Troponin T and Ca^{2+} Dependence of the Distance between Cys48 and Cys133 of Troponin I in the Ternary Troponin Complex and Reconstituted Thin Filaments†

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ABSTRACT: Contraction of vertebrate striated muscle is regulated by the interaction of Ca^{2+} with the heterotrimeric protein troponin (Tn), composed of troponin-C (TnC), troponin-I (TnI), and troponin-T (TnT). Although much is known about the Ca^{2+}-induced conformational changes in TnC, the Ca^{2+}-binding subunit of Tn, little is known about how TnI, the inhibitory subunit, responds to the binding of Ca^{2+} to TnC. In this work, we used resonance energy transfer to measure the distance between probes attached at Cys48 and Cys133 in the N- and C-terminal domains, respectively, of TnI. A mutant rabbit skeletal TnI, $TnI^{48/133}$ (C64S), was constructed by converting Cys64 into Ser. The remaining two thiols at Cys48 and Cys133 were labeled with the fluorescent donor 1,5-IAEDANS, and the nonfluorescent acceptor, DAB-Mal. We found an interprobe distance of ∼41 Å for both uncomplexed TnI and TnI in the binary complex with TnC. This distance increased to 51 Å in the ternary Tn complex with TnT. These distances did not change significantly on binding of Ca^{2+} to TnC. In the reconstituted thin filament, this distance remained to be 50 Å in the presence of saturating Ca^{2+}, but increased to ∼66 Å on removing Ca^{2+} with EGTA in the presence of Mg^{2+}. Our results indicate firstly that while TnC has only small effects on the global conformation of TnI, the presence of TnT in the ternary Tn complex gives rise to an apparent elongation of TnI. Secondly, whereas there is no detectable Ca^{2+}-dependent change in the global conformation of TnI in the Tn complex free in solution, the removal of Ca^{2+} caused a substantial separation of the N- and C-terminal TnI regions in the reconstituted thin filament, owing to the interaction between the C-terminal region of TnI and actin in the relaxed state.

The contraction of vertebrate striated muscle is regulated by Ca^{2+} interacting with the heterotrimeric regulatory protein troponin (Tn[1]), composed of the Ca^{2+}-binding, inhibitory, and Tm-binding subunits (TnC, TnI, and TnT, respectively). X-ray crystallographic studies have shown that TnC is a dumbbell-shaped molecule with its N- and C-terminal domains connected by a long central α-helical region (Herzberg & James, 1985; Sundaralingam *et al*., 1985). There are four functional Ca^{2+}-binding sites in skeletal muscle TnC, one pair in each globular domain. The Ca^{2+}-binding sites in the C-terminal domain have higher affinity for Ca^{2+} than those in the N-terminal one. The former also bind Mg^{2+}, with a binding constant about 1000 times smaller than that for Ca^{2+}. The lower affinity Ca^{2+}-specific sites have been identified as being directly involved in triggering the activation of contraction. Little is known about the structure of TnI or that of the TnC·TnI complex. Various pieces of evidence indicate that the peptide chains in TnC and TnI run in opposite directions in their complex (Farah *et al*., 1994; Kobayashi *et al*., 1994). Low-angle X-ray and neutron scattering data have led to a model in which the middle portion of TnI is wound around the central helix of TnC, while the remaining N- and C-terminal masses of TnI would be disposed as two caps on the two globular TnC domains (Olah *et al*., 1994; Olah & Trewhella; 1994).

Current models for the mechanism of Ca^{2+} regulation (Farah & Reinach, 1995; Grabarek *et al*., 1992; Zot & Potter, 1987) propose that in the low Ca^{2+} or relaxed state of muscle, TnI interacts strongly with actin and maintains Tm at a position in the thin filament that inhibits the cyclic interaction between myosin heads and actin. In the activated state, the binding of Ca^{2+} to TnC causes the strengthening of the interaction between TnC and TnI, and weakening of the interaction between TnI and actin, giving rise to the

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[1] Abbreviations: Tn and Tm, rabbit skeletal troponin and tropomyosin, respectively; TnC, TnI, and TnT, the Ca^{2+}-binding, inhibitory, and Tm-binding subunits of Tn, respectively; 1,5-IAEDANS, *N*-iodoacetyl-*N*′-(5-sulfo-1-naphthyl)ethylenediamine; DAN, the dansyl moiety of 1,5-IAEDANS; DAB-Mal, 4-dimethylaminophenylazophenyl-4′-maleimide; DAB, the dimethylaminophenylazophenyl moiety of DAB-Mal; $TnI^{48/133}$, mutant rabbit skeletal TnI with Cys64 replaced by Ser, retaining Cys48 and Cys133; d-$TnI^{48/133}$, $TnI^{48/133}$ labeled with the fluorescence donor DAN on the cysteines; da-$TnI^{48/133}$, $TnI^{48/133}$ labeled with both donor and acceptor (DAB); MES, 2-(*N*-morpholino)-ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); DTT, dithiothreitol; EDTA, ethlenediaminetetraacetic acid; PMSF, phenylmethanesulfonylfluoride; ATP, adenosine 5′-triphosphate; PCR, polymerase chain reaction; RET, resonance energy transfer; MOM, method-of-moments; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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detachment of TnI from actin and the movement of Tm to a noninhibitory position. Over the years, much effort has been directed toward deciphering the detailed mechanism of this complex process. Many workers have studied the interaction between TnC and TnI and between TnI and actin [see reviews by Farah and Reinach (1995), Leavis and Gergely (1984), and Zot and Potter (1987)]. Resonance energy transfer experiments provided evidence for a change in distance between TnC and TnI (Tao *et al*., 1989) and for the proposed movement of TnI with respect to actin in response to Ca^{2+} (Tao *et al*., 1990).

In recent years, the Ca^{2+}-induced conformational transition in TnC that initiates the chain of events leading to the activation of the cyclic acto-myosin interaction has become better understood (Herzberg *et al*., 1986; Grabarek *et al*., 1990; Fujimori *et al*., 1990; Wang *et al*., 1992; Gagne *et al*., 1995). In contrast, only a few studies have been carried out to characterize the conformational changes that occur within TnI in response to Ca^{2+} binding to TnC. Excimer emission of pyrene groups attached to Cys48 and Cys64, both in the N-terminal region, (Strasburg *et al.*, 1985) as well as the tryptophan fluorescence (Wang & Gergely, 1986) of TnI in the TnC·TnI complex is decreased by Ca^{2+} binding to the low-affinity sites of TnC. Lakowicz *et al.* (1988) found no effect of TnC on the distribution of the distance between Trp158 and Cys133 of TnI, both in the C-terminal region, without Ca^{2+}. In the presence of Ca^{2+}, the distribution was narrower. These studies indicate that some Ca^{2+}-induced local conformational changes do take place in TnI. Little is known, however, about the nature of the global conformational changes that occur in TnI when Ca^{2+} binds to TnC.

In this study, we used resonance energy transfer (RET) measurements to characterize the relative disposition of the N- and C-terminal domains of TnI, marked by probes on Cys48 and Cys133, respectively, in various forms of complexation and metal ion states. For this purpose, we constructed a mutant TnI that contains only these two of the three indigenous Cys residues ($TnI^{48/133}$). Using 1,5-IAEDANS as the fluorescent donor and DAB-Mal as the nonfluorescent acceptor, we measured the distance between these two residues in uncomplexed TnI, the binary TnC·TnI complex, the ternary TnC·TnI·TnT (Tn) complex, and in the reconstituted Tn·Tm·actin thin filament. The measurements were carried out both in the presence of saturating Ca^{2+}, simulating the *in vivo* activated state, as well as in the presence of EGTA and Mg^{2+}, simulating the *in vivo* relaxed state. Our results show, firstly, that whereas TnC had little effect on the distance between residues 48 and 133 of TnI, the addition of TnT to the TnC·TnI complex increased this distance by ~10 Å. Secondly, whereas the removal of Ca^{2+} has only negligibly small effects on this distance in the TnC·TnI and TnC·TnI·TnT complexes, there is an increase of 16 Å in this distance in the reconstituted thin filament upon the removal of Ca^{2+}.

MATERIALS AND METHODS

Chemicals. Unless otherwise specified, common reagents were obtained from Sigma (St. Louis, MO). HEPES was obtained from Research Organics (Cleveland, OH), reagents for recombinant DNA procedures from New England Biolabs (Beverly, MA), and chemicals for gel electrophoresis from Bio-Rad (Richmond, CA).

Proteins. Tn subunits (Greaser & Gergely, 1973), Tm (Greaser & Gergely, 1971), and actin were prepared from rabbit skeletal muscle according to established methods (Spudich & Watt, 1971). Rabbit skeletal myosin was prepared as described previously (Wagner & Yount, 1975) and stored in 50% glycerol at −20 °C. Chymotryptic S1 was prepared as described by Weeds and Taylor (1975) and was assumed to have a molecular weight of 115 000 and $\epsilon_{280} = 0.75$ $(mg/mL)^{-1}$ cm^{-1} (Wagner & Weeds, 1977). Mutant TnI cDNA was produced using the PCR method of overlap extension (Ho *et al*., 1989). Starting from the wild type rabbit fast skeletal cDNA (Jha *et al.*, 1994), cDNA coding for $TnI^{48/133}$ was constructed by converting the TGC codon for Cys64 to TCC for Ser. The construct was completely sequenced using the Sequenase kit (United States Biochemical Corp.). The expression vector pAED4 and *Escherichia coli* strain BL21(DE3) were used for expression of the mutant protein as described by Gong *et al.* (1993). The cells were harvested by centrifugation, suspended in a solution containing 10 mM MES, 1 mM DTT, and 1 mM EDTA, pH 5.5, and centrifuged again following several cycles of freezing and thawing. The pellets were washed with a solution containing 25 mM MES, 1 mM DTT, 1 mM EDTA, and 3 M urea, pH 6.0, and resuspended in a solution containing 50 mM Tris-HCl, 1 mM DTT, 1 mM EDTA, 0.2 mM PMSF, and 7 M urea, pH 8.8, for extraction of recombinant TnI. The cell debris was pelleted, and the supernatant was loaded onto a 2.5 × 10 cm DE-52 (Whatman, preswollen microgranular anion exchanger) column pre-equilibrated with the extraction solution. The flow-through, to which 30 mM sodium acetate was added and whose pH was adjusted to 4.5 with 1 N HCl, was loaded onto a 2.5 × 10 cm SP-Sepharose Fast Flow (Pharmacia Biotech., strong cation exchanger) column pre-equilibrated with the separation solution (30 mM sodium acetate, 1 mM DTT, 1 mM EDTA, 4 M urea, and 0.28 M NaCl, pH 4.5). On elution with the separation solution using a linear gradient of 0.28 to 0.7 M NaCl, recombinant TnI appeared at about 0.35 M NaCl. The purified TnI was dialyzed against distilled water, lyophilized, and stored at −20 °C. The purity was assessed by SDS−PAGE and UV spectrophotometry.

Protein Labeling. Two different procedures, random labeling and specific labeling, were used to label $TnI^{48/133}$ with the donor and the acceptor. Random labeling was carried out with the protein in the denatured state, so that the donor and the acceptor were randomly distributed between the two residues. The second procedure exploits the fact that Cys48 is nonreactive while Cys133 remains reactive in the ternary Tn complex (Chong & Hodges, 1982).

For random labeling, lyophilized $TnI^{48/133}$ was dissolved, ~40 μM, in a solution containing 6 M urea or 4 M GdmCl, 2 mM EDTA, 0.1 M NaCl, and 20 mM HEPES, pH 7.5 (denaturing solution), plus 5 mM DTT. After incubation at room temperature for 30 min, the reduced $TnI^{48/133}$ was dialyzed against the DTT-free denaturing solution and then incubated with 1,5-IAEDANS at a probe:protein ratio of 0.75:1 (mol/mol) for 20 min at room temperature. The reaction was quenched with 5 mM DTT and dialyzed against the denaturing solution. The extent of DAN incorporation was determined spectroscopically using A_{280} (1 mg/mL, 1 cm path) = 0.4 for TnI, $\epsilon_{337} = 6000$ M^{-1} cm^{-1}, and $\epsilon_{280} = 1060$ M^{-1} cm^{-1} for DAN (Hudson & Weber, 1973). The donor-labeled material, d-$TnI^{48/133}$, was then incubated with

DAB-Mal at a probe:protein ratio of 10:1 (mol/mol) for 3 h at room temperature, and then overnight at 4 °C. After quenching and dialysis as described above, the mutant TnI labeled randomly with donor and acceptor, designated as da-TnI$^{48/133}$, was stored at −20 °C in the denaturing solution. Before using, the denaturant was removed by dialysis. The spectroscopically determined labeling ratios were DAN:DAB:TnI = 0.4:0.9:1 using the extinctions given above and ϵ_{460} = 24 800 M^{-1} cm^{-1}, ϵ_{280} = 8400 M^{-1} cm^{-1} for DAB (Tao *et al*., 1983).

For specific labeling, the reduced TnI$^{48/133}$ was recombined with TnC and TnT at a molar ratio of 1:1:1 in the denaturing solution, and dialyzed against a solution containing 0.1 M NaCl, 0.2 mM $CaCl_2$, and 10 mM HEPES, pH 7.5 (Tn buffer). The Tn complex, 20−30 μM, was incubated with DAB-Mal for labeling of Cys133 at a probe:protein ratio of 5:1 (mol/mol) for 3 h at room temperature, and continued overnight at 4 °C. The alkylation reaction was quenched by 5−10 mM DTT. The reaction mixture was spun and dialyzed against the denaturing solution to remove the unreacted probe and to dissociate the Tn complex. The DAB-labeled TnI$^{48/133}$ was separated from the other two subunits of Tn by a procedure used to purify Tn subunits from extracted rabbit skeletal Tn (Greaser & Gergely, 1973). The modified TnI$^{48/133}$ was then incubated with 1,5-IAEDANS at a probe:protein ratio of 1:1 (mol/mol) for 30 min. After quenching with DTT, the reaction mixture was dialyzed against the denaturing solution and stored at −20 °C. Typical labeling ratios were DAN:DAB:TnI = 0.45:0.8:1. A donor-alone control sample was also prepared with iodoacetamide substituted for DAB-MAL in the first step before labeling with 1,5-IAEDANS.

Upon reconstitution with other subunits, the labeled or unlabeled TnI$^{48/133}$ was mixed stoichiometrically with TnC or TnC and TnT, all in denaturing solution, and dialyzed against a solution containing 0.1 M NaCl, 0.2 mM $CaCl_2$, and 10 mM HEPES, pH 7.5. To make regulated thin filaments, the reconstituted Tn was mixed with Tm and F-actin in a molar ratio of 1:1:7 (the actual concentration of Tn was adjusted so that [DAN] = 1 μM for all the fluorescence measurements). This mixture was dialyzed against solution containing 50 mM NaCl, 2 mM Tris-HCl, pH 8.0, 0.2 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.2 mM ATP.

ATPase Activity Assays. The regulatory activity of labeled and unlabeled TnI$^{48/133}$ was assessed by a myofibrillar ATPase assay and actin activated S1-MgATPase regulated by the reconstituted troponin. Rabbit skeletal myofibrils were prepared by homogenizing fresh psoas or adductor muscle in a medium containing 80 mM KCl, 1 mM HEPES, pH 7.0, and 0.1 mM DTT, followed by five cycles of sedimentation and resuspension (Solaro *et al*., 1971). The endogenous TnC and TnI were extracted by incubating the myofibrils with rabbit skeletal TnT (myofibril:TnT = 4:1 by weight) in a solution containing 0.25 M KCl, 20 mM MOPS/KOH, 5 mM EGTA, 5 mM $MgCl_2$, 0.5 mM DTT, pH 6.5, at 25 °C for 1 h (Shiraishi *et al*., 1992). Following low-speed sedimentation, the extracted myofibrils were rehomogenized in the ATPase assay solution containing 60 mM KCl, 2 mM $MgCl_2$, 30 mM imidazole, pH 7.0. Labeled or unlabeled TnI$^{48/133}$, either alone or complexed with TnC, was then added to the extracted myofibrils to a final concentration of 4 μM (Shiraishi *et al*., 1992; Ball *et al*., 1994). The ATPase reactions of the intact, extracted and reconstituted myofibrils, ∼0.5 mg/ml, were initiated by the addition of 2 mM ATP (Ball *et al*., 1994). At each of three or four time points 2 min apart, an aliquot of the reaction mixture was removed and quenched with equal volume of 10% cold trichloroacetic acid. After centrifugation, the phosphate content of the supernatant was determined by measuring the absorbance at 700 nm following the addition of ammonium molybdate and fresh $FeSO_4$ to 0.66% each and H_2SO_4 to 0.5 N. The regulated actin-activated S1-MgATPase activities were determined using 1.5 μM S1 to initiate the reaction in an assay mixture containing 5 μM F-actin, 1 μM Tm, TnC, TnT, and TnI or modified TnI mutant and 2 mM ATP. Other conditions are specified in the legends.

Fluorescence Measurements. Fluorescence and anisotropy decay curves were obtained using a modified ORTEC9200 nanosecond fluorometer as described previously (Tao & Cho, 1979). Fluorescence decay curves were analyzed by the method-of-moments (MOM) using the program FLUOR (Small & Isenberg, 1976) to yield τ_{da} and τ_d, the donor lifetime in the presence and absence of acceptor, respectively. Energy transfer efficiencies and distances were calculated with the use of Förster's equations:

$$E = 1 - \tau_{da}/\tau_d \quad (1)$$

$$R = R_o (E^{-1} - 1)^{1/6} \quad (2)$$

where R_o is the critical transfer distance, corrected for the variation in donor fluorescence quantum yield using the equation:

$$R_o = R_o'(\tau_d/\tau_d')^{1/6} \quad (3)$$

with R_o' = 39.9 Å and τ_d' = 13.5 ns measured for the DAN-DAB couple (Tao *et al*., 1983).

Steady-state fluorescence intensity and polarization measurements were carried out on a ISS K2 spectrofluorometer in the L-format with the excitation and emission wavelengths set at 337 and 480 nm, respectively.

RESULTS

ATPase Activities. The ability of the various TnI forms to modulate Ca^{2+} regulation of acto-myosin ATPase activity was assessed using both the reconstituted myofibrillar system and the regulated acto-S1 system (see Materials and Methods). The results are shown in Figure 1. As expected, the intact myofibrils exhibited high ATPase activity in the presence of Ca^{2+}, which decreased by 82% in the absence of Ca^{2+} (Figure 1A, column 1). Also, as expected, the ATPase activity of myofibrils whose TnC and TnI had been extracted exhibited no Ca^{2+} dependence (Figure 1A, column 2). The Ca^{2+}-regulated activity was restored to the same extent by muscle TnI. All three forms of TnI, TnI$^{48/133}$, d-TnI$^{48/133}$, and da-TnI$^{48/133}$, exhibited the same Ca^{2+} regulatory activities as muscle TnI (Figure 1A, columns 4−6). The acto-S1-MgATPase activities regulated by Tn containing these TnI forms were the same, both in the presence and absence of Ca^{2+}, as that using TnI from rabbit muscle (Figure 1B). All these results indicated that neither the conversion of Cys64 to Ser nor the labeling with DAN or both DAN and DAB significantly impair the functional integrity of TnI.

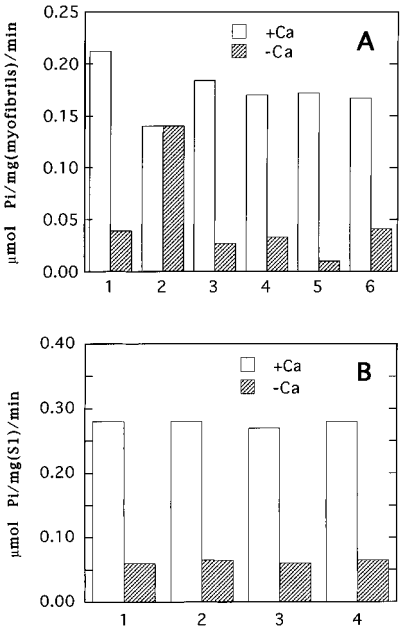

FIGURE 1: (A) ATPase activities of (1) rabbit skeletal myofibrils, (2) extracted myofibrils (with the endogeneous TnI and TnC removed), (3) extracted myofibrils reconstituted with rabbit skeletal TnI and TnC, (4) extracted myofibrils reconstituted with wild-type TnC and $TnI^{48/133}$, (5) same as 4, but with d-$TnI^{48/133}$, and (6) same as 4, but with da-$TnI^{48/133}$. A typical assay mix contained 0.4−0.6 mg/mL of myofibrils, 4 μM exogenous TnC and TnI or TnI derivatives, 60 mM KCl, 30 mM immidazole, pH 7.0, 2 mM $MgCl_2$, plus 0.2 mM $CaCl_2$ for the Ca^{2+} state (empty columns) or 2 mM EGTA for the Ca^{2+}-free state (shaded columns). (B) Acto-S1-MgATPase activities regulated by troponin consisting of rabbit skeletal TnC, TnT, and (1) TnI, (2) recombinent $TnI^{48/133}$, (3) d-$TnI^{48/133}$ or (4) da-$TnI^{48/133}$. The assay mix contained 1.5 μM S1, 1 μM reconstituted troponin and Tm, 5 μM F-actin, ~17 mM NaCl, 5 mM $MgCl_2$, and 2 mM ATP, plus 0.2 mM $CaCl_2$ for the Ca^{2+} state (empty columns) or 2 mM EGTA for the Ca^{2+}-free state (shaded columns). All ATPase experiments were performed at 22 °C.

Fluorescence Polarization. The binding in the presence of Ca^{2+} of the labeled $TnI^{48/133}$ with TnC, TnC, and TnT, as well as to thin filaments, was examined using time-resolved fluorescence anisotropy measurements. Under conditions identical to those used for the energy transfer measurements, the anisotropy decay rate of d-$TnI^{48/133}$ was significantly decreased by TnC (Figure 2). This is consistent with the lower rotational mobility of the binary TnC·TnI complex compared with free TnI owing to its larger volume. A further decrease in the anisotropy decay rate was observed when TnT was present, consistent with a further decrease in rotational mobility of the ternary Tn complex. The anisotropy was virtually nondecaying for the reconstituted thin filament, reflecting the virtual immobility of the F-actin filaments on the nanosecond time scale of fluorescence lifetimes. The shapes of the anisotropy decay curves were not affected by the presence of the acceptor (Figure 2, dashed lines), which can be expected to increase the fluorescence decay rate of the donor but not to affect the anisotropy decay rate if the hydrodynamic volume and shape remain the same. These results indicate that both the singly donor labeled and the doubly donor−acceptor labeled $TnI^{48/133}$ are fully able to form the binary TnC·TnI complex, the ternary Tn complex, and the reconstituted thin filament.

Values of the anisotropy at time zero, A_o, vary from 0.13 for free TnI to 0.24 for TnI incorporated into thin filaments

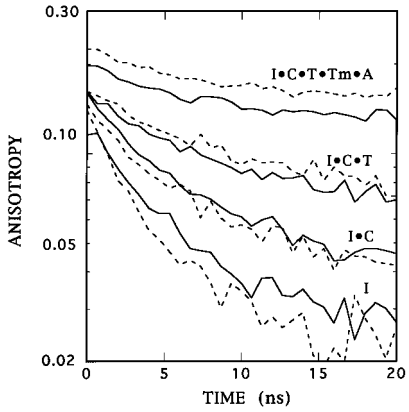

FIGURE 2: Fluorescence anisotropy decay of d-$TnI^{48/133}$ (solid curves), or da-$TnI^{48/133}$ (dashed curves): Modified TnI free in solution (I), complexed with TnC (I·C), complexed with TnC and TnT (I·C·T), and in reconstituted thin filaments (I·C·T·Tm·A). Solution conditions, free TnI: 0.5 M NaCl, 10 mM HEPES, pH 7.5, 2 mM EDTA. Binary and ternary complexes: 0.1 M NaCl, 10 mM HEPES, pH 7.5, 0.1 mM $CaCl_2$. Reconstituted thin filaments: 50 mM NaCl, 2 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$, 0.2 mM ATP, 0.2 mM $CaCl_2$. All measurements were made with [DAN] = 1 μM and at 20 °C.

(Figure 2). These A_o values are considerably lower than the theoretical maximum value of 0.4, suggesting a considerable degree of rapid depolarization owing to either rapid segmental rotational mobility of the probe or electronic depolarization of the excited state.

Cosedimentation. The incorporation of the labeled $TnI^{48/133}$ into reconstituted thin filaments was also checked by ultracentrifugation (Beckman TL100 Ultracentrifuge, 300000*g*, 20 min) followed by SDS−PAGE analysis on aliquots of the samples used for RET measurements. In the presence of Ca^{2+}, all Tn subunits, including the labeled $TnI^{48/133}$, cosedimented with Tm and actin, indicating no dissociation of the subunits or dissociation of the Tn complex from F-actin. In the absence of Ca^{2+}, all the labeled $TnI^{48/133}$ and TnT and 80−90% of TnC cosedimented with actin.

Resonance Energy Transfer. We recorded the fluorescence decay curves of d-$TnI^{48/133}$ and da-$TnI^{48/133}$ under four complexation conditions: (1) free TnI; (2) in the binary TnC·TnI complex; (3) in the ternary Tn complex; and (4) in the reconstituted Tn·Tm·F-actin complex. Measurements were made first on samples containing 0.2 mM $CaCl_2$ for the Ca^{2+}-bound state, then on the same sample to which 2 mM EGTA and 8 mM $MgCl_2$ were added. In the latter condition, Mg^{2+} binds only to the high-affinity sites in the C-terminal domain, simulating the Ca^{2+} free relaxed state in muscle.

Figure 3 shows the decay curves from one set of data (set 1 in Table 2) for free TnI, the binary complex, and the ternary complex, all in the presence of Ca^{2+}. It can be seen that the decays of the donor−acceptor samples (lower curves) are faster than those of the donor alone samples (upper curves) owing to quenching of the donor fluorescence via energy transfer to the acceptor. The extent of quenching is similar for free TnI and that in the binary complex, but notably less in the ternary complex, indicating that the interprobe distance in TnI is little affected by the presence of TnC, but is increased in the presence of both TnC and TnT.

The fluorescence decay curves were subjected to MOM analysis to obtain the lifetimes, followed by application of the Förster equations to obtain information on probe separa-

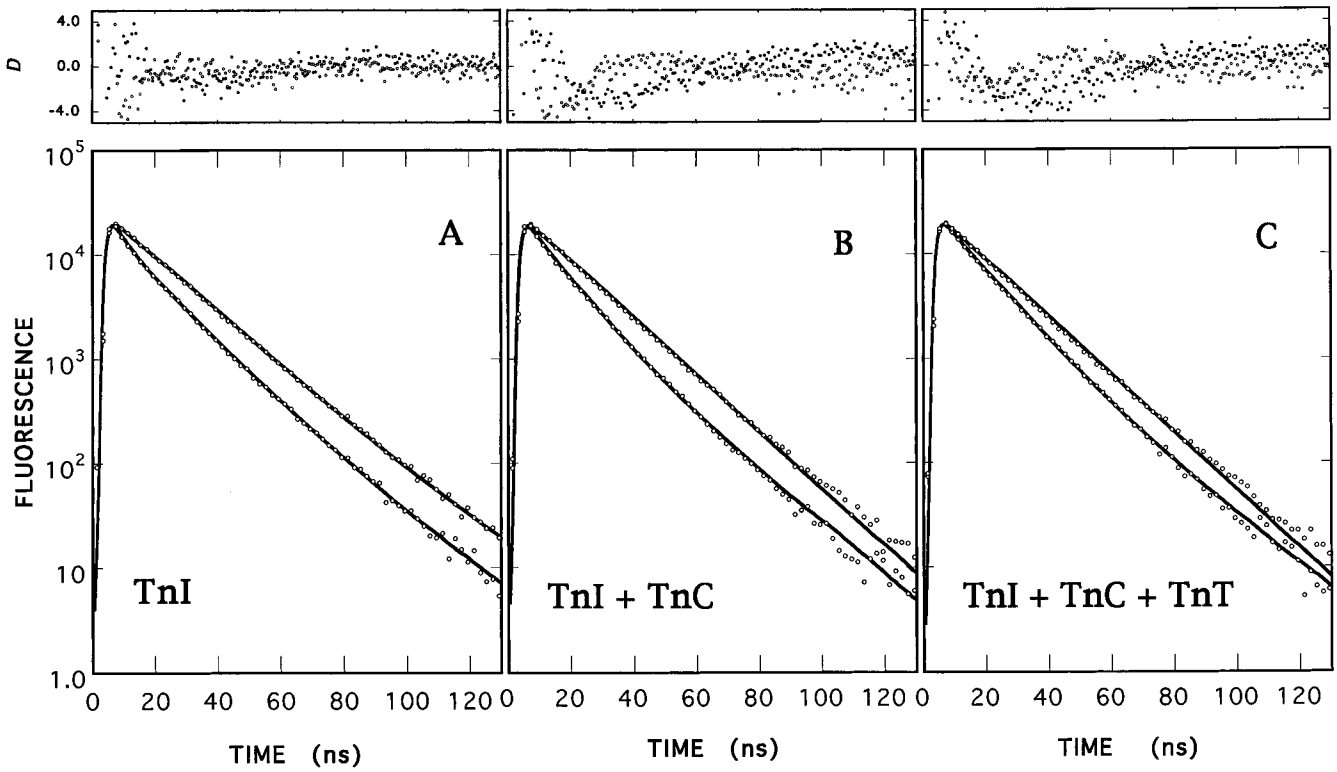

FIGURE 3: Fluorescence decay curves of d-TnI$^{48/133}$ (upper curves, monoexponential) and da-TnI$^{48/133}$ (lower curves, biexponential). (A) Labeled TnI$^{48/133}$ was free in solution, (B) complexed with TnC, and (C) complexed with TnC and TnT. Conditions were the same as in Figure 2 for the corresponding complexation states. Circles are experimental points, shown one for every three; solid lines are calculated curves using paramenters derived from MOM analyses (Table 1). Upper panels show the deviation functions for d-TnI$^{48/133}$ (filled circles) and da-TnI$^{48/133}$ (open circles) decay curves. The deviation function is defined as $D_i = (c_i - e_i)/(e_i)^{1/2}$, where c_i and e_i are the calculated and experimental fluorescence intensities, respectively, at the ith data point. All measurements were made at 20 °C.

tion distances (see Materials and Methods). In what follows, we illustrate the procedure using the data for randomly labeled TnI$^{48/133}$ in the free state. For d-TnI$^{48/133}$, monoexponential analysis yielded a lifetime of 16.5 ns (Table 1). Biexponential analysis yielded a major component with a lifetime of 16.0 ns and a minor component with a negligibly small fractional amplitude (0.015). Residual analysis showed that the quality of fit was only marginally improved when the biexponential parameters were used to describe the data. We conclude that the decay of d-TnI$^{48/133}$ is essentially monoexponential and that the value of 16.5 ns can be taken as τ_d, the fluorescence lifetime of the donor in the absence of the acceptor. The fact that the decay curve of d-TnI$^{48/133}$ exhibits only one significant lifetime indicates that probes at either site are in similar environments. This is also supported by the finding that the τ_d values for randomly and specifically labeled samples are similar.

The fluorescence decay of da-TnI$^{48/133}$ is distinctly multiexponential. Biexponential MOM analysis yielded a major and a minor component with lifetimes of 8.28 and 17.5 ns, respectively. Triexponential analysis yielded a third component of negligible fractional amplitude (0.005). Thus, we considered the decay of da-TnI$^{48/133}$ to be essentially biexponential. The 17.5 ns value is comparable to the 16.5 ns lifetime of d-TnI$^{48/133}$ and most likely corresponds to the unquenched donor decay in the da-TnI$^{48/133}$ preparation that contains no acceptor moiety. The 8.3 ns component clearly corresponds to donor moieties whose fluorescence decay rate is enhanced owing to energy transfer to the acceptor, and can therefore be taken as τ_{da}, the fluorescence lifetime of the donor in the presence of the acceptor. Substitution of these values for τ_d and τ_{da} into eq 1−3 yielded a transfer efficiency E of 0.499 and a separation distance of 41 Å (Table 1).

The properties of the decay curves for the binary and ternary complexes in both Ca^{2+} and Ca^{2+}-free (presence of Mg^{2+} and EGTA) states are similar to those for free TnI, so that we could apply the same analysis to obtain transfer efficiency and distance information (Table 1). As we deduced from qualitative considerations, we found that TnC had no effect on the donor-acceptor separation, while the addition of TnT increased this distance by ∼7 Å. The distance between probes attached to residues 48 and 133 in TnI (hereafter referred to as the 48/133 distance) was insensitive to Ca^{2+} for the binary TnI·TnC complex and for the ternary TnI·TnC·TnT complexes, as is the case for free TnI.

Figure 4 shows the decay curves for d-TnI$^{48/133}$ and da-TnI$^{48/133}$ incorporated into the TnI·TnC·TnT·Tm·actin synthetic thin filament in the Ca^{2+} (panel A) and Ca^{2+}-free (panel B) states. It can be seen that in the Ca^{2+} state the extent of quenching is similar to that for the ternary complex (Figure 3C), while in the Ca^{2+}-free state the quenching is virtually abolished. The addition of excess Ca^{2+} to the Ca^{2+}-free sample restored the quenching observed for the original Ca^{2+}-saturated sample (panel C), indicating that the effect is reversible. These qualitative observations suggest that unlike the binary and ternary complexes, the 48/133 distance in TnI undergoes a substantial increase from the Ca^{2+}-saturated to the Ca^{2+}-free state. The decay curves for these samples were also subjected to the analysis procedure described above, and the results are shown in Table 1. It

Table 1: Fluorescence Decay and RET Parameters for TNI$^{48/133}$ Labeled Randomly with Donor 1,5-IAEDANS and Acceptor DAB-MaI (Detailed Data for set 1 in Table 2)[a]

proteins	metal	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	χ^2/N	E	R_o (Å)	R (Å)
I^d	Ca^{2+}	16.52 (1.00)			2.48		41.3	
		16.01 (.985)	33.02 (.015)		1.48			
I^{da}	Ca^{2+}	8.28 (.676)	17.49 (.324)		4.31	0.499		41.3
		5.69 (.454)	14.81 (.541)	34.60 (.005)	2.83			
I^d	Mg^{2+}	16.77 (1.00)			3.05		41.4	
		16.57 (.998)	49.24 (.002)		2.04			
I^{da}	Mg^{2+}	8.30 (.684)	17.61 (.316)		3.75	0.505		41.3
		4.90 (.415)	14.26 (.576)	30.81 (.010)	1.69			
I^d·C	Ca^{2+}	15.43 (1.00)			2.64		40.8	
		15.18 (.997)	43.36 (.003)		1.69			
I^{da}·C	Ca^{2+}	8.42 (.736)	16.93 (.264)		5.71	0.454		42.1
		5.94 (.461)	13.77 (.543)	31.34 (.006)	7.48			
I^d·C	Mg^{2+}	16.61 (1.00)			2.70		41.3	
		16.36 (.997)	45.70 (.003)		1.99			
I^{da}·C	Mg^{2+}	7.29 (.638)	16.75 (.362)		3.01	0.561		39.6
		5.36 (.481)	14.51 (.503)	25.67 (.016)	1.64			
I^d·C·T	Ca^{2+}	15.40 (1.00)			3.37		40.8	
		15.16 (.997)	42.49 (.003)		2.77			
I^{da}·C·T	Ca^{2+}	11.09 (.860)	19.40 (.140)		2.15	0.280		48.2
		10.54 (.744)	16.87 (.253)	32.64 (.003)	2.04			
I^d·C·T	Mg^{2+}	16.56 (1.00)			3.23		41.3	
		16.48 (1.00)	64.26 (.000)		3.00			
I^{da}·Ca·T	Mg^{2+}	11.06 (.660)	17.55 (.340)		1.29	0.332		46.4
		9.40 (.402)	15.61 (.594)	31.47 (.004)	1.18			
I^d·CaT·Tm·A	Ca^{2+}	15.70 (1.00)			2.86		40.9	
		15.54 (.998)	43.13 (.002)		2.45			
I^{da}·C·T·Tm·A	Ca^{2+}	11.66 (.689)	18.05 (.311)		1.42	0.257		48.8
		11.21 (.596)	17.09 (.400)	27.88 (.004)	1.41			
I^d·C·Tm·A	Mg^{2+}	16.86 (1.00)			2.04		41.4	
		16.37 (.973)	27.59 (.027)		1.82			
I^{da}·C·T·Tm·A	Mg^{2+}	15.94 (.947)	27.30 (.026)		1.49	0.055		66.6
		14.35 (.665)	19.51 (.313)	−2.71 (.023)				

[a] I^d is TnI$^{48/133}$ labeled with the DAN donor alone, i.e., d-TnI$^{48/133}$; I^{da} is TnI$^{48/133}$ labeled with both the DAN donor and the DAB acceptor, i.e., da-TnI$^{48/133}$. C is TnC, T is TnT, Tm is tropomyosin, and A is actin. The τs are fluorescence lifetimes obtained by MOM analysis; the quantity in parenthesis following each lifetime is the fractional amplitude of each decay component. χ^2/N is the mean residual, defined as $\chi^2/N = [\sum_{i=1}^{N}(f_i - f_{ci})^2/f_i]/N$, where N is the number of data points, f_i is the measured intensity of the ith data point, and f_{ci} is the calculated intensity using the derived parameters. E is the transfer efficiency, calculated according to eq 1 (Materials and Methods). R_o is the corrected critical transfer distance, calculated according to eq 3. R is the interprobe distance, calculated uisng eq 2. The solution conditions for Ca^{2+} state of all complexes were those given for Figure 2. To change to the Mg^{2+} state, 2 mM EGTA, and 8 mM $MgCl_2$ were added.

Table 2: Distances (Å) between Cys48 and Cys133 of TnI by RET Measurements[a]

protein	metal	set 1	set 2	set 3	set 4	ave	sem
I	Ca^{2+}	41.3	38.5	40.9	43.2	41	±1
	Mg^{2+}	41.3	39.2	41.6	41.2	41	±1
I·C	Ca^{2+}	42.1	39.1	45.8	44.3	43	±1
	Mg^{2+}	39.6	41.3	43.8	40.5	41	±1
I·C·T	Ca^{2+}	48.2	47.9	56.7		51	±2
	Mg^{2+}	46.4	53.2	54.0		51	±2
I·C·T·Tm·A	Ca^{2+}	48.8		45.6	53.6	50	±2
	Mg^{2+}	66.6		64.1	67.2	66	±1

[a] Data sets 1 and 2 were obtained using randomly labeled d-TnI$^{48/133}$ and da-TnI$^{48/133}$. Data sets 3 and 4 used specifically labeled d-TnI$^{48/133}$ and da-TnI$^{48/133}$ with donor on Cys48 and acceptor on Cys133. ave is the unweighted average. sem is the standard error of mean.

can be seen that the 48/133 distance in TnI increased by ~17.8 Å when Ca^{2+} was removed from the synthetic thin filaments.

In Table 2, we present the results of three additional data sets as well as set 1 that has been discussed in detail above. Sets 1 and 2 are randomly labeled, sets 3 and 4 are specifically labeled with donor on Cys48 and acceptor on Cys133. Data were collected and analyzed as was done for set 1 reported in detail in Table 1. Despite the differences in the sample preparation, the measured distances for each protein combination and metal condition are not significantly different, justifying their being averaged. The averaged values show that the 48/133 distance is 41 Å for both uncomplexed TnI and TnI complexed with TnC. In the ternary Tn complex containing TnT as well, it increases to 51 Å. All these values are Ca^{2+} insensitive. In the reconstituted thin filament, this distance is 50 Å, in the presence of Ca^{2+}, virtually identical to that for Tn by itself. In the absence of Ca^{2+}, this distance increases by 16 Å to 66 Å.

We should add that two protein combinations were used as controls for the measurements of the 48/133 distance in the reconstituted thin filaments. One is TnI·Tm·actin, and the other is TnI·TnC·TnT·Tm, in which the distance is 46 and 51 Å, respectively, regardless of Ca^{2+} concentration. These results indicate that mere binding of TnI to Tm·actin or Tn binding on Tm alone without actin does not result in the observed large distance increase within TnI in thin filaments.

DISCUSSION

In this work we measured the distance between residues 48 and 133 of TnI (48/133 distance) under a variety of conditions to gain new insights into structural changes in TnI that underlie the regulation of vertebrate muscle contraction. We stress that all the labeled mutant TnIs retain their

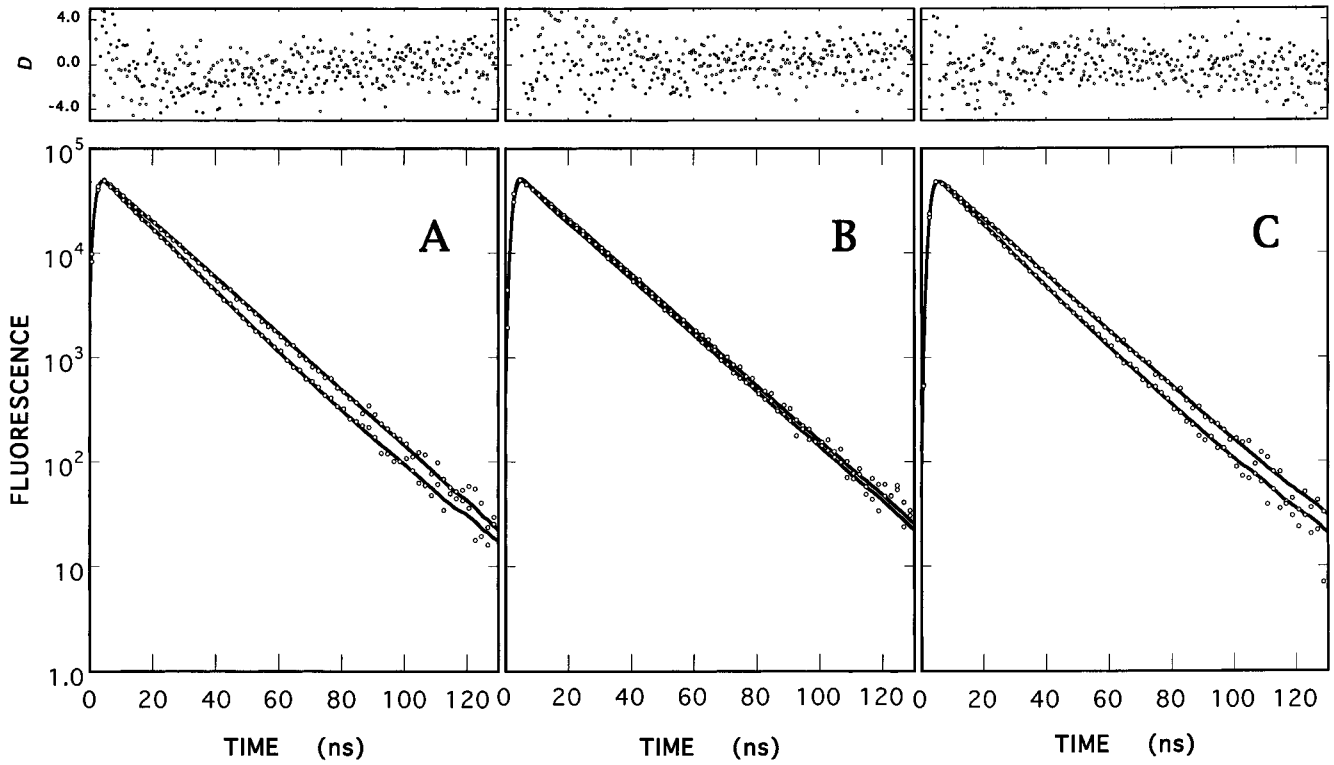

FIGURE 4: Fluorescence decay curves of d-TnI$^{48/133}$ (upper curves, monoexponential) and da-TnI$^{48/133}$ (lower curves, biexponential) combined with TnC, TnT, Tm, and F-actin to form reconstituted thin filaments. Conditions, Ca^{2+} saturated (panel A): 50 mM NaCl, 2 mM Tris-HCl, pH 8.0, 0.2 mM ATP, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, Ca^{2+} free (panel B): as for A plus 2 mM EGTA and 8 mM $MgCl_2$. Ca^{2+} re-added (panel C): as for panel B plus 2.5 mM $CaCl_2$. Other conditions and definitions as in Figures 2 and 3.

native biological properties with respect to Ca^{2+} regulation of reconstituted myofibrillar MgATPase activity.

We found that the 48/133 distance is 41 Å in TnI itself or when TnI forms a binary complex with TnC. When TnT binds to the TnI·TnC complex, this distance becomes 51 Å, an increase of 10 Å, and remains at this value in the Tn·Tm complex. While none of the above distances are sensitive to Ca^{2+}, there is an increase by 16 Å or more upon the removal of Ca^{2+} in the reconstituted thin filaments.

It is not likely that this increase in distance inferred from a decrease in transfer efficiency is due to a change in the orientation factor, κ^2. Firstly, the values of A_o (the zero time anisotropy) for da-TnI$^{48/133}$·TnC·TnT·Tm·actin in the Ca^{2+} and Mg^{2+} states are similar (0.22 and 0.24, respectively), so that there does not appear to be any Ca^{2+}-dependent change in the rotational mobility of the donor. Secondly, the fact that the randomly and the specifically labeled samples gave the same results suggests that the value of κ^2 is the same regardless of the location of the donor and the acceptor, most likely the isotropically averaged value of $^2/_3$. Finally, these values are considerably smaller than the theoretical maximum of 0.4, indicating substantial rapid reorientation of the donor's transition moment via either mixing of electronic states (Haas *et al.*, 1978; Perkins *et al.*, 1984) or rapid probe rotation or both, further justifying our assumption that κ^2 is $^2/_3$ under all conditions.

Our finding that the 48/133 distance is unaffected by the binding of TnC can be compared with results from a previous RET measurement of an intra-TnI distance; it was reported that, while the distribution of the distance between Trp158 and a probe attached at Cys133 of TnI was affected upon the binding of TnC, the average distance increased only nominally from 24 to 27 Å (Lakowicz *et al.*, 1988). It should be pointed out that both Trp158 and Cys133 are in the C-terminal domain of TnI, while we measured a larger distance (41 Å) between residues that are in different N- and C-terminal domains.

The 48/133 distance of 41 Å in the TnI·TnC complex reported here is much shorter than that the model of Olah and Trewhella (1994) would predict. According to this model, TnC is in the same conformation as that in the crystal structure, while residues 1−49 and residues 131−179 of TnI constitute the cap regions at either end of the TnC molecule. This means that the 48/133 distance must be at least as large as the length of the TnC molecule, *viz.* 70 Å, in the crystal structure. Our results would place both, or at least one of, residues 48 and 133 between the cap regions. Indeed, in the complex of TnC with a TnI mutant, of which the native Cys133 is the sole cysteine, Cys133 was found to be located between the N- and C-terminal lobes of TnC (Luo *et al.*, 1996). These apparent discrepancies indicate that whereas the general features of the Olah and Trewhella (1994) model are probably correct, the precise placement of specific TnI residues remains to be revealed by higher resolution techniques.

The fact that we found no change in the 48/133 distance in free TnI as a function of Ca^{2+} is to be expected since TnI by itself is known not to have any high-affinity metal-binding sites. That this distance is independent of Ca^{2+} in the binary TnI·TnC and ternary Tn complex is somewhat surprising since it is expected that the Ca^{2+}-induced conformational change in TnC is transmitted to TnI during thin-filament regulation. Also, early RET measurements of inter- and intrasubunit distances in the TnI·TnC complex have led to the conclusion that extensive structural rearrangements occur both within and between the subunits as a function of Ca^{2+}

(Wang & Cheung, 1986). However, later distance distribution measurements of Lakowitz *et al.* (1988) showed little or no Ca^{2+}-induced change in the average distance between Trp158 and Cys133 of TnI as a function of Ca^{2+}. Our RET measurement of the distance between Cys98 of TnC and Cys133 of TnI in the ternary Tn complex also showed only a small Ca^{2+}-dependent change of ~5 Å (Tao *et al.*, 1989). Studies of intersubunit cross-linking using labeled TnC or TnI in the Tn complex found no Ca^{2+}-dependent change in the cross-linking pattern (Sutoh, 1980; Sutoh & Matsusaki, 1980). Taken together with our present findings, the weight of the evidence seems to indicate that there is no large scale Ca^{2+}-induced structural rearrangements either within or between the subunits in the Tn complex.

It should be noted that it is possible for TnI to undergo a Ca^{2+}-induced conformational change without there being a change in the 48/133 distance. This would occur if the change was (e.g.) a rotation about one or the other of the two cysteine residues. We will in our future work attempt to resolve this issue using TnI mutants with cysteines at locations other than 48 and 133.

Our finding that TnT significantly affects the conformation of TnI upon binding to the TnI·TnC complex has not been reported before. There are many pieces of evidence for interactions between TnT and TnI. It has been pointed out that residues 53−106 of TnI and residues 205−255 of TnT possess a heptad hydrophobic repeat, suggesting that TnI and TnT may be interacting by means of coiled coils (Parry, 1981, Pearlstone & Smillie, 1985). Such interactions may be the origin of the TnT-induced increase in distance between the N- and C-terminal regions of TnI found in this work. The possibility of a direct interaction between TnT and the regions containing Cys48 and Cys64 of TnI in native Tn was suggested by Chong and Hodges (1982) on the basis of studies on the reactivity of TnI's thiol groups with iodoacetamide. In any case, these results indicate that the conformation of TnI in the ternary complex is different from that in the binary complex of TnI·TnC.

The most striking result of this study is the observation of a large increase in the 48/133 distance upon the removal of Ca^{2+} in the reconstituted thin filament. Since this increase does not occur in the Tn·Tm complex, it must arise owing to the presence of actin. Our current understanding of thin filament regulation is that in entering relaxed (absence of Ca^{2+}) state the N-terminal domain of TnC undergoes a transition from an open to a closed state (Herzberg *et al.*, 1986), whereupon TnI, or some portion of TnI, becomes attached to actin (Tao *et al.*, 1990). Our present findings indicate that the attachment is accompanied by a significant separation between portions of the C- and N-terminal regions of TnI. This result is consistent with cross-linking studies which showed that significant Ca^{2+}-dependent changes in cross-linking patterns occurred only in the thin filament state and not in the ternary Tn complex (Sutoh, 1980; Sutoh & Matsusaki, 1980).

The increase in the 48/133 distance is most likely the consequence of depleting Ca^{2+} from the low-affinity triggering sites of TnC, although the effect of replacing Mg^{2+} by Ca^{2+} at the high-affinity sites cannot be ruled out. We believe, however, that the latter is not as likely because it is known that the effect of replacing Ca^{2+} with Mg^{2+} causes only subtle structural changes in EF-hand domains (Strydnaka & James, 1989). In contrast, a large conformational change in the N-terminal domain of TnC has been predicted (Herzberg *et al.*, 1986) and observed (Wang *et al.*, 1992; Slupsky *et al.*, 1995; Slupsky & Sykes, 1995) in response to binding of Ca^{2+} to the low-affinity sites of TnC.

The origin of this Ca^{2+}-dependent structural change in TnI in the presence but not in the absence of actin is not clear. It has been suggested that in the TnI·TnC complex the polypeptides run in an antiparallel manner, so that the N-terminal structural domain and the C-terminal regulatory domain of TnI interact with the C- and N-terminal domains of TnC, respectively, (Farah & Reinach, 1995; Farah *et al.* 1994; Kobayashi *et al.*, 1994). In this context, our results can be rationalized in the following model: in the Ca^{2+} state the C-terminal domain of TnI has high affinity for the N-terminal domain of TnC and remains attached to TnC regardless of the absence or presence of actin. In the Ca^{2+}-free state, the affinity of TnI's C-terminal domain for TnC's N-terminal domain is reduced, so that in the presence of actin attachment of TnI's C-terminal domain (or a portion thereof) to actin is now thermodynamically favored. This C-terminal regulatory region of TnI containing Cys133 then moves to attach to actin, while the N-terminal structural domain of TnI containing Cys48 remains attached to the C-terminal domain of TnC and the rest of the Tn·Tm complex, giving rise to our observed increase in the 48/133 distance. In the absence of actin, in spite of the lowered affinity, most of TnI's C-terminal segment remains attached to TnC, with TnI retaining its overall conformation. This would account for our observed lack of change with Ca^{2+} in the 48/133 distance in the Tn complex alone.

In closing, we would point out that this is one of the first studies on the conformation of a Tn subunit in the complete synthetic thin filament, so that interpretation of these results is necessarily difficult. Here we present a possible scenario that is compatible with our findings and current knowledge. We anticipate that this and other hypotheses will be tested in the future by ourselves and others. We would also add that the ~16 Å change in distance in TnI might make a contribution to the change in the X-ray diffraction pattern of thin filaments in response to Ca^{2+} binding, which has been mainly attributed to tropomyosin's movement [see Holmes (1995)].

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