Regulatory Properties of the NH₂- and COOH-terminal Domains of Troponin T

ATPase ACTIVATION AND BINDING TO TROPONIN I AND TROPONIN C

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The contraction of skeletal muscle is regulated by Ca²⁺ binding to troponin C, which results in an internal reorganization of the interactions within the tropomyosin-tropomyosin complex. Troponin T is necessary for Ca²⁺-dependent inhibition and activation of actomyosin. Troponin T consists of an extended NH₂-terminal domain that interacts with tropomyosin and a globular COOH-terminal domain that interacts with tropomyosin, troponin I, and troponin C. In this study we used recombinant troponin T and troponin I fragments to delimit further the structural and regulatory interactions with the thin filament. Our results show the following: (i) the NH₂-terminal region of troponin T activates the actomyosin ATPase in the presence of tropomyosin; (ii) the interaction of the globular domain of troponin T with the thin filament blocks ATPase activation in the absence of Ca²⁺; and (iii) the COOH-terminal region of the globular complex anchors the troponin C–troponin I binary complex to troponin T through a direct Ca²⁺-independent interaction with the NH₂-terminal region of troponin I. This interaction is required for Ca²⁺-dependent activation of the actomyosin ATPase activity. Based on these results we propose a refined model for the troponin complex and its interaction with the thin filament.

The mechanism through which TnT exerts its role in the regulatory function of the troponin complex is not fully understood. TnT interacts with TnC, TnI, and Tm and holds the TnC/TnI dimer in the thin filament irrespective of the Ca²⁺ concentration (9, 15–17).

Each Tm dimer spans seven actins, strongly suggesting that the regulatory function of the troponin complex is transduced through Tm to the actin molecules. TnT is the troponin subunit that most strongly binds to Tm (1, 2). Two separate sites of attachment for TnT on Tm have been identified. The first site is near the head-to-tail overlap of sequential Tm molecules along the filament (16) and interacts with the T1 fragment of TnT (Fig. 1). This interaction is independent of Ca²⁺ binding to TnC (18). The second site is within fragment T2 of TnT (Fig. 1) which binds near residues 150–180 of the Tm molecule (16, 19). The T2 fragment of TnT also interacts with TnC and TnI in vitro and to Tm in a Ca²⁺-sensitive manner in the presence of TnC (18).

In this work we used different combinations of TnT and TnI fragments to map the regions involved in both structural and regulatory interactions present in the trimeric troponin complex within the thin filament in the presence and in the absence of calcium.

MATERIALS AND METHODS

Proteins—Actin (20) and myosin (21) were prepared from the pectoralis major muscle of adult chickens. α-Tm (22) was prepared from adult chicken heart muscle. Recombinant TnI and TnC were isolated as described (23, 24). Deletion mutants of TnI were prepared as described (13).

Construction of TnT Deletion Mutants—Chicken skeletal muscle TnT-3 cDNA (GenBankTM accession number M22156) (25) was used as a template for site-directed mutagenesis (26). An NdeI site was inserted at codon Met1 (13). M13mp18-TnT-3 was mutated with the oligonucleotide 5′-GTTATACCAGTAGCAGACTGA-3′ to change the codon Leu217 into a stop codon (underlined), with the oligonucleotide 5′-GCGCGAAGTAATGACATTGAA-3′ to change the Pro250 codon into a stop codon, and with the oligonucleotide 5′-AGGCGCCAAGTACATTGAAATTGAA-3′ to introduce an NdeI site (underlined) at codons 192 and 193. The NdeI-EcoRI fragments of the M13mp18-TnT-3 containing a stop codon at positions 192 or 217 were cloned into the same restriction sites of the pET-3a (27). These vectors express, respectively, the fragments TnT192–216 (the first 216 amino acids of TnT) and TnT1–191 (the first 191 amino acids of TnT). The small NdeI-EcoRI fragment (produced by the second NdeI site, inserted at positions 192 and 193) was subcloned in the same sites of pET-3a, producing a vector for the expression of the fragment TnT194–263 (amino acids 194–263). For the production of the fragment TnT157–263, the NcoI-BamHI fragment of the expression vector pET-TnT* (11) was subcloned in the same sites of pET-3d (27).

Expression and Purification of the Recombinant TnT Fragments—Escherichia coli BL21(DE3) pLysS (27) was used to express wild-type TnT, TnT1–216, TnT1–191, TnT157–263, and TnT194–263. Cultures (4 liters) of E. coli harboring the different plasmids grown in 2×YT were induced with isopropyl-1-thio-β-D-galactopyranoside (0.4 mm final) in mid-log phase (A₆₀₀ = 0.8) and incubated for 3 h at 37 °C. For the purification of wild-type TnT, TnT1–216, TnT1–191, and TnT157–263, the cells were
recovered by centrifugation at 3,000 × g (15 min), resuspended in 100 ml of 50 mM Tris-Cl, pH 8.0, 1 mM EDTA, 6 μM urea, 1.4 mM β-mercaptoethanol and lysed in a French press at 16,000 p.s.i. The extract was centrifuged (109,200 × g, 40 min), and the supernatant was loaded onto a DEAE-Sepharose Fast-Flow column (Pharmacia XK16/70), and eluted with a 0–600 mM NaCl gradient. The fractions containing TnT were pooled and dialyzed against 50 mM sodium acetate, pH 5.0, 1 mM EDTA, 6 μM urea, 1.4 mM β-mercaptoethanol and loaded onto a CM-Sepharose Fast-Flow XK16/40 column equilibrated with the same buffer. Proteins were eluted with a 0–600 mM NaCl gradient in the same buffer, dialyzed against 50 mM Tris, pH 8.0, 1 mM EDTA, 1 mM KCl, 1 mM DTT and stored at −20 °C. For the purification of the TnTΔp263 fragment the E. coli cells were resuspended in 50 mM Tris, pH 8.0, 1 mM EDTA, 1.4 mM β-mercaptoethanol, lysed in the French press at 16,000 p.s.i., and centrifuged at 75,800 × g (40 min). The supernatant was loaded into a CM-Sepharose Fast-Flow XK16/40 column equilibrated with the same buffer. The protein was eluted with a 0–500 mM NaCl gradient, dialyzed against 50 mM Tris, pH 8.0, 6 μM urea, 1.4 mM β-mercaptoethanol and loaded into a DEAE-Sepharose Fast-Flow column. The protein eluted in the flow-through was dialyzed against 50 mM Tris, pH 8.0, 1 mM EDTA, 1 mM KCl, 1 mM DTT and stored at −20 °C. One molar KCl is necessary to maintain the TnTs in solution at high concentrations (100 μM), TnT−121 soluble in lower salt concentrations. Protein concentrations were determined (28), and the samples were analyzed in 15% SDS-PAGE. The deletion fragments presented the expected molecular masses: 25.5 kDa (TnTΔp263), 22.5 kDa (TnTΔ193), 12.5 kDa (TnTΔ157–263), and 8.5 kDa (TnTΔp263).

Reconstitution of the Troponin Complex—Tn subunits (20 μM final concentration) were combined in a 1:1:1 molar ratio in 6 mM urea, 1 mM KCl, 50 mM CaCl₂, 20 mM imidazole, pH 7.5, 1 mM DTT. Successive dialysis (4 °C, 12 h each) against the same buffer containing 5.6 μM urea, 2 μM NaCl, 100 μM KCl, and CaCl₂ were used to gradually reduce the urea and salt concentrations. After dialysis the reconstituted complexes were centrifuged (12,000 × g, 10 min), and the supernatant was aliquoted and stored at −70 °C.

Actomyosin ATPase Measurements—The actomyosin ATPases were measured as described previously (11, 13). Actin (4 μM), Tn (0.58 μM), Tm (0.2 μM) and CaCl₂ (7 M) were combined in 20 mM imidazole, pH 7.0, 60 mM NaCl, 0.5 mM CaCl₂, 2 mM β-mercaptoethanol (+Ca²⁺), or in the same buffer with 0.5 mM EGTA replacing the CaCl₂ (+Ca²⁺). The mixtures were centrifuged at 315,000 × g for 10 min at 4 °C in a Beckman Optima TLX ultracentrifuge. The pellets were rinsed and resuspended in the original volume. Equivalent volumes of the mixture before centrifugation and of the supernatants and pellets after centrifugation were analyzed by 15% SDS-PAGE and 12.5% Tricine/SDS-PAGE (30) for the small fragments TnTΔp263 and TnTΔ157–263. Densitometric quantification was performed using a dual wavelength scanner (Shimadzu CS-9000) at 550 nm. Since TnT and Tn and their fragments have a low solubility at low ionic strength, control experiments in the absence of actin-Tn were performed to ensure that at these relatively low protein concentrations, all the Tn components were soluble.

RESULTS

TnT Deletion Mutants—A schematic representation of the TnT mutants is shown in Fig. 1. The precise sites for the insertion of stop codons were chosen so that the predicted α-helices would not be interrupted (Fig. 1). The mutants were expressed as non-fusion proteins and purified to homogeneity. When Tn complexes containing wt-TnC, wt-TnI, and wt-TnT or the different TnT fragments were reconstituted from the isolated components. After reconstitution, the TnT mutants became soluble at low ionic strength at high protein concentrations, indicating that they were incorporated into stable complexes. When analyzed by gel filtration in the presence of Ca²⁺, all complexes eluted as a single peak (data not shown). In the absence of Ca²⁺, TnT1–216 was dissociated from the ternary complex, which suggests that residues 217–263 in the COOH-terminal region of TnT are necessary for the incorporation of TnT into the trimeric complex in the absence of Ca²⁺ (see below).

Regulation of Actomyosin ATPase—We analyzed the regulatory properties of complexes containing the different TnT mutants (Fig. 2A). Under physiological molar ratios of actin, Tm, and Tn (7:1:1), we confirmed that the control Tn complex containing wt-TnT confers full Ca²⁺ sensitivity to actomyosin ATPase, i.e., it inhibits the ATPase activity in the absence of Ca²⁺ and activates the ATPase activity in the presence of Ca²⁺ (10) (activation is defined as the ability of troponin, in the presence of Ca²⁺, to increase the actomyosin/tropomyosin ATPase activity to levels above its activity in the absence of troponin). We also confirmed that the binary Tn/TnC complex was not able to inhibit or to activate the ATPase activity at physiological ratios of actin to troponin (Fig. 2A) (10, 31).

Inhibitory Function of the Mutant TnT Complexes—Deletions of the COOH-terminal region of TnT or deletion of the T₁ region reduces the inhibitory function of the troponin complex (Fig. 2A): TnT1–216 and TnT157–263 complexes inhibit the ATPase activity to about 60% and TnT1–191 complex to about 75%, whereas the wt-TnT complex inhibits to about 35%. The complex containing TnT194–263 conferred no Ca²⁺ sensitivity to the actomyosin ATPase, a behavior identical to the TnTNC binary complex (Fig. 2A). Since the partial inhibition observed for the TnT mutant complexes could be explained by a lower affinity for the thin filament, we performed ATPase experiments using increasing molar ratios of the troponin complex to actin. We observed full inhibitory activity of complexes containing TnT1–216, TnT157–263 and TnT194–263 at actin/troponin molar ratios of 4:7, 3:7, and 3:3 (data not shown). These results imply that the region shared by these mutants, namely amino acids 157–216, bind to the thin filament and may have an inhibitory role.

Activation Function of the Mutant TnT Complexes—The complexes containing TnT1–216, TnT157–263, and TnT194–263 were not capable of activating the ATPase activity (Fig. 2A) even when increasing ratios of Tn to actin were used (data not shown). The complex containing TnT1–191 was the only one capable of activating the ATPase activity like wt-Tn in the presence of Tm (Fig. 2A). This suggests that the NH₂-terminal end of TnT (residues 1–191) contains the region responsible for the activation of the ATPase activity. The evidence presented so far suggests that regions 1–191, 157–216, and 217–263 of TnT participate, respectively, in activation, inhibition/thin filament binding, and Tn/TnC binding. The experiments described below were designed to test and essentially confirm these hypotheses.

Effects of Isolated TnT Fragments on the Actomyosin ATPase—The direct effects of the different regions of TnT on the inhibition and activation of the ATPase activity was analyzed in the presence of Tn but in the absence of TnI and TnC (Fig. 2B). TnT1–191 is able to activate the ATPase activity, whereas wt-TnT and the mutants TnT157–263 and TnT194–263 are not. TnT1–216, which is not able to activate the ATPase activity in the context of the ternary complex (Fig. 2A), is able to activate it in the absence of TnC and TnI, although to a lesser extent than TnT1–191. These results indicate that residues 191–263 of TnT are blocking the ability of region 1–191 to activate the ATPase activity and may explain why no activation is observed with isolated full-length TnT.

TnC/TnI Regions Involved in the Activation of the ATPase—Since full-length TnT alone does not activate the ATPase, but does so in the presence of TnI/TnC, we determined which regions within TnC/TnI are required for activation. We used

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TnI103–182, which binds to TnC-Ca$^{2+}$ and is capable of inhibiting the ATPase activity but does not bind to TnT, and the TnI1–98 mutant, which binds to TnT and TnC but shows no inhibitory ability (13). The regulatory properties of the ternary complexes containing TnT, TnC, and the different TnI fragments are shown in Fig. 3A. Although the complex containing TnI103–182 inhibited the ATPase activity as well as the complex containing TnI, it was not able to activate the ATPase activity. The complex containing TnI1–98 activates the ATPase to the same extent as the complex containing wt-TnI (Fig. 3A). This

FIG. 1. Schematic representation of troponin T and the deletion mutants. The chymotryptic fragments (T1, TnT1; T2, TnT2) and cyanogen bromide fragments (CB2, CB5, CB4, and CB6) are indicated (1, 2). Two highly conserved regions present in TnTs from different species (from Drosophila melanogaster to Homo sapiens) are represented by shaded boxes. The first of these regions corresponds closely to CB2, which is predicted to be highly helical and the site of interaction of the T1 fragment with Tm. The second, within T2, coincides with a region containing a series of heptad repeats, typical of coiled-coil structures (2, 5, 36, 54). The exons present in the cDNA of isoform 3 (25) are numbered. The predicted α-helices (55) are represented by boxes below wt-TnT. Two COOH-terminal deletion mutants and two NH$_2$-terminal deletion mutants were constructed. TnT157–263 corresponds closely to the T2 proteolytic fragment (TnT157–216 is four amino acids longer at the NH$_2$ terminus than T2).

FIG. 2. Ca$^{2+}$-dependent regulation of the actomyosin ATPase activity by Tn complexes. A, complexes containing the TnT deletion mutants. The effect of the reconstituted complexes on the actomyosin ATPase activity was measured as a function of pCa. The results are expressed as a percentage of the actin-Tm-activated Mg$^{2+}$-ATPase of myosin obtained in the absence of Tn. The averages (±S.E.) of at least five independent determinations at each pCa are shown. Control experiments were performed with the wt-Tn complex and with the binary TnC–TnI complex. Assay conditions are as follows: actin (4 μM), Tm (0.57 μM), Tn (0.57 μM), and myosin (0.2 μM) in 20 mM imidazole HCl, pH 7.0, 60 mM KCl, 3.5 mM MgCl$_2$, 1 mM DTT, 0.5 mM EGTA, 2 mM NaATP, pH 7.0. CaCl$_2$ was added to give the free Ca$^{2+}$ concentration indicated. Ternary Tn complexes are abbreviated by the name of the TnT mutant followed by Tn. B, the effect of TnT mutants in the absence of TnI and TnC on the actomyosin ATPase activity. The actomyosin ATPase activity was determined at increasing concentrations of the different TnTs (indicated in the abscissa). 100% corresponds to the activity of actin/Tm/myosin in the absence of Tn. Assay conditions are as in A.
activation was independent of the Ca$^{2+}$ concentration, although slightly higher levels of activation were produced in the absence of Ca$^{2+}$ as observed previously (13). These results indicate that the NH$_2$-terminal region of TnI but not the COOH-terminal region is necessary for the activation of the ATPase activity.

Next we analyzed if the NH$_2$-terminal half of TnI is sufficient to activate the ATPase activity or if TnC is also required. The ATPase activity in the presence of filaments containing the different TnT mutants was titrated with increasing amounts of TnI$_{1–98}$ in the absence of TnC (Fig. 3B). TnI$_{1–98}$ in combination with wt-TnT does not activate the ATPase in the absence of TnC. TnI$_{1–98}$ in combination with TnT$_{157–263}$ was also not able to activate. The effects of TnT$_{1–191}$ and TnT$_{1–216}$ on the ATPase activity were not altered by the addition of TnI$_{1–98}$ (indicated in the abscissa), in the absence of TnC. The symbols used are: ○, WT TnT; ■, TnT$_{1–216}$; ▲, TnT$_{1–191}$; ◊, TnT$_{157–263}$.

In conclusion, our ATPase results show that residues 1–191 of TnT contain an activation domain, whereas the region between residues 191 and 263 has an inhibitory effect. Thus activation can only be observed with intact TnT in the presence of TnC/TnI and Ca$^{2+}$. Furthermore, the NH$_2$-terminal regions of TnI in conjunction with TnC are necessary for activation by the ternary complex.

Incorporation of the Troponin Complexes into the Thin Filament—

The interactions between TnT fragments, TnI and TnC, were analyzed in an actin/tropomyosin co-sedimentation assay. The TnC$_{z}$TnI complex is partially retained in the thin filament in the absence of Ca$^{2+}$ but remains soluble if Ca$^{2+}$ is present (Fig. 4A). In the presence of wt-TnT, all three components of the Tn complex remained in the thin filament, irrespective of the Ca$^{2+}$ concentration (Fig. 4B) (15, 31). If trimeric complexes containing TnT$_{1–216}$ and TnT$_{1–191}$ are incubated with actin/Tm, these two TnT mutants remained associated with the thin filament in the presence or absence of Ca$^{2+}$ but were not able to retain the TnC/TnI dimer in the thin filament in the presence or absence of Ca$^{2+}$.
ence of Ca\(^{2+}\) (Fig. 4, C and D). These controls confirm the previous observations that TnT is required to anchor the TnC/TnI complex to the thin filament in the presence of Ca\(^{2+}\) (15, 31) and that only the T\(_2\) region of TnT interacts with the TnC/TnI dimer (16, 32). These results specifically implicate the COOH-terminal half of T\(_2\) (residues 217–263) in this interaction.

**Interaction between the COOH-terminal Region of TnT and NH\(_2\)-terminal Region of TnI**—It has been previously demonstrated that the NH\(_2\)-terminal region of TnI (TnI\(_{1–98}\)) is required for the incorporation of TnT into the ternary complex (12, 13). Fig. 4E shows that the binary TnC/TnI\(_{1–98}\) complex does not associate with the thin filament in the absence of TnT irrespective of the Ca\(^{2+}\) concentration. In contrast, complexes containing TnC/TnI\(_{1–98}\)-wt-TnT are always associated with the thin filament (Fig. 4F). Since TnI\(_{1–216}\) and TnI\(_{1–191}\) lack residues 216–263 which interact with the TnC/TnI binary complex, they were not expected to, and indeed did not, retain TnI\(_{1–98}/TnC\) bound to the thin filament (Fig. 4, G and H).

Control complexes containing TnI\(_{103–182}\) (which does not bind TnT) were analyzed. TnI\(_{103–182}\) remained associated with the filament in the absence of Ca\(^{2+}\) and was removed by TnC in the presence of Ca\(^{2+}\), both in the presence and absence of TnT (13, data not shown). These findings demonstrate that the NH\(_2\)-terminal region of TnI interacts with the region between residues 216 and 263 of TnT and that this interaction is the major point of calcium-independent anchoring of the TnC/TnI dimer to TnT.

Since isolated TnC has been shown to interact with TnT in the presence of Ca\(^{2+}\) (reviewed in Refs. 1, 2, and 5), we analyzed the binding of the binary TnC/TnT complex (without TnI) to actin/Tm. The amount of TnC associated with the thin filaments was measured as a function of total TnC added in the assay to obtain approximately a 1:1 ratio of TnC:TnI bound to the thin filament (Fig. 5). In the presence of TnI, a 2-fold excess of TnC (6 \(\mu\)M) was sufficient. This suggests that there are two different sites of interaction between TnC and the thin filament in the presence of Ca\(^{2+}\). The first, through binding to TnI which in turn binds TnT, is a strong binding site (reviewed in Refs. 1, 2, and 5). The second, through direct interaction of TnC with TnT, is much weaker.

![Figure 5](Image)  
**Fig. 5.** Incorporation of TnC into the thin filament in the presence of Ca\(^{2+}\). Co-sedimentation was performed using actin and Tm as described in Fig. 4. Thin filaments were reconstituted with the wt-TnT and increasing concentrations of TnC or with wt-TnTw-TnI (1:1) and increasing concentrations of TnC (the 7:1 actin to troponin T ratio was kept constant). The ability of these complexes to retain TnC in the thin filament was analyzed. The amount of TnC retained in the pellet was quantified by densitometric scan of the 15% SDS-polyacrylamide gels.

![Figure 6](Image)  
**Fig. 6.** Interaction of TnT\(_{157–263}\) with the thin filament, and the effect of the TnI mutants on this interaction. The ability of TnT\(_{157–263}\) to bind to actin/Tm was analyzed in co-sedimentation assays in the presence of wt-TnI and TnC (A), in the presence of TnC (C), in the presence of TnC and TnI\(_{103–182}\) (D), and in the presence of TnC and TnI\(_{103–182}\) (E). A ternary complex containing TnT\(_{157–263}\), TnC, and TnI\(_{1–98}\) was analyzed (B). A, B, a 3-fold excess of the complex had to be used to visualize the TnT\(_{157–263}\) complex. Note that in this case, the excess of the TnT\(_{157–263}\) containing complex will remain in the supernatant. The experiments were performed as described in Fig. 4. All the samples were analyzed in Tricine/SDS gels. The molecular weights are as follows: TnT\(_{157–263}\), M\(_r\) 12,532; TnT\(_{194–263}\), M\(_r\) 8,570; TnI\(_{1–98}\), M\(_r\) 11,433; and TnI\(_{103–182}\), M\(_r\) 9,526.

**The Interaction of the Region between Residues 157 and 263 of TnT with the Thin Filament**—In the presence and absence of Ca\(^{2+}\), the affinity of the TnT\(_{194–263}\)/TnC/TnI complex for the thin filament is reduced (Fig. 6B) when compared with the affinity of the TnT\(_{157–263}\)/TnC/TnI complex (Fig. 6A). This result suggests that a binding site to actin/Tm may reside in the NH\(_2\)-terminal region of TnT\(_{157–263}\) (residues 157–191) and that this binding is influenced by Ca\(^{2+}\) binding to TnC. When the control TnT\(_{157–263}/TnC\) complex was incubated with actin/Tm in the absence of Ca\(^{2+}\), TnT\(_{157–263}\) was incorporated into the filament while TnC remained in solution (Fig. 6C). This confirms previous observations that TnT\(_{157–263}\) itself presents a binding site to actin/Tm (16, 32). In the presence of Ca\(^{2+}\), both subunits remained in the supernatant indicating that TnC is able to remove TnT\(_{157–263}\) (TnT\(_{2}\)) from the thin filament in the presence of Ca\(^{2+}\) (Fig. 6C).

Even though TnT\(_{2}\) fragment alone is able to interact with Tm (18) and with actin/Tm, it is still not clear how strong this interaction is in the context of the ternary troponin complex. Our results do not rule out the possibility that the interaction of TnT\(_{157–263}\) with the thin filament in the absence of Ca\(^{2+}\) is mainly mediated by TnI, which itself binds to the thin filament.
To address this question we investigated how the interaction of TnT157–263 with the thin filament is modulated by the TnI fragments (Fig. 6, D and E). The TnT157–263, TnI1–98, TnC and TnT157–263, TnI103–182, TnC complexes were reconstituted, and their ability to be incorporated into the thin filament was analyzed. TnI103–182 binds actin/Tm and TnI1–98 does not (13). The TnT157–263, TnI1–98, TnC complex was not incorporated into the filament irrespective of the Ca\(^{2+}\) concentration (Fig. 6D). When the trimeric TnT157–263, TnI103–182, TnC complex was incubated with actin/Tm in the absence of Ca\(^{2+}\), TnI103–182 and TnT157–263 were incorporated into the filament, whereas TnC remained in solution (Fig. 6E). In the presence of Ca\(^{2+}\), the three subunits remained in solution (Fig. 6E). These results indicate that although Ca\(^{2+}\) binding to TnC was able to remove both TnT157–263 and TnI103–182 from the filament, the interaction of TnI1–98/TnC with TnT reduces the affinity of TnT157–263 for the thin filament irrespective of the Ca\(^{2+}\) concentration. Therefore, we conclude the following. (i) TnI1–98 seems to predispose the dissociation of TnT157–263 from the thin filament independent of Ca\(^{2+}\) concentration. Therefore, in the context of the ternary complex, the direct interaction between TnT2 and the thin filament is likely to be diminished. This observation needs to be confirmed with direct evidence obtained from equilibrium binding experiments. This explains the Ca\(^{2+}\)-independent activation by TnT/TnI1–98/TnC. (ii) The main function of Ca\(^{2+}\) binding to TnC is the dissociation of TnI103–182 from the thin filament.

**DISCUSSION**

Troponin inhibits the actomyosin-tropomyosin Mg\(^{2+}\)-ATPase in the absence of Ca\(^{2+}\) and activates the ATPase in the presence of Ca\(^{2+}\). The original model of two-site binding between troponin and actin-Tm (32, 33) distinguished the Ca\(^{2+}\)-independent interaction between the NH\(_2\)-terminal domain of TnT (TnI\(_1\)) and Tm-actin from the Ca\(^{2+}\)-dependent interaction of the globular troponin domain (TnT-TnI-TnC) with actin-Tm. In the two-site binding model, Ca\(^{2+}\)-induced dissociation of the globular domain removes inhibition by TnI; this model does not address how activation is achieved. In this paper we have identified a TnT fragment that possesses intrinsic activation activity. We also showed that the anchoring of the TnC/TnT dimer to TnT occurs via an interaction between the amino-terminal domain of TnI and the last 50 residues of TnT. These two new aspects of the troponin complex have been incorporated into a refined model of thin filament regulation.

*The Amino-terminal Region of TnT Activates Actomyosin ATPase—Ca\(^{2+}\)-induced activation of actomyosin ATPase activity is a well known property of muscle tropoen (10, 34) and recombinant troponin (11). In the absence of the inhibitory domain of TnI, the troponin complex activates the ATPase independently of the Ca\(^{2+}\) concentration (Ref. 13 and this work). Although TnT1 is required for activation in the context of the whole complex, very few studies have analyzed the regulatory properties of isolated TnT and TnI fragments. Studies of the effects of isolated TnT on the ATPase have varied from no significant effect (this work), to a slight inhibitory effect ascribed to contaminating TnI (10), to a significant inhibitory effect (35). In this report we demonstrate that two amino-terminal fragments of TnT (TnT11–191 and TnT11–216) can activate the ATPase in the presence of Tm to levels observed for the whole troponin complex in the presence of Ca\(^{2+}\). Although this effect is probably mediated by Tm, to our knowledge, this is the first report that activation of the actomyosin ATPase can be obtained with a fragment derived from a single troponin subunit.

In the context of the whole troponin complex, only full-length TnT and TnT11–191 activated the ATPase, whereas a larger NH\(_2\)-terminal fragment (TnT11–216) or a COOH-terminal fragment (TnT11–191) failed to activate. Troponin complexes reconstituted with these TnT fragments bound to the thin filament and did inhibit in the absence of Ca\(^{2+}\), although full inhibition was achieved only at Tn:actin ratios higher than those required by the wild-type complex. Troponin reconstituted with the smallest COOH-terminal TnT fragment (TnT94–263) did not inhibit or activate and showed very low affinity for the filament, suggesting that residues 156–193 are involved in either binding or inhibition (see also “Discussion” in Ref. 36). Other workers have analyzed the regulatory properties of TnT fragments incorporated into troponin complexes. Ohtsuki and co-workers (37, 38) have presented the only other studies of the regulatory properties of an amino-terminal fragment of TnT, namely TnT1 (residues 1–158; see Fig. 1) obtained by chymotryptic digestion of rabbit skeletal muscle TnT. The behavior of TnT1 was significantly different from what we observed for TnT11–191. Troponin reconstituted with TnT1 inhibited the ATPase at low Ca\(^{2+}\) concentrations and did not activate the ATPase to levels above those observed in the absence of TnT. A direct comparison of the results obtained with rabbit TnT and chicken TnT11–191 is complicated by their different lengths (our chicken TnT11–191 corresponds to residues 1–186 of the rabbit TnT sequence, Ref. 39). Their different sequences and the fact that the rabbit TnT fragments presumably contain a mixture of isoforms with significant heterogeneity (25, 40) must also be taken into consideration. The region between residues 162 and 191 alone is not responsible for activation since TnT157–263 did not activate on its own or in reconstituted troponin. Analysis of the regulatory properties of smaller amino-terminal fragments of TnT isoforms is necessary to localize the precise sequences responsible for this activation.

A number of studies have analyzed the regulatory properties of COOH-terminal fragments (or small NH\(_2\)-terminal deletions) of TnT in the context of the whole complex. Ohtsuki and co-workers (37, 38, 41) analyzed the regulatory properties of TnT2a (residues 159–259; see Fig. 1) derived from chymotryptic digestion of rabbit skeletal muscle TnT. In the two earlier studies (37, 41), troponin complexes reconstituted with TnT2a inhibited the ATPase but did not activate above levels observed in the absence of troponin, in agreement with our observations for complexes reconstituted with TnT94–263. In a later study (38), both activation and inhibition was observed at levels comparable to that observed for wild-type troponin. These differences were attributed to contamination by TnT2b fragments (residues 159–242) in the earlier reports. Recent reports from other laboratories have concentrated on larger COOH-terminal fragments. Fragments corresponding to residues 39–284 of bovine cardiac TnT and residues 46–259 of rabbit skeletal TnT both activated and inhibited the acto-S1 ATPase, but in both cases the level of activation was significantly reduced (see Fig. 3 in Ref. 42 and Fig. 6 in Ref. 43). These results are consistent with the conclusion that the amino-terminal region of TnT is involved in the activation activity of TnT.

*The Carboxyl Terminus of TnT Anchors the TnC/TnI Dimer by Way of the Amino Terminus of TnI—*It is well established that the TnC-TnI binary complex binds to the COOH-terminal globular domain of TnT, although the exact sequences involved in the interactions with TnC and TnI are not fully elucidated (reviewed in Refs. 2 and 44). Although TnC can bind to TnI on its own (45–49), comparison of the free energies of formation of troponin binary and ternary complexes shows that the TnT-TnC interaction within troponin may be weaker than that observed in the binary complexes (47, 48). We also found that the TnC-TnI interaction is relatively weak when TnT is bound to the thin filament and that stoichiometric binding at micro-
Ca\(^{2+}\) residues 216–263 of TnT abolishes the binding of TnI 1–98-TnC to the thin filament. The activation function of the NH\(_2\)-terminal domain of TnT is implied since it is not explicitly stated in the two-site binding model (32, 33), an amino-terminal domain of TnI (TnI1–98), and the last 50 residues of TnT. This leads to activation of the ATPase activity in the presence of Ca\(^{2+}\) alone. In the presence of Ca\(^{2+}\), TnT switches to a strong interaction with N-TnC and there is also a weak interaction with TnC. The precise regions of TnC or TnT involved in this interaction in the presence of Ca\(^{2+}\) were not determined. The Ca\(^{2+}\)-induced changes result in the removal of the inhibitory region of TnT and the region between residues 157–216 of TnT from the thin filament. This leads to activation of the actomyosin ATPase activity by the activation domain of TnT (located within residues 1–191). The attachment of the NH\(_2\)-terminal half of TnT (residues 1–98) to residues 216–263 of TnT and to N-TnC are necessary for the release of this activation function present within the amino-terminal domain of TnT.

molar protein concentrations only occurs in the presence of TnI. The amino-terminal domain of TnI interacts with TnT (13, 36, 50–52) and is involved in the Ca\(^{2+}\)-independent interaction with TnC (13, 53). In this work we presented the following evidence supporting the conclusion that residues 216–263 of TnT are involved in the interaction with TnI1–98: (i) deletion of residues 216–263 of TnT abolishes the binding of TnI1–98-TnC to the thin filament; and (ii) the deletion of residues 1–102 of TnI abolishes the binding of TnC/TnI103–182 to actin-TnT in the presence of calcium. We conclude that the three subunits are held together by a core of structural, Ca\(^{2+}\)-independent interactions between the COOH-terminal domain of TnC, the amino-terminal domain of TnI (TnI1–98), and the last 50 residues of TnT. Consistent with this finding, Jha et al. (36) reported that a deletion of residues 202–258 of human fast skeletal TnT reduces its interaction with rabbit TnI-TnC and that an amino-terminal fragment of Tn (TnI1–120) forms a stable complex with TnT.

Transmission of the Ca\(^{2+}\) Binding Signal to TnT—Although not explicitly stated in the two-site binding model (32, 33), an activation function of the TnI1 fragment is implied since it would be the only fragment interacting with actin-Tm in the presence of Ca\(^{2+}\). In this work we have explicitly shown that a TnT fragment containing the TnT1 region can activate the ATPase on its own. In the context of the two-site binding model, Ca\(^{2+}\)-induced dissociation of the globular domain would liberate the activation function of the NH\(_2\)-terminal domain of TnT. What interactions are responsible for this liberation? In other words, is the Ca\(^{2+}\) binding signal transmitted to TnT directly through TnC-TnT interactions, through the TnI1–98-TnT interaction, or both? It has been suggested that the activation of the ATPase comes solely from a specific Ca\(^{2+}\)-dependent interaction between TnT and TnC (12). This was based on ATPase studies using a TnI deletion mutant (TnI157–263, deletion of residues 1–57) which interacts with TnC but not with TnT in binary complexes (12, 52, 53). On the other hand, a role for TnI in activation is suggested by our observation that TnT-TnI1–98-TnC activates the ATPase whereas TnC-TnT does not. It is noteworthy that both the COOH-terminal region of TnT and the NH\(_2\)-terminal region of TnI have conserved hydrophobic heptad repeats (54). The TnI heptad is present in both TnI157 and TnI1–98. If these repeats are involved in the TnI-TnT interaction as has been suggested (Ref. 54 and reviewed in Refs. 2, 5, and 36), this interaction would be present in troponin complexes containing TnI157 and TnI1–98. This could explain why troponin ternary complexes containing TnI157 or TnI1–98 are able to activate the ATPase (Fig. 2A and Ref. 12). Finally, as neither TnC-TnT nor TnI1–98-TnC complexes have been observed to activate on their own, a synergistic activation effect by TnI1–98-TnC is suggested. Consistent with this view is our observation that the affinity of TnT157–263 for the thin filament is reduced by its association with TnI198-TnC (±Ca\(^{2+}\)).

The intersubunit interactions in troponin can be divided into two classes (Fig. 7). The first class consists of interactions that are not significantly changed with the alterations of the Ca\(^{2+}\) concentrations and are “structural” in nature. These core interactions, best represented by the last 50 residues of TnT (residues 216–263), the NH\(_2\)-terminal domain of TnI (residues 1–98), and the COOH-terminal domain of TnC, are responsible for maintaining the structure of the troponin complex and its interaction with the actin-tropomyosin filament in both the “on” and “off” states (Fig. 7). The second class of interactions are dependent on the presence of Ca\(^{2+}\) and are responsible for the regulatory function of the troponin complex; the domains involved in these interactions switch their preferred interaction from one domain to another as a function of Ca\(^{2+}\) binding to the regulatory domains of TnC. In the absence of Ca\(^{2+}\), the inhibitory/carboxyl region of TnI interacts strongly with the thin filament and weakly with TnC. In addition, the COOH-terminal domain of TnT may be interacting with the thin filament blocking the activation function of the amino-terminal domain of TnT. In this situation, the ATPase activity is inhibited. In the presence of Ca\(^{2+}\), the inhibitory domain of TnI interacts strongly with the amino-terminal domain of TnC and weakly with the thin filament, thereby removing inhibition. The COOH-terminal region of TnT then interacts more strongly with TnI1–98-TnC than with the thin filament, thereby liberating the activation function of amino-terminal domain of TnT (Fig. 7). This activation may be mediated by a change in TnI1–191-Tm interactions (Fig. 7) which would affect the equilibrium between two or more actin-Tm binding states (3, 56).

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