongated region of attachment between troponin T and tropomyosin.

The contraction of vertebrate skeletal muscle is regulated by changes that take place in the thin filament. These changes are initiated by the binding of Ca^{2+} ions to the troponin complex which is located at intervals of approximately 40 nm along the thin filament (Ebashi & Endo, 1968). The mechanism of regulation is believed to involve a change in the position occupied by tropomyosin in the actin long-pitch helical grooves (Haselgrove, 1972; Huxley, 1972; Parry & Squire, 1973; Wakabayashi et al., 1975; Gillis & O'Brien, 1975). The tropomyosin molecule is a coiled coil formed from two α-helical polypeptide chains 284 residues long (Stone & Smillie, 1978; Mak et al., 1980). Knowledge of the interactions between troponin and tropomyosin is necessary to understand how such changes may be brought about.

Troponin is thought to bind to tropomyosin principally through its troponin T (TnT) component (Greaser et al., 1972). There may also be an interaction between troponin I (TnI) and tropomyosin (Drabowska et al., 1976), though the evidence for this is less clear. It has been suggested that troponin attaches to a position on tropomyosin between residues 197 and 217 (McLachlan & Stewart, 1976), which corresponds to a distance of about 3 nm. However, recent evidence, obtained by electron microscopy, indicates that the bound TnT molecule extends a greater distance along the thin filament and hence along the tropomyosin molecule (Ohtsuki, 1979). In this work, Ohtsuki showed that antibodies directed against N-terminal and C-terminal chymotryptic fragments of TnT were located at positions displaced from each other by about 13 nm along the thin filament axis.

We have investigated the nature of the binding of troponin to tropomyosin by attaching a fluorescent probe, *N*-(1-anilinonaphth-4-yl)maleimide (ANM) (Kanaoka et al., 1973), to the cysteine-190 residues of tropomyosin and observing the changes in fluorescence caused by the binding of troponin, TnT, and the chymotryptic TnT fragments. ANM has previously been used as a probe of changes in tropomyosin conformation and interactions in the thin filament (Ohyashiki et al., 1976). Our results show that both troponin and TnT cause substantial increases label fluorescence when they bind and indicate that the stoichiometry of the interaction is 1:1. An N-terminal fragment of TnT binds with very little effect upon the fluorescence, indicating that it is located away from cysteine-190. A C-terminal fragment causes a substantial increase in fluorescence, indicating that it binds near cysteine-190.

Materials and Methods

Preparation of Proteins. All myofibrillar proteins used in this study were prepared from rabbit skeletal muscle. Troponin was prepared by the method of Ebashi et al. (1971) and purified by that of van Eerd & Kawasaki (1973). TnT prepared by the method of Greaser & Gergely (1973) was the generous gift of Dr. P. Leavis and E. Gowell. Actin was prepared by the method of Spudich & Watt (1971). Reduced tropomyosin was prepared as described by Lehrer (1975) with modifications described by Lehrer & Morris (1982).

Digestion of TnT and Purification of Its Fragments. Rabbit skeletal TnT was digested with chymotrypsin by using a method based on that of Ohtsuki (1979). TnT was dissolved in 0.4 M NaCl, 0.02 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), and 2 mM $MgCl_2$, pH 7.5 at 5 °C, at a concentration of about 1.5 mg/mL and digested at 0 °C

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with a 1/500 weight ratio of α-chymotrypsin (Worthington) for periods between 5 min and 3 h. The reaction was stopped by the addition of phenylmethanesulfonyl fluoride (100 mM) in acetone to give a final concentration of 1 mM. The product of digestion was was dialyzed 3 times for 12 h against 1 L of 1 mM HCl at 5 °C.

The digested TnT was dialyzed against 7 M urea–50 mM sodium formate, pH 4.0 at 5 °C, and applied to a 3 × 30 cm column of Whatman CM52 carboxymethylcellulose equilibrated in the same buffer. The column was eluted at 5 °C with a gradient of 0–0.5 M NaCl over 600 mL in the urea buffer. Fractions containing pure fragments were identified by sodium dodecyl sulfate (SDS) gel electrophoresis, pooled, dialyzed extensively against 1 mM HCl, and freeze-dried. For whole troponin, the digestion procedure was modified to minimize cleavage of components other than TnT. The troponin was digested at 25 °C with a weight ratio of 1/20 000 chymotrypsin in a solution containing 0.1 M NaCl, 20 mM Tris-HCl, and 2 mM $MgCl_2$, pH 7.5, for 30 min. The digested products were fractionated by ion-exchange chromatography on Whatman diethylaminocellulose (DE53), column size 3 × 30 cm. The fractions were applied in 25 mM Tris-HCl, pH 7.5 at 5 °C, and eluted with a gradient of 0–0.4 M KCl in the same buffer over 500 mL.

Fluorescent Labeling of Tropomyosin. Reduced tropomyosin, dissolved at 5 mg/mL in 1 M NaCl–50 mM sodium phosphate, pH 6.0 at 25 °C, was reacted at 37 °C for 2 h with a 20-fold molar excess of ANM (obtained from the Teika Seiyaku Co., Japan, and from Polysciences) added from a solution of 5 mM ANM in *N,N*-dimethylformamide. The reaction was stopped by the addition of β-mercaptoethanol in a 10-fold molar excess over ANM. The reaction product was dialyzed 3 times for 12 h against 1000 volumes of 0.1 M NaCl–20 mM sodium phosphate, pH 6.5 at 5 °C, in order to remove ANM not covalently attached to the tropomyosin. The solution was then clarified at 100000*g* for 90 min in order to remove small amounts of tropomyosin possibly denatured during the reaction.

Estimation of Protein Concentrations. The concentration of the following proteins was estimated by ultraviolet spectrophotometry using the given extinction coefficients (in units of milliliters per milligram per centimeter): G-actin, 0.63 at 290 nm (Lehrer & Kerwar, 1972); troponin, 0.45 at 278 nm (Hartshorne & Mueller, 1969); tropomyosin, 0.24 at 277 nm (Lehrer, 1978); TnT, 0.5 at 280 nm (Wilkinson, 1974). The concentration of the labeled tropomyosin was estimated by the biuret reaction of Gornall et al. (1949) calibrated with unlabeled tropomyosin.

Spectral Measurements. Ultraviolet absorption measurements were made with a Beckman 25 double-beam spectrophotometer. Fluorescence measurements were made with a Perkin-Elmer MPF-4a spectrofluorometer with a thermostated cell holder. The effects of troponin, TnT, and the TnT fragments upon the fluorescence of ANM–tropomyosin were observed by titrating these proteins into a solution containing 0.05 mg/mL ANM–tropomyosin, 0.1 KCl, 10 mM sodium phosphate, and 2 mM $MgCl_2$, pH 7.0 at 25 °C. A correction for light scattering was made by using a solution containing unlabeled tropomyosin in the same buffer and the relevant amount of troponin, TnT, or TnT fragments. This was subtracted from the ANM–tropomyosin fluorescence. The correction never exceeded 10% of the total signal. Appropriate corrections were made for dilution during the titrations.

Analysis of the Binding Profiles. The binding profiles obtained as a function of total added ligand were analyzed by using a computer program to find the values for the binding constant, K, the fluorescence change at saturation, F, and the stoichiometry, n, which best fitted the data. It can be shown that the binding of two species (A and B) to form a complex, AB_n, can be described by the equation

$$AKv^2 - (1 + nKA + KB)v + nKB = 0$$

where A and B are the total concentrations of the species A and B, v is the binding density of B to A, and n is the apparent stoichiometry of the saturated complex. This analysis assumes that for stoichiometries other than 1 the binding of different molecules to the same complex is independent. The binding profiles were fitted to this equation by a nonweighted nonlinear least-squares method.

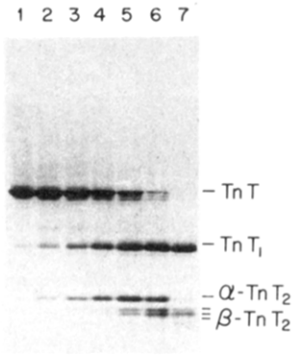

FIGURE 1: Time course of TnT digestion by chymotrypsin visualized on an SDS-gradient polyacrylamide gel. Digestion times were the following: (1) t = 3; (2) t = 5 min; (3) t = 10 min; (4) t = 30 min; (5) t = 60 min; (6) t = 120 min; (7) t = 240 min.

Binding Experiments. The ability of TnT and its fragments to bind to tropomyosin in the F-actin–tropomyosin complex was tested by the type of sedimentation experiment described by Potter & Gergely (1974) and Hitchcock (1975). To F-actin at a concentration of 0.5 mg/mL was added ANM–tropomyosin or tropomyosin and the TnT or its fragments in a 1/7 molar ratio in a solution containing 0.1 M NaCl, 5 mM $MgCl_2$, and 10 mM sodium phosphate, pH 7.0 at 5 °C. The samples were centrifuged at 100000*g* at 5 °C for 90 min. The resulting pellets were dissolved in 1% SDS–10 mM sodium phosphate, pH 7.0. The compositions of the pellets and supernatants were analyzed by SDS–polyacrylamide gel electrophoresis.

SDS–Polyacrylamide Gel Electrophoresis. The method of Laemmli (1970) was used for SDS–polyacrylamide gel electrophoresis. Gels were formed with a linear gradient of acrylamide from 9% to 18% with the highest polyacrylamide concentration closest to the anode.

Amino Acid Sequence Analysis. The N-terminal sequences of the TnT fragments were analyzed by Edman degradation using a Beckman 890C sequencer and by thin-layer chromatography as described by Lu (1980) with the exception that the sequencer was operated with program 030176. The C-terminal sequences of the fragments were analyzed by carboxypeptidase digestion using a mixture of carboxypeptidases A and B (Ambler, 1967): the release of amino acids was monitored by using a Beckman 119CL amino acid analyzer.

Results

Purification and Identification of TnT Fragments. The digestion of TnT with chymotrypsin yielded several new polypeptide chains as indicated by SDS gel electrophoresis (Figure 1). The least mobile of the new bands has a slightly lower mobility than TnI; the other bands are much more mobile than TnC (the mobilities of the fragments can be compared with those of TnI and TnC in Figure 3). The bands

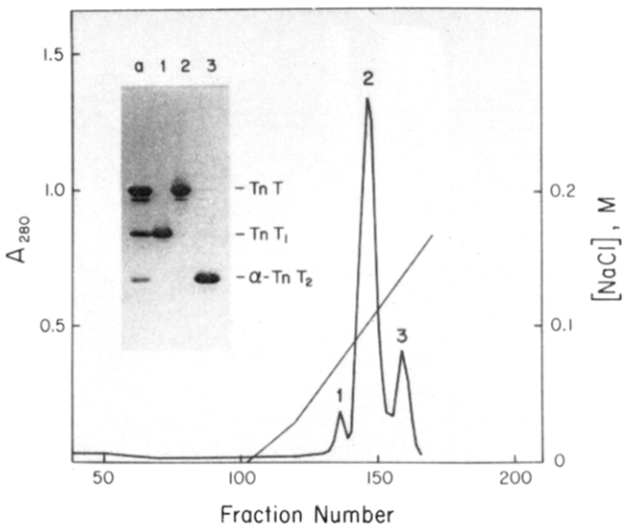

FIGURE 2: Chromatographic separation of chymotryptic fragments of TnT. Column profile of the separation of the fragments produced by digesting TnT for 10 min with chymotrypsin by ion-exchange chromatography on CM-52 in the presence of 7 M urea. A gradient of 0–0.2 M NaCl was applied. The inset shows an SDS-gradient polyacrylamide gel of (a) the starting material (TnT digested for 10 min), (1) fraction 137 (first peak), (2) fraction 147 (second peak), and (3) fraction 159 (third peak).

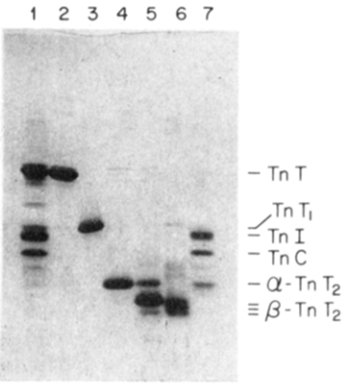

FIGURE 3: SDS–polyacrylamide gel of the protein preparations derived from troponin used in this study: (1) troponin; (2) TnT; (3) TnT_1; (4) α-TnT_2; (5) mixture of α- and β-TnT_2; (6) β-TnT_2; (7) complex of α-TnT_2, TnI, and TnC.

	1	155	158 159	227	259
Tn-T	Ac-Ser----------Tyr-Ser-Ser-Tyr-Leu-------Lys-Tyr-------Trp-Lys				
Tn-T_1	Ac-Ser----------Tyr(Ser-Ser-Tyr)				
α-Tn-T_2		(Ser-Ser-Tyr)Leu-------Lys-Tyr-------Trp-Lys			
β-Tn-T_2 (most abundant of the β fragments)		(Ser-Ser-Tyr)Leu-------Lys-Tyr			

FIGURE 4: Schematic representation of the positions in the intact TnT sequence of the chymotryptic TnT fragments as identified in this study.

appear to correspond to fragments described by Ohtsuki (1979) and Tanokura et al. (1982) which were referred to as TnT_1, α-TnT_2, and a third group of fragments, β-TnT_2. We will use this nomenclature. During the course of digestion (shown in Figure 1), the TnT_1 and α-TnT_2 bands were the first to appear: at later stages, the β-TnT_2 bands replaced the α-TnT_2 band as noted by Ohtsuki (1979). In the β-TnT_2 region, we observe three bands; these probably correspond to the three β fragments identified by Tanokura et al. (1982).

The TnT_2 fragments were separated from TnT_1 and undigested TnT by ion-exchange chromatography. A column profile illustrating the separation of the fragments obtained after a short (10-min) digestion is shown in Figure 2. Three peaks were identified and analyzed by SDS gel electrophoresis (shown as the inset in Figure 2). The first peak contained TnT_1, the second undigested TnT, and the third TnT_2. In the column profile, the first peak is considerably smaller than the third: this is because the TnT_1 fragment contains tyrosine but no tryptophan and hence absorbs less at 280 nm than the TnT_2 fragment which contains both tryptophan and tyrosine. With such short digestions (5 or 10 min), it was possible to obtain a TnT_2 peak which was quite pure, α-TnT_2 (see lane 3 of Figure 2). To obtain a sample enriched in β-TnT_2, longer digestions (1–2 h) were used. Further digestion reduced the amount of α-TnT_2 so that it became undetectable. In the following experiments, three types of TnT_2 were used: (1) α-TNT_2 prepared by using short digestions; (2) a mixture of α- and β-TnT_2 prepared by digestions of intermediate times (the β/α weight ratio was estimated by gel densitometry at 4/1); (3) a preparation obtained by using a long digestion which contained β-TnT_2 and no α-TnT_2. The different samples are shown in the SDS gel in Figure 3.

To determine the precise location of the cleavage points, an analysis was made of the N-terminal sequence of α-TnT_2. This showed that there was a major species with an N-terminal sequence corresponding to residues 159–166 of the TnT sequence given by Pearlstone et al. (1976). There was also a minor species with an N-terminal sequence corresponding to residues 156–163 of TnT. The amount of the minor species was estimated as one-third of the major species. This heterogeneity of TnT_2 was noted by Pearlstone & Smillie (1981) and Tanokura et al. (1982). The N-terminal analysis of the mixture of α- and β-TnT_2, which had a ratio of about 4 β to 1 α, gave the same result. Thus, α- and β-TnT_2 would appear to share the same N-terminal sequence, which in each case is heterogeneous.

The difference between the α and β forms of TnT_2 was identified by carboxypeptidase digestion. The C-terminal sequence of α-TnT_2 was found to be the same as that of TnT. The additional C-terminal sequence in the mixture of α- and β-TnT_2 was found to correspond to residues 222–227 of TnT. This indicates that the major C-terminus in these preparations of β-TnT_2 is tyrosine-227. The most prominent band in the gels of the α- and β-TnT_2 mixture used for this analysis was the middle one of the β-TnT_2 triplet (Figure 3, lane 5). This probably corresponds to the TnT_2 β-11 of Tanokura et al. (1982), the C-terminus of which they identified as tyrosine-227. Therefore, our analysis appears to be consistent with theirs. Pearlstone & Smillie (1981) also obtained results which inferred that tyrosine-227 was the C-terminal residue of their β-TnT_2 preparation. The location of the TnT fragments in the complete TnT sequence indicated by our results is shown in Figure 4.

Chymotryptic digestion of whole troponin under similar conditions caused the TnT component to be cleaved into two fragments with SDS gel electrophoretic mobilities similar to those of TnT_1 and α-TnT_2. An SDS gel of a digested sample is shown in lane b of Figure 5. It was possible to separate the TnT_1-like fragment from a complex of the TnT_2-like fragment with TnI and TnC by ion-exchange chromatography using Whatman DE53 diethylaminocellulose as described under Materials and Methods. A column profile is shown in Figure 5 together with an SDS–polyacrylamide gel of selected fractions. The first and second peaks (peaks 1 and 2 in Figure 5) in the column profile contain the TnT_2-like protein TnI, and TnC. Two peaks were consistently obtained in this region, with the same protein coposition as that determined by SDS–polyacrylamide gel electrophoresis. The reason for this

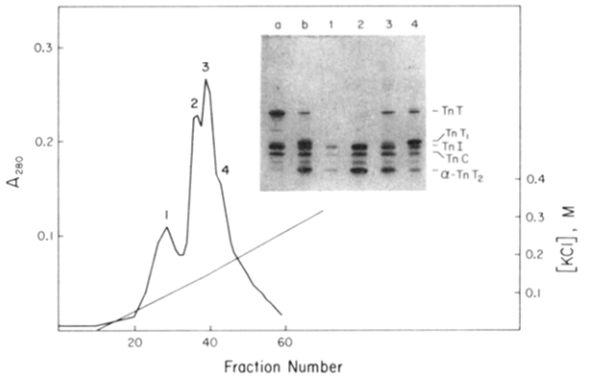

FIGURE 5: Column profile of the separation of the products of troponin digested with chymotrypsin by ion-exchange chromatography on DE53. A gradient of 0–0.4 M KCl was applied. The inset shows an SDS-gradient polyacrylamide gel of (a) undigested troponin, (b) digested troponin, (1) fraction 26 (first peak), (2) fraction 36 (second peak), (3) fraction 39 (third peak), and (4) fraction 43 (shoulder on trailing edge of third peak).

behavior is not clear. The third peak (peak 3 of Figure 5) contains undigested TnT together with the species noted for peaks 1 and 2. The TnT_1 fragment is eluted as a shoulder on the trailing edge of the third peak (Figure 5, 4). This fraction is considerably enriched in TnT_1 but also contains the species noted for the third peak. N-Terminal sequence analysis of the complex obtained in the first and second peaks showed that the TnT_2-like fragment has the same N-terminal sequence as the major species of α-TnT_2. Since the two fragments have the same mobility on SDS–polyacrylamide gels, it seems likely that they are the same polypeptides. We therefore refer to the complex isolated in the first and second peaks of the DE53 column as TnT_2–TnI–TnC. The presence of TnC and TnI appears to have little effect upon the chymotryptic cleavage of TnT after tyrosine-158. however, we noticed that the β-TnT_2 species is not produced even after long digestions: thus, TnI + TnC appears to protect the cleavage site on TnT at tyrosine-227 and the other cleavage sites which give rise to the β-TnT_2 fragments.

Properties of ANM–Tropomyosin. The absorption spectrum of ANM–tropomyosin had a peak at 345 nm, in agreement with earlier work (Ohyashiki et al., 1976). Different samples showed labeling ratios ([ANM]/[tropomyosin]) in the range 1.8–2.6. The labeling ratios were calculated by using an extinction coefficient for ANM of 10 800 cm^{-1} M^{-1} at 345 nm (Ohyashiki et al., 1974), while the tropomyosin concentration was estimated by the biuret reaction calibrated with unlabeled tropomyosin. Since there are 2.3 ● 0.2 cysteine residues for each molecule of rabbit skeletal tropomyosin as estimated by reaction with 5,5′-dithiobis(2-nitrobenzoate) (Nbs_2) (Lehrer, 1975), the cysteine labeling was essentially complete. The specificity with which the ANM was attached to cysteine residues was checked by using tropomyosin in which the sulfhydryl groups were blocked by the reaction with Nbs_2. This sample was reacted with ANM under the same conditions as the unblocked sample. The reaction product had a labeling ratio of 0.1 ± 0.05 estimated by fluorescence measurements. Thus, ANM attached at residues other than cysteine or bound noncovalently should account for no more than 8.5% of the labels attached to tropomyosin.

The fluorescence emission spectrum of ANM–tropomyosin has a maximum at 435–440 nm. This can be compared to that obtained by Ohyashiki et al. (1976) since a similar spectrofluorometer was used. The wavelength maximum which we obtained is 10–20 nm higher than that reported by these authors; the reason for this apparent discrepancy is not clear.

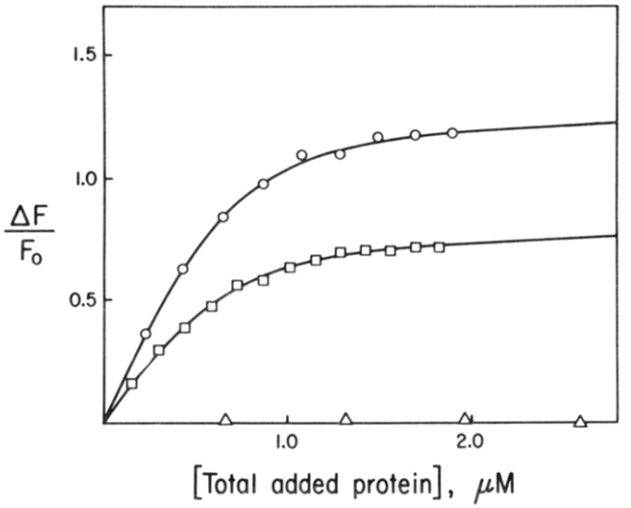

FIGURE 6: Effects of troponin and derivatives on the fluorescence of ANM-labeled tropomyosin. Effects of troponin (□), TnT (○), and TnT_1 (△). Solid curves are fitted to values for the binding constant (K), the stoichiometry (n), and the maximal fluorescence (F). Troponin, TnT, and TnT_1 were added to 0.76 μM ANM–tropomyosin in 0.1 M KCl, 10 mM sodium phosphate buffer, pH 7.0, and 2 mM $MgCl_2$ at 25 °C.

Interaction of Troponin, TnT, and Fragments of TnT with ANM–Tropomyosin. The effect of the fluorescence intensity at 400 nm of the addition of increasing amounts of troponin, TnT, and TnT_1 to ANM–tropomysin is illustrated in Figure 6. The TnT_1 fragment has essentially no effect upon the fluorescence. Troponin and TnT, however, cause substantial increases in the intesity of the fluorescence without any appreciable change in the wavelength maximum. The points obtained from the titrations with troponin and TnT were fitted to binding curves as described under Materials and Methods.

For troponin binding to ANM–tropomyosin, the fitted value for the binding constant was 6.7×10^6 M^{-1}, the stoichiometry was 0.83, and the maximal fractional increase in fluoresece was 0.81. The corresponding values for TnT binding to ANM–tropomyosin were 8.8×10^6 M^{-1}, 0.91, and 1.29. In order to determine the significance of these values, the fitting procedure was repeated, holding one parameter constant at a series of values close to the fitted value and allowing the other two parameters to assume values which gave the minimum variance. The F statistic (Bevington, 1969) was calculated from the ratio between the variance obtained in this way and the variance obtained when all three parameters were fitted freely. From tabulated values of the F statistic, ranges within which there was a 90% probability or less for each parameter were established. For troponin binding to ANM–tropomyosin, the range for the binding constant was 1.2×10^6–3.0×10^7, the lower limit of the stoichiometry was not determined and the upper limit was 1.15, and the maximum fluorescence change range was 0.73–1.09. The corresponding ranges for TnT binding were 2.0×10^6–3.7×10^7, 0.28–1.15, and 1.18–1.59.

In both the fitted curves, the best obtained fit for the stoichiometry was slightly less than 1. In each case, however, the value of 1 was included in the 90% probability range determined from the F statistic, while the value of 2 was excluded. Therefore, for a model in which it is considered that all binding sites have the same affinity, these data are consistent with a stoichiometry of one troponin or TnT molecule per tropomyosin, while a stoichiometry as great as two troponin or TnT molecules is quite improbable (e.g., the probability of a value of 1.5 for the stoichiometry was less than 0.1% for both

data sets). We have also considered a model in which the stoichiometry is 2 troponin or TnT molecules/tropomyosin with different affinities for the two binding sites. In fitting this model to the data, it was found that in both cases the best fit for the affinity of the weaker site was 0 M^{-1}. For troponin binding, the range of affinities of the weaker site, within a probability of 95%, was (0–6) × 10^5 M^{-1}, while the corresponding range for TnT was (0–3.5) × 10^5 M^{-1}. The values for the stronger binding constant at the upper ranges of the weak constants were about 5 × 10^7 M^{-1} in each case. Thus, if there is a second binding site, it is unlikely that its affinity is greater than 0.01 of the main binding site.

Additions of equimolar quantities of α-TnT_2 and TnT_2–TnI–TnC caused increases in the intensity of the fluorescence of about 20%, but because the increase did not saturate with further additions, the data were not analyzed. A preparation containing just the β-TnT_2 fragments had essentially no effect on the ANM–tropomysin fluorescence, consistent with a lack of binding of the β fragment as suggested by Ohtsuki (1979).

The ANM label is probably attached to two distinct sites on the tropomyosin molecule, cysteine-36 on the β chain of tropomyosin and cysteine-190 on both the α and β chains of tropomyosin. To determine which of the labeled sites were responsible for the observed effects, tropomysin was labeled at the cysteine-36 residue as follows. $\alpha\beta$-Tropomyosin, prepared by hydroxyapatite chromatography (Eisenberg & Kielley, 1974), was subjected to Cu^{2+}-catalyzed air oxidation as described by Lehrer (1978) to introduce a disulfide cross-link between the cysteine-190 residues. The oxidized tropomyosin was then reacted with ANM as described for reduced tropomyosin under Materials and Methods. The labeling ratio of the product was estimated as 0.5. Tropomyosin labeled in this way with ANM was relatively insenesitive to the binding of either troponin or TnT, showing less than 10% fluorescence enhancement in either case. Thus, it would seem that the labels attached at cysteine-190 are responsible for the large changes in fluorescence observed with TnT and troponin.

The addition of TnT_1 has no detectable effect upon the fluorescence of the label (Figure 6). To determine whether this was because because TnT_1 did not bind to labeled tropomyosin or whether it bound in such a way that the fluorescence of the label was not affected, we measured the binding of these preparations to actin-tropomyosin by sedimentation.

Interaction of TnT and Its Fragments with Actin–ANM–Tropomysin. The binding of TnT, TnT_1, α-TnT_2, and the mixture of α- and β-TnT_2 to actin–ANM–tropomyosin was studied by SDS gel electrophoretic analysis of the protein composition of the pellets and supernatants formed by sedimentation of the filaments (Figure 7). Tropomyosin was always present in the pellets regardless of the presence of TnT or its fragments, showing that the labeled tropomyosin is still able to bind to F-actin. The gels of the pellets show that TnT, TnT_1, and α-TnT_2 are each sedimented with the filaments. In the case of the mixture of α- and β-TnT_2, a small amount of both α- and β-TnT_2 is sedimented. The gels also show that the amount of ANM-tropomyosin in the pellets is increased and the amount in the supernatants is reduced when TnT or TnT_1 is present, indicating that these species assist the binding of tropomyosin to actin, whereas in the case of the TnT_2 fragments the amount of tropomyosin bound to the actin is essentially unaffected.

Fluorescence titrations of ANM–tropomyosin bound to actin with TnT and TnT_1 are shown in Figure 8. The results are

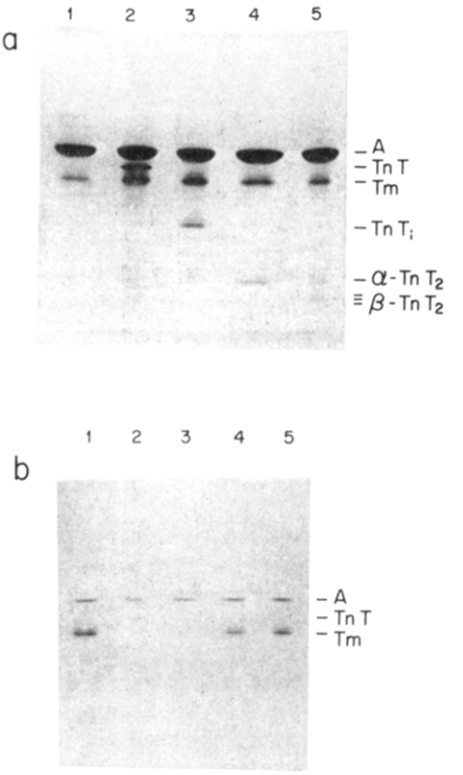

FIGURE 7: Binding of TnT and its fragments to actin–ANM–tropomyosin measured by sedimentation. SDS–polyacrylamide gradient gel of (a) pellets and (b) supernatants: (1) actin–ANM–tropomyosin; (2) actin–ANM–tropomyosin + TnT; (3) actin–ANM–tropomyosin + TnT_1; (4) actin–ANM–tropomyosin + α-TnT_2; (5) actin–ANM–tropomyosin + α-TnT_2 and β-TnT_2 mixture.

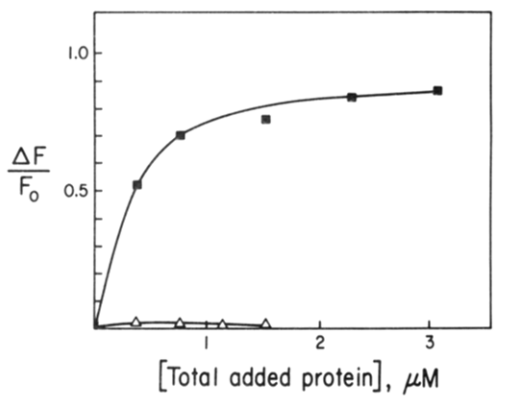

FIGURE 8: Effects on the fluorescence of ANM-labeled tropomyosin when it is attached to actin: TnT (■); TnT_1 (△). Conditions are the same as those described for Figure 6 except that actin was present at a concentration of 0.22 mg/mL.

qualitatively the same as in the case of labeled tropomyosin alone, showing a relatively large effect with TnT and very little effect with TnT_1. When α-TnT_2 was used, the fluorescence did increase, but as in the absence of actin, the curves did not saturate (data not shown). With TnT, the maximum fluorescence change appeared to be somewhat reduced in the presence of actin. A curve-fitting analysis of the titration with TnT was not attempted in this case due to possible complex effects of increased binding of ANM–tropomyosin to actin during the titration, as was observed in the sedimentation experiments.

Discussion

Nature of the TnT Fragments and the Structure of the Troponin Complex. The three TnT fragments obtained in this

study were identified in the amino acid sequence as indicated in Figure 4. This corresponds to the assignment recently made by Pearlstone & Smillie (1981). However, this was an important point to establish since Tanokura et al. (1982) have recently identified six different chymotryptic fragments in the C-terminal region of TnT. From the order of the apperance of fragments in the digestion, it would appear that the region around residues 155–159 in TnT is particularly susceptible to chymotryptic cleavage. Susceptibility in this region appears to be maintained in the intact troponin complex since similar digestion products are obtained. Interestingly, the presence of the other two troponin compnents tended to protect the TnT_2 fragment from further cleavage to produce the β form.

The ability to isolate a α-TnT_2–TnI–TnC complex by ion-exchange chromatography after chymotrypsin treatment under quite mild native conditions indicates that there is relatively little interaction between the TnT_1 region of TnT with TnI and TnC. This is in agreement with earlier indications that the TnT_2 region of TnT interacts with TnI–TnC (Ohtsuki, 1979; Katayama, 1979). Pearlstone & Smillie (1980) observed some binding between the CB3 fragment of TnT (residues 1–70) and TnI. It would seem likely from our results that this interaction is either very weak or nonexistent in the complete troponin complex.

Recent electron microscope observations of Flicker et al. (1983) show that troponin is an elongated molecule of some 26.5-nm length with both a globular and a rodlike domain and that the rodlike domain comprises part of the TnT component while the globular domain comprises TnI, TnC, and probably part of TnT. The results of Ohtsuki (1979) and Katayama (1979) would indicate that the part of TnT in the globular domain is the TnT_2 region of TnT, while the rodlike domain presumably contains the TnT_1. The susceptibility to chymotryptic digestion of the region joining TnT_1 and TnT_2 in the intact troponin complex and the relative ease with which the TnT_1 fragment can be separated from the TnT_2–TnI–TnC complex suggest that this region is located in the rodlike domain of troponin.

Enhancement of the Fluorescence of ANM–Tropomyosin Caused by Troponin Binding. The data which we obtained show that an enhancement of ANM fluorescence is associated with the binding of troponin, TnT, and α-TnT_2. Kanaoka et al. (1973) have shown that the flourescence intensity of the ANM adduct of *N*-acetylcysteine increases as the solvent polarity decreases. A likely interpretation of the enhancement of ANM fluorescence attached to tropomyosin is, therefore, that the label becomes less accessible to the polar solvent when troponin is bound. This most likely arises from direct contact between troponin and the label but may alternatively be the result of a conformational change in the region of tropomyosin to which the label is attached. The smaller maximal enhancement obtained with troponin as compared to TnT indicates that the presence of TnI and TnC alters the interaction between TnT and tropomyosin. Enhancement of fluorescence on the binding of troponin to tropomyosin has been observed with several different labels attached to cysteine-190 of tropomyosin (Tao et al., 1981; Lamkin et al., 1983).

Stoichiometry of the Interaction between Troponin and Tropomyosin. Analysis of the titrations of troponin and TnT into ANM–tropomyosin showed that the binding profiles could best be fitted to a stoichiometry of close to 1:1 and that a stoichiometry any higher than 1.5 was highly improbable. It was also shown that if there is a second binding site for troponin or TnT on tropomyosin, it is unlikely that its affinity is any greater than 0.01 of the main binking site. A 1:1 stoichiometry of troponin binding to tropomyosin has also been indicated by Lamkin et al. (1983) and Tao et al. (1981) on the basis of results obtained with different fluorescent labels. In the intact thin filament, it is fairly clearly established that the troponin/tropomyosin molar ratio is 1/1. However, since tropomyosin is composed of a coiled coil of two identical or near-identical α-helical polypeptide chains in register, for any potential binding region on one side of the molecule, there will be a second site related by 2-fold rotational symmetry. In the intact thin filament, this symmetry is lost because of the presence of the actin filament on one side of the tropomyosin, and this loss of symmetry might be used to explain the 1:1 stoichiometry of troponin–tropomyosin in the thin filament. Since our results favor a 1:1 stoichiometry in the absence of actin, this explanation is not satisfactory. One possible explanation is that the region of interaction between one troponin and one tropomysin molecule may include both sides of the tropomyosin, so at least part of the interaction region on each side is occupied by a single troponin molecule and additional binding is excluded. A second possible explanation is that binding of a single troponin molecule to one side of the tropomyosin alters its conformation in such a way as to discourage binding on the other side.

Location of Troponin Binding Regions on the Tropomyosin Molecule. A simple explanation of the enhancement in ANM fluorescence observed when troponin or TnT binds to tropomyosin is that a region of TnT binds close to the label and reduces the accessibility of the label to the polar solvent either by direct contact or by a local conformational change in the tropomyosin. It is conceivable that conformational changes are transmitted over longer distances in the tropomyosin molecule. However, our interpretation in terms of local effects is supported by independent evidence, so we will discuss the results in these terms.

The observation that the TnT_1 fragment of TnT binds with essentially no effect upon the ANM fluorescence suggests that it binds away from the label and hence away from cysteine-190 of tropomyosin. This is consistent with the observation of Mak & Smillie (1981) that binding of the CB1 fragment of TnT (residues 1–151) reduces the accessibility to iodination of two tyrosine residues (261 and 267) close to the C-terminus of α-tropomyosin, indicating that this part of TnT is attached in this region. Our fluorescence results suggest that another part of the TnT molecule is located close to cysteine-190 on tropomyosin, a conclusion supported by the original electron microscope results (Nonomura et al., 1968) when combined with the location of the cysteine-190 in the paracrystals (Stewart, 1975): this evidence is summarized by McLachlan & Stewart (1976). By elimination, it would seem likely that part of the TnT_2 region of TnT binds close to cysteine-190 on tropomysin. This is confirmed by recent experiments of Chong & Hodges (1982) in which a cross-link was introduced between cysteine-190 on tropomyosin and the TnT_2 region of TnT.

The lack of saturation of the fluorescence of ANM–tropomyosin with increasing additions of the α-TnT_2 fragment suggests that this fragment binds to multiple sites including the cysteine-190 region. Further studies are needed to clarify this suggestion. Whether or not the cysteine-190 region is the only site for the α-TnT_2 interaction, it is likely that in the intact TnT molecule the TnT_2 region is limited to this region by its attachment to the rest of the molecule, which presumably does bind in a specific manner. The β-TnT_2 fragment had essentially no effect upon the fluorescence of ANM–tropomyosin. This is consistent with the observations of Ohtsuki (1979) and Pearlstone & Smillie (1981) that this fragment, which lacks

32 residues from its C-terminus, binds to tropomyosin much more weakly than α-TnT_2.

In conclusion, our observations suggest that troponin and TnT bind to labeled tropomyosin with a 1:1 molar stoichiometry. They provide additional evidence that the C-terminal region of the TnT component binds close to cysteine-190 of tropomyosin and that TnT binds to tropomyosin over a considerable portion of the length of the tropomyosin molecule.

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Registry No. *N*-(1-Anilinonaphth-4-yl)maleimide, 50539-45-2.

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