Investigation of the Structural Requirements of the Troponin C Central Helix for Function†

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ABSTRACT: The two globular Ca^{2+}-binding domains of troponin C are connected by a three-turn, exposed central helix. The requirements of this helical linker for regulatory function are not fully understood. In the present work we investigated the structural requirement of the linker using a series of insertion mutations that differ in predicted flexibility. TnCinrc has a nine-residue flexible random coil insert, TnCinpp has a nine-residue rigid polyproline insert (three turns), and TnCinαh has a seven-residue insert with high potential of forming α-helix. TnCinrc and TnCinpp were defective in the activation of the regulated actomyosin ATPase activity in the presence of Ca^{2+} when compared to wild type or TnCinαh, suggesting that altering the flexibility of the central helix impairs the regulatory function of troponin C. TnCinαh, TnCinrc, and TnCinpp had 87% ± 3, 62% ± 3, and 58% ± 2 of the wild type activity, respectively (n = 6). All insertions in the central helix resulted in elongation of molecule compared to wild type TnC as determined by Stokes' radius. The Ca^{2+}-affinity, the Ca^{2+}-dependence of the actomyosin ATPase, and the stability of the insertion mutants were similar to wild type. Deletions of up to two turns of the central helix have little effect on troponin C function [Dobrowolski, Z., Xu, G-Q., & Hitchcock-DeGregori, S. E. (1991) *J. Biol. Chem. 266,* 5703−5710]. In another mutant (TnCd11) the entire central helix, ^{87}KEDAKGKSEEE^{97}, was deleted. With TnCd11, activation of the actomyosin ATPase activity in the presence of Ca^{2+} was normal, but inhibition in the absence of Ca^{2+} was less effective. Interaction of TnCd11 with TnI was altered. There was a 2-fold excess of TnCd11 in reconstituted Tn complex, consistent with another report [Babu, A., Rao, V. G, Su, H., & Gulati, J. (1993) *J. Biol. Chem. 268,* 19232−19238]. Our results suggest that the native length and structure of the central helix are optimal for normal regulatory function and that connectivity alone is insufficient for TnC function.

Troponin C (TnC)[1] is an intracellular Ca^{2+} receptor involved in the transmission of Ca^{2+} signals for muscle contraction, in cooperation with the other regulatory components of the thin filament, troponin I (TnI), troponin T (TnT), and tropomyosin (TM) (Zot & Potter, 1987; Grabarek *et al.*, 1992). TnC is a member of the EF-hand protein family that includes calmodulin (CaM), calbindin, parvalbumin, and recoverin (Strynadka & James, 1989; Flaherty *et al.*, 1993).

TnC and CaM are homologous in their primary and three-dimensional crystal structures. In the X-ray structure, TnC is an elongated dumbbell-shaped molecule, consisting of two Ca^{2+}-binding domains connected by a long central helix. The central helix of TnC has a nine-turn α-helix with three turns in the middle (the D/E linker) exposed to solvent in the crystal structure (Babu *et al.*, 1985; Herzberg & James 1988; Satyshur *et al.*, 1988). Fluorescence resonance energy transfer studies suggest that uncomplexed TnC undergoes a pH dependent transition from an elongated conformation, at acidic pH, compatible with the crystal structure, to a more compact conformation at neutral pH. When complexed with TnI, TnC adopts an intermediate conformation (Wang *et al.*, 1987; Cheung *et al.*, 1991). Small-angle X-ray scattering studies also revealed that TnC is extended when complexed with TnI or TnI inhibitory peptide (residues 96−115) (Blechner *et al.*,1992; Olah *et al.*, 1994; Olah & Trewhella, 1994).

A high degree of flexibility in the central helix of CaM has been suggested to be responsible for its ability to bind a diverse array of target molecules (Persechini & Kretsinger, 1988a). X-ray, NMR, and small angle scattering studies further support the suggestion that a flexible central helix allows interdomain interaction when CaM is complexed with certain target molecules (Trewhella *et al.*, 1990; Ikura *et al.*, 1992; Raghunathan *et al.*, 1993; Meador *et al.*, 1993). There is no comparable information available for TnC.

Communication between the two Ca^{2+}-binding domains in TnC is well established (Levine *et al.*, 1977, 1978; Johnson *et al.*, 1978; Carew *et al.*, 1980; Iio & Kondo 1981; Grabarek *et al.*, 1986; Heidorn & Trewhella, 1988; Blechner *et al.*, 1992; Wang *et al.*, 1993). In addition, the connectivity between domains has also been shown to be essential for the regulatory function (Grabarak *et al*, 1981; Swenson & Fredrickson, 1992) suggesting that the central helix plays a crucial role in Ca^{2+}-signaling. However, the exact mechanism and the structural requirements of the central helix (flexible or rigid) for the regulatory function of TnC remain obscure.

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[1] Abbreviations: Tn, troponin; TnC, troponin C; TnI, troponin I; TnT, troponin T; TM, tropomyosin; CaM, calmodulin; CD, circular dichroism; DTT, dithiothreitol.

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Table 1: Design of Troponin C Central Helix Mutants

TnC	amino acid sequence[a]	predicted changes in the central helix	Stokes' radius (Å)[b]
WT	^{87}kedakgkseee^{97}		26.0
d11	^{87} ——— ^{97}	deletion of three turns of an α-helix (−16.5 Å[c])	22.5
inαh	^{87}kedakgkSEEELAKseee^{97}	insertion of two turns of an α-helix (+10.5 Å[c])	28.5
inrc	^{87}kedakgkGSGKGEGWGseee^{97}	insertion of a flexible random coil	27.5
inpp	^{87}kedakgkPPPPPPPPPseee^{97}	insertion of three turns of a rigid left-handed polyproline II helix (28Å[c])	29.5

[a] The amino acid sequence of the central helix is shown in lower case, and the insertion sequence is shown in upper case. [b] The Stokes's radii of TnCs were determined by analytical gel filtration in 10 mM imidazole, pH 7.0, 150 mM NaCl, 5 mM $MgCl_2$, 2 mM EGTA, 1 mM DTT (Ackers, 1975). [c] These values are based on the assumption that the central helix is an α-helix with 1.5 Å/residue, polyproline with 3.1 Å/residue.

In this work we address the structural requirement of the central helix for the regulatory function of TnC by inserting amino acid sequences of predicted conformations in this region. Two of the insertion linkers, a flexible random coil and three turns of a rigid polyproline II helix, should alter the flexibility of the central helix and increase the length between the Ca^{2+}-binding domains. A seven residue insertion with high helical potential should not alter the flexibility of the linker region but would increase the length between the Ca^{2+}-binding domains by two turns. Mutants containing flexible or rigid polypeptide inserts were defective in the thin filament activation in the presence of Ca^{2+}.

In addition, we made a deletion mutant lacking the entire D/E linker (three turns). Previous work showed that deletion of up to two turns (seven residues) did not greatly affect the regulatory function (Dobrowolski *et al.*, 1991a,b; Sheng *et al.*, 1991; Babu *et al.*, 1993). However, Babu *et al.* (1993) showed deletion of more than seven residues results in serious loss of function as tested on skinned muscle fibers. Here, we show our 11-residue deletion mutant is defective in TnI interaction and in inhibition of actomyosin ATPase in the absence of Ca^{2+}. This work has been previously reported in a preliminary form (Ramakrishnan & Hitchcock-DeGregori, 1993, 1994).

MATERIALS AND METHODS

Mutant Design and Construction. A synthetic cDNA for the chicken fast skeletal muscle troponin C was used in this study (Xu & Hitchcock-DeGregori, 1988). This recombinant protein has two amino acid differences from the TnC expressed in chicken pectoral muscle: residue 99 is Glu (instead of Ala) and residue 100 is Asp (instead of Asn) (Golosinska *et al.*, 1991). In addition, the amino terminus is unmodified. Previous comparison of the structure and function showed no significant differences between these two forms (Xu & Hitchcock-DeGregori, 1988; Dobrowolski *et al.*, 1988).

The mutants were constructed using oligonucleotide-directed mutagenesis using an *in vitro* mutagenesis kit from Bio-Rad (Zoller & Smith, 1983; Kunkel, 1985; Xu & Hitchcock-DeGregori, 1988; Dobrowolski *et al.*, 1991a). The mutation was confirmed by complete sequencing of the DNA (Sanger *et al.*, 1977). The alterations in the amino acid sequence and the predicted changes in the central helix are given in Table 1. The mutagenic oligonucleotides are complementary to the noncoding strand of the cDNA and are as follows (inserted DNA sequence is underlined): TnCd11 (3′-CAGGCAGTCTAC/GAACTTCTGACG-5′); TnCinαh (3′-GATTTCCATTTAGGCTCCTTCTCGAACGA-TTTAGACTTCTTCTTGAAC-5′); TnCinrc (3′-CTTCTGC-GATTTCCATTTCCAAGAGCTTTTCCGCCT-TCCACCCCAAGGCTCCTTCTTGAAC-5′); TnCinpp (3′-CTTCTGCGATTTCCATTTGGAGGAGGCGGCGGA-GGCGGTGGAGGCAGG′CTCCTTCTTGAAC-5′).

Routine molecular biological techniques, such as preparation of plasmid, M13 single and RF DNA, agarose gel electrophoresis, restriction enzyme digestion, dephosphorylation, and ligation, were performed as described by Sambrook *et al.* (1989) or as recommended by the supplier.

Expression and Purification of Proteins. The mutant cDNA was over-expressed in *Escherichia coli* BL21 (DE3) or BL21(DE3)pLysS cells using pET3d or pET11d vectors (Studier *et al.*, 1990). The cells were grown in 2× TY broth (16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl per liter) in a 37 °C shaking incubator to an OD_{550} of 0.6, and the protein production was induced by adding isopropyl thio-β-D-galactopyranoside (to 0.4 mM). The cells were then grown for 4−5 h. The expression of TnC was tested by analyzing the samples on 8% polyacrylamide gels containing urea (Head & Perry, 1974).

All mutant TnCs with the exception of TnCd11 were purified by phenyl-Sepharose column chromatography and reverse-phase HPLC as previously described (Xu & Hitchcock-DeGregori, 1988; Dobrowolski *et al.*, 1991a) with the modification that the buffer used for the reverse-phase HPLC contained 10 mM imidazole, pH 6.5, 0.1 mM $CaCl_2$, and 1 mM DTT. Since TnCd11 irreversibly bound to the phenyl-Sepharose column, this mutant was purified using anion-exchange chromatography (DE52 cellulose, Whatman; Dobrowolski *et al.*, 1991a). The yield of the protein varied between 10 and 30 mg/liter of culture. The purity of the proteins was evaluated by SDS−polyacrylamide gels.

Circular Dichroism (CD) Spectroscopy. Far-UV CD Measurements. CD measurements were carried out on a spectropolarimeter (Aviv model 62 DS) fitted with a temperature-controlled cell holder (Haake model CH constant temperature bath fitted with a Haake model F3 regulator). The far-UV spectrum was analyzed from 250 to 200 nm, every 0.5 nm, with 2 s collection time in a 1 or 2 mm rectangular quartz cell. The α-helix content was calculated according to Greenfield and Fasman (1969).

Thermal Denaturation. Thermal unfolding was recorded at 222 nm by increasing the temperature from 2 to 90 °C in two-degree intervals with 1 min equilibration time and 2 s collection time.

Calcium Titration. The protein samples were dialyzed against 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 1.0 mM DTT, 0.9 mM EGTA, and 0.9 mM nitrilotriacetic acid (NTA) for 48 h with two changes. Titrations were performed by addition of increasing amounts of 9.01 mM $CaCl_2$ (Banco) and an equimolar amount of KOH to maintain the pH. A 1 min CD-scan was taken at 250 and 222 nm after each addition. The free Ca^{2+} concentrations were determined as previously described (Dobrowolski *et al.*, 1991a) based on

the binding constants published by Sillen and Martel (1964) and calculated according to Perrin and Sayce (1967).

The ellipticity at 250 nm was subtracted from that at 222 nm in order to correct for displacement of the cell and the values were corrected for dilution. The experimental data were normalized on a 0−1 scale by calculating the fraction of the total conformational change as a function of Ca^{2+}. The data were fit using SigmaPlot, a curve fitting and statistical analysis program, to the following equation

$$\Theta_i = \frac{d\Theta_{max1}\,[Ca^{2+}]_{(i)}^{H1}K_{a1}^{H1}}{1 + [Ca^{2+}]_{(i)}^{H1}K_{a1}^{H1}} + \frac{d\Theta_{max2}\,[Ca^{2+}]_{(i)}^{H2}K_{a2}^{H2}}{1 + [Ca^{2+}]_{(i)}^{H2}K_{a2}^{H2}}$$

for Ca^{2+}-dependent changes in the ellipticity of TnC where Θ_i = ellipticity increase for ith calcium concentration (in percent of initial ellipticity), $d\Theta_{max1}$ and $d\Theta_{max2}$ = maximal ellipticity increase upon Ca^{2+}-binding to the high- and low-affinity sites, respectively; K_{a1} and K_{a2} are the Ca^{2+}-binding constants to the high and low affinity sites of TnC, respectively; $H1$ and $H2$ are the Hill coefficients of transitions for the high- and low-affinity Ca^{2+}-binding sites, respectively; $[Ca^{2+}]_{(i)}$ is the concentration of free Ca^{2+} ion after the ith addition.

ATPase Measurements. The thin filament was reconstituted by mixing actin, TM, and TnIT or TnI in a 7:1.5:2.5 molar ratio in 10 mM imidazole, pH 7.0, 1 mM DTT, and 0.15 M NaCl (final concentration). TnIT complex was prepared by combining TnI and TnT (in urea) in 1:1 molar ratio and dialyzing against 10 mM imidazole, pH 7.0, 0.25 M NaCl, 1 mM DTT. The actin mixture was sedimented at 60 000 rpm, 4 °C, for 30 min (Beckman TL-100 ultracentrifuge). The actin pellet was rinsed and resuspended in the same buffer. The same method was followed for preparing reconstituted thin filaments with TnI.

The actomyosin ATPase assays were performed in a total volume of 75 μL, in the condition described in the figure legends, in a thermoequilibrated microplate reader (Molecular Devices) at 28 °C. The reaction was started by the addition of MgATP to a final concentration of 5 mM and terminated after 15 min by adding of 25 μL of 13.4% SDS, 0.12 M EDTA. Time courses of the ATPase activity were carried out to establish that phosphate liberation was linear over the time of the experiment. The amount of inorganic phosphate released was determined colorimetrically according to White (1982) in microtiter plates and were read with a 650 nm filter. The data were fit to the Hill equation using SigmaPlot.

The Ca^{2+}-dependence of the actomyosin ATPase was carried out as described above except for the presence of 0.45 mM CaEGTA. The ratio of Ca^{2+} to EGTA was varied to obtain the desired free $[Ca^{2+}]$, calculated using the titration curves based on the binding constants published by Sillen and Martel (1964) and by Perrin and Sayce (1967). The data were fit to the Hill equation using SigmaPlot.

Analytical Gel Filtration. The Stokes' radius (R_s) of wild type and mutant proteins was determined by gel filtration (Ackers, 1975) using a Shodex protein WS-802.5 column, 8 × 300 mm (Showa Denko K. K.) attached to a Hewlett Packard HPLC 1050 with a flow rate of 0.5 mL/min, at room temperature. The buffer contained 10 mM imidazole, pH 7.0, 150 mM NaCl, 5 mM $MgCl_2$, 2 mM EGTA, and 1 mM DTT. Calibration of the column was carried out using protein standards with known R_s purchased from Sigma (ribonuclease, 12 000 Da; cytochrome C, 12 400 Da; myoglobin, 16 950 Da; bovine hemoglobin, 28 000 Da; ovalbumin, 43 000 Da; bovine serum albumin, 67 000 Da; transferrin, 76 000 Da; γ-globulin, 150 000 Da). The void volume (V_o) was determined with fibrinogen (330 000 Da) and the internal volume (V_i) with DTT.

The partition coefficient (σ) was calculated using the following equation:

$$\sigma = \frac{V_r - V_o}{(V_i - V_o)}$$

where V_r is the retention volume of the sample. Using the standards, the R_s values were determined by plotting $(-\log \sigma)^{1/2}$ versus R_s (Ackers, 1975).

Polyacrylamide Gel Electrophoresis. 8% polyacrylamide gels containing 6 M urea were used for the analysis of complex formation of TnC with TnI, and non-denaturing 8% polyacrylamide gels were used to detect the complex formation between TnC and TnT, as described by Head and Perry (1974). SDS−polyacrylamide gel electrophoresis was performed according to Laemmli (1970).

Reconstitution and Purification of Tn Complex. To determine the stoichiometry between Tn components in reconstituted Tn complexes with wild type and mutant TnCs, ternary complexes were prepared by mixing equimolar concentrations of TnI and TnT (in urea) and about a 2-fold molar excess of TnC. This complex was dialyzed against 10 mM imidazole, pH 7.0, 150 mM NaCl, 0.1 mM $CaCl_2$, 1 mM DTT. After dialysis, the complex was centrifuged at 10 000 rpm for 15 min. The complex was isolated by gel filtration using Protein Pak 300sw column, 7.5 × 300 mm (Waters Associates) with a flow rate of 0.5 mL/min, at room temperature. The Tn complex peak eluted at 26 min as did native skeletal troponin from chicken pectoral muscle. The fractions containing the Tn complex were combined, dialyzed, concentrated by lyophilization, and analyzed on 12% SDS−polyacrylamide gels stained with Coomassie Blue. The amount of the Tn components in the reconstituted Tn complex were quantified using a computing densitometer (Molecular Dynamics, model 300A).

General Methods. Contractile proteins were prepared from chicken pectoral muscle. Troponin was prepared according to Potter (1982) with the modification that protease inhibitors were added during extraction. α-Tropomyosin was purified from the isoelectric precipitate of the Tn preparation (Hitchcock-DeGregori *et al.*, 1985). Actin was purified from an acetone powder according to Hitchcock-DeGregori *et al.* (1982); myosin was prepared as described by Margossian and Lowey (1982).

Protein concentrations were determined using a biuret assay with bovine serum albumin standards (Goa, 1954) or spectrophotometrically using the following extinction coefficients: $A_{1\%}^{280}$ actin, 11.0; for tropomyosin, 3.0; for myosin, 5.3.

RESULTS

Mutant Design. To investigate the functional significance of the TnC central helix, we made a series of deletion and insertion mutations, as shown in Table 1 and modeled in Figure 1. Deletion of 11 residues (^{87}KEDAKGKSEEE^{97}) in TnCd11 would remove the entire exposed region of the central helix. The three insertion mutants were designed to have different predicted conformations. In TnCinαh, resi-

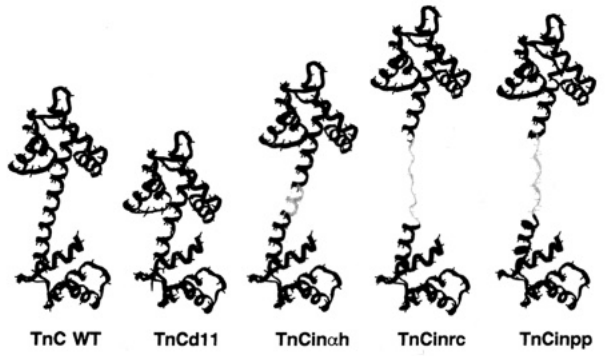

FIGURE 1: Molecular models of wild type and mutant TnCs. The atomic coordinates for the TnC were obtained from Brookhaven Protein Data Bank (Herzberg & James, 1988). The insertions in the central helix are shown in gray. The modeling of mutant TnCs and energy minimization (maximin2) were performed using the molecular modeling program Sybyl version 6.0 (Tripos). The backbone conformations of the α-helix insertion in TnCinαh and random coil insertion in TnCinrc were generated using the protein polymer building option available within the software (α-helix, $\varphi = -58°$, $\psi = -47°$; random coil, $\varphi = -60°$, $\psi = 60°$). The atomic coordinates for the backbone conformation of the polyproline II helix in TnCinpp were generated by application of dihedral angular rotations, $\varphi = -78°$, $\psi = 149°$, to a standard backbone model (Deber *et al.*, 1970). The energy-minimized model of TnCinpp shows that the central helix is distorted after the insertion of the polyproline II helix due to the altered dihedral angles where the polyproline II helix interfaces with the α-helix of TnC.

dues with a high helical probability were inserted to form two turns of an α-helix, assuming the residues in the linker form α-helix. Designed to duplicate seven residues of the D/E linker, it contains the conserved ^{94}SEEE^{97} charge cluster and three residues with a high potential to form an α-helix (LAK vs KGK). In TnCinpp, the nine-residue proline insertion should form three turns of a rigid, *all-trans*-polyproline II-like helix (Deber *et al.*, 1970). In these first three mutants, the lengths of the TnCd11 deletion and the TnCinαh and TnCinpp insertions were designed so that the orientation of the two Ca^{2+}-binding domains would be in a similar relative orientation as in wild type TnC, assuming the linkers assume the predicted conformations. In TnCinrc, the linker has nine residues with glycine present at every other residue and would be highly flexible.

All mutant proteins were well-expressed in *E. coli* and purified chromatographically, as previously described (Dobrowolski *et al.*, 1991a). Attempts to purify TnCd11 using phenyl-Sepharose affinity chromatography failed because it bound tightly to the column and did not elute with buffers containing EDTA or 5 M urea + 2 mM EDTA. This suggests that in TnCd11 the hydrophobic sites involved in the interaction with the phenyl-Sepharose are exposed even in the absence of Ca^{2+} (Brzeska *et al.*, 1983a). This property may account for its ability to bind to TnI in the absence of Ca^{2+} or defective inhibition of the actomyosin ATPase activity in the absence of Ca^{2+}. The hydrophobic site exposed in TnCd11 in the absence of Ca^{2+} may be different from that normally induced in wild type TnC by Ca^{2+} since the Ca^{2+} affinity and Ca^{2+} of the actomyosin ATPase are similar to that of wild type TnC (see below). Similar results were observed with two other central helix deletion mutants, d^{91}KGK^{93} and d^{87}KEDAKGK^{93} (Dobrowolski *et al.*, 1991a).

Conformation and Stability of Mutant Troponin Cs. To determine if the deletion and insertions in the central helix affected the secondary structure, the mutant TnCs were analyzed using circular dichroism spectroscopy. The CD spectra of all of the mutants, except TnCinpp, were nearly identical to wild type (Figure 2). TnCinpp differs from wild type and other mutants in that the minimum at 208 nm is always more prominent. This difference in the spectrum is most likely due to the contribution of the polyproline insert. Deconvolution of the spectral data of TnCinpp using α, β, random, turn (Brahms & Brahms, 1980), and polyproline II (Jenness et al., 1976) reference spectra indicated the presence of a polyproline II-type conformation, 5%, as expected based on the sequence.

Figure 2 also shows that all mutants became more helical upon binding of Mg^{2+} to the high-affinity sites, with a small further increase upon binding of Ca^{2+} to the low-affinity sites. The α-helix, calculated from the ellipticity measured at 222 nm, increased to 44%−49% and 55%−58% upon binding to Mg^{2+} and Ca^{2+}, respectively, comparable to published values (Kawasaki & van Eerd, 1972; Johnson & Potter, 1978; Dobrowolski *et al.*, 1991b; Babu *et al.*, 1993). The slightly lower ellipicity of TnCinαh compared to wild type TnC is most likely related to the difficulty to obtain highly accurate measurements of protein concentration. The Ca^{2+}-dependent conformational changes were also examined in polyacrylamide gels containing 6 M urea (Head & Perry, 1974). All mutant proteins migrated faster in the presence of Ca^{2+} relative to apo-TnC (results not shown).

To investigate if mutations in the central helix have a global effect on stability, we measured the ellipticity at 222 nm as a function of temperature in the presence of Mg^{2+}, EGTA (only high-affinity sites occupied). The T_m values for the mutant TnCs were similar to that of wild type (wild type, 68 °C; TnCinαh, 70 °C; TnCinrc, 68 °C; TnCinpp, 66 °C; TnCd11, 69 °C), close to values previously reported for rabbit skeletal TnC (Brzeska *et al.*, 1983b) and recombinant TnC (Dobrowolski *et al.*, 1991b; Smith *et al.*, 1994) under similar ionic conditions. These results suggest that changes in the central helix do not alter the thermal stability of TnC in a major way, consistent with our previous results (Dobrowolski *et al.*, 1991b).

Our models of mutant TnCs based on the X-ray structure indicated a possible change in the overall shape of the molecule. Insertions and deletions may result in extension and shortening of the molecule, respectively, though it must be recognized that the models of the mutants in Figure 1 are only derivatives of the wild type structure. To evaluate the consequences of the mutations on the shape in solution, we determined the Stokes' radius of the mutant TnCs. Table 1 shows that deletion or insertions in the central helix decreased or increased, respectively, the Stokes' radius. The order and the calculated difference in the radii between mutants and wild type are as follows: TnCd11 (−3.5 Å) < WT < TnCinrc (+1.5 Å) < TnCinαh (+2.5 Å) < TnCinpp (+3.5 Å). Except for TnCinrc, this order is consistent with the TnC models shown in Figure 1, although it is difficult to correlate the Stokes' radius with the radius of gyration (R_g) or actual length from the molecular models based on the X-ray structure. The number of inserted residues in TnCinrc is higher than in TnCinαh yet its radius is smaller. This suggests that the flexible random coil structure of the insert results in the collapse of the central helix and an overall more spherical shape than with the other inserts. We conclude that central helix mutations affect conformation of the central helix and therefore the overall length of TnC.

Regulation of Actomyosin ATPase. Relief of TnIT Inhibition. To learn if the alterations in the central helix affect the Ca^{2+}-dependent activation of the thin filament, we

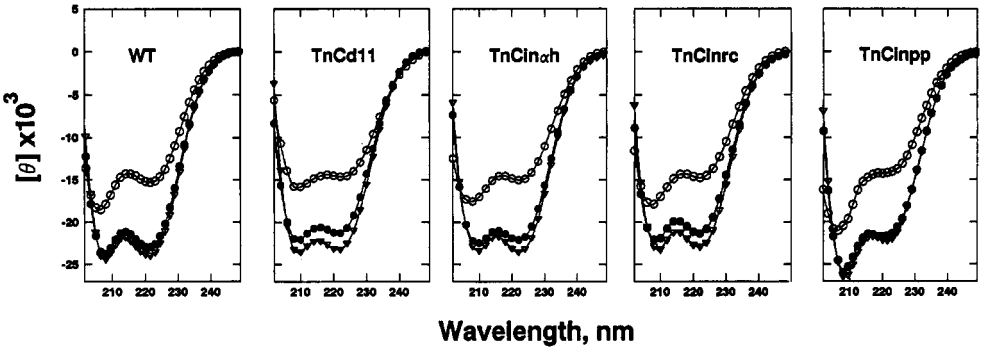

FIGURE 2: Far-UV circular dichroism spectra of wild type and mutant TnCs. The spectra were determined at 2 °C, in a 1 mm cuvette, 0.15−0.3 mg of protein/mL. Θ is in the units of deg cm²/dmol. Conditions: 2 mM HEPES, pH 7.0, 50 mM NaCl, 1 mM DTT and either 2 mM EDTA (○), 5 mM $MgCl_2$ and 1 mM EGTA (●), or 3 mM $CaCl_2$(▽).

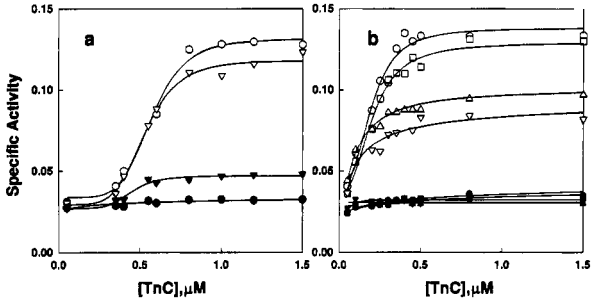

FIGURE 3: Regulation of the actomyosin ATPase activity by wild type and mutant TnCs. Increasing concentrations of TnC were added to a reconstituted thin filament complex containing actin, tropomyosin, TnI and TnT. The final concentrations were myosin (0.9 μM), thin filament (about 2.4 μM of actin), TnC (0−1.5 μM) in 10 mM imidazole, pH 7.0, 40 mM NaCl, 0.5 mM $MgCl_2$ and 0.5 mM ATP, and 0.1 mM $CaCl_2$ or 0.2 mM EGTA. The assay was performed for 15 min. at 28 °C. Specific activity is expressed as μmol of P_i/mg of myosin/min. The data in Figure 3a,b are representative, and the data are from experiments carried out at different times. The difference in the TnC concentration required for half-maximal activation reflects the fact that the concentration of the reconstituted thin filaments (and the amounts of TnI and TnT bound) could not be strictly controlled because of the way they were made (see Materials and Methods). a: ○ and ●, wild type; ▽ and ▼, TnCd11. b: ○ and ●, wild type; □ and ■, TnCinαh; △ and ▲, TnCinrc; ▽ and ▼, TnCinpp. Open symbols, 0.1 mM $CaCl_2$; filled symbols, 0.2 mM EGTA.

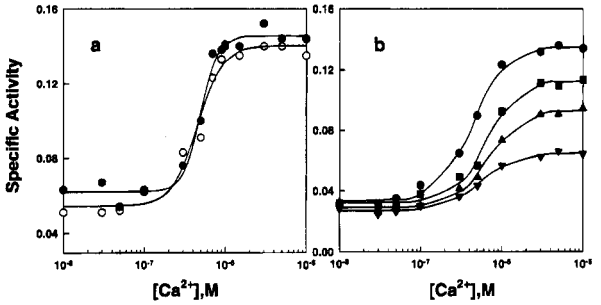

FIGURE 4: Ca^{2+}-dependence of the regulated actomyosin ATPase activity with wild type and mutant TnCs. Conditions: The assay was carried out as described in Figure 3 except at a saturating TnC concentration (1μM). The free Ca^{2+} concentration was controlled using CaEGTA buffer system as described under Materials and Methods. a: ●, wild type; ○, TnCd11. b: ●, wild type; ■, TnCinαh; ▲, TnCinrc; ▼, TnCinpp.

assayed the ability of the mutants to regulate the actomyosin MgATPase in a reconstituted thin filament system. Troponin complex incorporating TnCd11 was indistinguishable from wild type TnC in its ability to activate the actomyosin ATPase activity in the presence of Ca^{2+} (97% ± 4%, n = 5) but was less effective in inhibiting in the absence of Ca^{2+} (Figure 3a), as were the d^{87}KEDAKGK^{93} and d^{91}KGK^{93} mutants previously described (Dobrowolski *et al.*, 1991a). The sigmoidal shape of the curves (seen also in Figure 5) most likely relates to the cosedimentation of excess TnI or TnIT with the reconstituted thin filaments.

Figure 3b shows TnCinrc and TnCinpp were defective in activation of the actomyosin ATPase activity in the presence of Ca^{2+} and had only 62% ± 3% and 58% ± 2% of the wild type maximal activity, respectively (n = 6). In TnCinαh, activation of actomyosin ATPase activity was nearly identical to wild type TnC (87% ± 3%, n = 6). Percent maximal activation was calculated by dividing maximal activation of the mutant TnC by the maximal activation of wild type, multiplied by 100.

To establish if the mutant proteins were able to form ternary complexes with TnIT, we measured the stoichiometry of the Tn components in HPLC-purified reconstituted Tn complexes in the presence of saturating Ca^{2+} (see Materials and Methods). In five independent experiments, the ratio of TnC to TnI in reconstituted troponin complexes containing insertion mutants normalized to that in Tn with wild type TnC was one (TnCinαh, 1.0 ± 0.04; TnCinrc, 1.0 ± 0.13; and TnCinpp, 1.0 ± 0.05). These results indicate that the failure of TnCinrc and TnCinpp to fully activate the thin filament in the presence of Ca^{2+} cannot be explained by a failure of the mutant TnCs to form a ternary troponin complex with the correct stoichiometry. In TnCd11−IT complex, the TnC:TnI ratio was twice (2.00 ± 0.4) that in complexes made with wild type TnC, agreeing with the report by Babu *et al.* (1993) on a comparable deletion mutant of rabbit skeletal TnC.

Ca^{2+}-Dependence of the Actomyosin ATPase. There were small differences between mutants in the Ca^{2+} concentration required for the half-maximal activation (K_d) of the actomyosin ATPase (Figure 4a,b) [WT, (4.0 ± 0.3) × 10^{-7} M; TnCd11, 6.0 × 10^{-7} M; TnCinαh, (6.3 ± 0.8) × 10^{-7} M; TnCinrc (5.8 ± 0.3) × 10^{-7} M; and TnCinpp (6.8 ± 0.5) × 10^{-7} M, K_d values were obtained from 8−10 different data sets and were averaged ±SD. The K_d for TnCd11 is the average of the values from two experiments].

Interaction with TnI and TnT. To investigate if the impaired regulatory function of mutant TnCs is due to altered subunit interaction with TnI or TnT, we analyzed the Ca^{2+}-dependent binary complex formation of TnC with TnI and TnC with TnT in polyacrylamide gels (Head & Perry, 1974). All mutants TnCs formed complexes with TnI and TnT in the presence of Ca^{2+} (results not shown), but, surprisingly, TnCd11 formed a complex with TnI even in the absence of Ca^{2+} (Figure 5, inset).

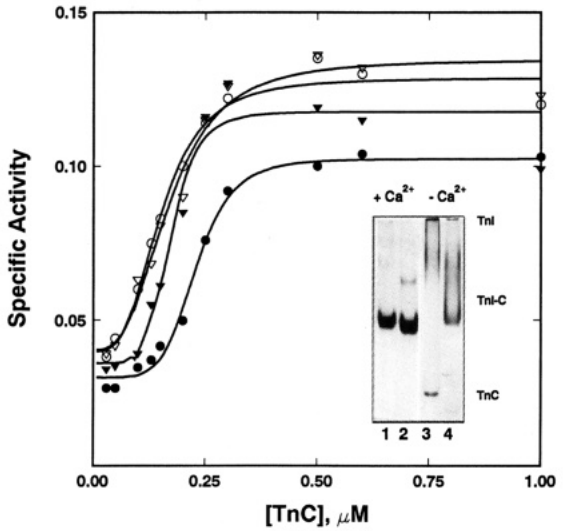

FIGURE 5: Relief of troponin I inhibition of the actomyosin ATPase by wild type and TnCd11. Conditions: The assay was carried out as described in Figure 3 except the reconstituted filament contained actin, tropomyosin, and TnI (no TnT). ○ and ●, wild type; ▽ and ▼, TnCd11. Open symbols, 0.1 mM $CaCl_2$; filled symbols, 0.2 mM EGTA. *Inset*: Electrophoresis of TnI-C in polyacrylamide gels: TnI and TnC were combined in 2.5 M urea/10 mM Tris-HCl, pH 8.0, 50 mM NaCl, 5 mM EGTA or 2.5 mM $CaCl_2$, and analyzed on 8% polyacrylamide gels. Lanes 1 and 3, wild type; lanes 2 and 4, TnCd11.

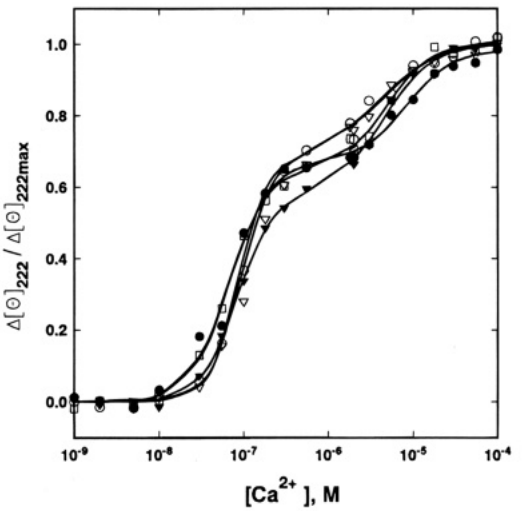

FIGURE 6: Ca^{2+} titration of the change in ellipticity at 222 nm. Wild type and mutant TnCs (0.1 mg/ml, in 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.9 mM EGTA, and 0.9 mM nitrilotriacetic acid, 25 °C in a 2 mM cuvette) were titrated with increasing Ca^{2+} as described under Materials and Methods. The curves have been fitted for two classes of sites. A representative data set is shown here. The parameters averaged from two data sets are presented in Table 2. Symbols: ○, wild type; ●, TnCd11; ■, TnCinαh; ▲, TnCinrc; ▽, TnCinpp.

To further characterize TnC-TnI interaction, we investigated the ability of mutant TnCs to relieve the TnI-inhibition of actomyosin ATPase activity in the presence and absence of Ca^{2+}. The relief of TnI inhibition by TnC in the absence of Ca^{2+} is TnC-specific in that CaM cannot substitute (Amphlett *et al.*, 1976). The relief of inhibition of actomyosin activity in the presence of Ca^{2+} by TnCd11, was comparable to wild type TnC (Figure 5). However, in the absence of Ca^{2+}, the relief of inhibition by TnCd11 was 1.32-fold (±0.09, $n = 6$) higher than wild type, consistent with results with the fully regulated actomyosin ATPase activity (Figure 4a), reflecting the tight binding to TnI in the absence of Ca^{2+} (Figure 5, inset). Greater relief of TnI ($-Ca^{2+}$) was also observed with d^{87}KEDAKGK^{93} and d^{91}KGK^{93} mutants previously reported from this laboratory (Dobrowolski *et al.*, 1991b). All insertion mutants were equally effective in relieving TnI inhibition in the presence and absence of Ca^{2+} as compared to wild type TnC (results not shown).

Calcium Affinity of Central Helix Mutants. We measured the Ca^{2+} binding to the low- and high-affinity sites indirectly using circular dichroism by taking advantage of the increase in ellipticity at 222 nm upon Ca^{2+} binding (Hincke *et al.*, 1978; Golosinska *et al.*, 1991; Pearlstone *et al.*, 1992; Smith *et al.*, 1994). Figure 6 shows the Ca^{2+} titration curves of wild type and other mutant TnCs. A major increase in ellipticity (60%−70%) occurs in all cases when Ca^{2+} binds to the high-affinity sites, consistent with previous reports (Hincke *et al.*, 1978; Golosinka *et al.*, 1991; Pearlstone *et al.*, 1992; Smith *et al.*, 1994). The mutations had little effect on the Ca^2 affinity of the low- and high-affinity sites (Table 2). In TnCinrc, the percentage change in ellipticity was lower when the high affinity sites are occupied by Ca^{2+} (Table 2). In TnCd11, the affinities of both classes of sites were consistently different than wild type TnC, but this difference was not observed in the Ca^{2+}-dependence of the actomyosin ATPase (Figure 4a).

DISCUSSION

The central helix connecting the calcium-binding domains of TnC is highly conserved in length and sequence (Collins, 1991), yet an understanding of its functional significance has remained elusive. Here we have shown that altering the predicted conformation of the linker connecting the two calcium-binding domains impairs the ability of TnC to activate the thin filament in the presence of Ca^2, independent of the increase in length of the molecule as indicated by the Stokes' radius. Length increase due to insertion of an α-helical linker in TnCinαh did not significantly affect the regulatory function, as compared to TnCinrc and TnCinpp. In contrast, insertion of four residues in the central helix of CaM resulted in functional abnormalities independent of the composition of the insert (Putkey *et al.*, 1988).

It is well-established that the N- and C-terminal domains of TnC bind independently to different sites on TnI (Leszyk *et al.*, 1990; Kobayashi *et al.*, 1991; Swenson & Fredricksen, 1992; Farah *et al.*, 1994). Connectivity between the domains does appear to be important for efficient regulatory function in that a mixture of the N- and C-terminal fragments is inefficient in activating the thin filament (Grabarek *et al.*, 1981; Swenson & Fredricksen, 1992). Similarly, in CaM none of the single-domain peptides can activate the target enzymes (Newton *et al.*, 1984) except at extremely high concentration of single-domain peptides (Persechini *et al.*, 1994). One hypothesis could be that the central helix serves as a flexible linker to allow the two Ca^{2+}-binding domains to bind to the target sites, as has been postulated for CaM (Persechini & Kretsinger, 1988a,b). Persechini & Kretsinger (1988a) have shown that as long as the two domains are connected, either by the polypeptide chain of the central helix or by a disulfide bond between residues in the two domains, activity is preserved. However, recent studies using small-angle scattering techniques indicate TnC is elongated when it binds to TnI (Olah *et al.*, 1994; Olah & Trewhella, 1994).

Our results imply that connectivity alone is insufficient for TnC function and that the structure of the TnC central

Table 2: Calcium Binding to Troponin Cs[a]

	high affinity sites			low affinity sites		
protein	K_d (M × 10^8)	fractional change ($\Delta\theta_1$)	Hill coefficient, *H1*	K_d (M × 10^6)	fractional change ($\Delta\theta_2$)	Hill coefficient, *H2*
WT	9.2	0.68	2.3	4.4	0.32	1.2
TnCd11	5.6	0.65	1.8	8.0	0.35	1.4
TnCinαh	9.8	0.66	2.3	3.8	0.34	1.2
TnCinrc	9.2	0.57	2.0	4.6	0.43	1.4
TnCinpp	7.8	0.65	1.9	4.3	0.35	1.4

[a] The values are the mean of two of the parameters reported by SigmaPlot. A representative data set is shown in Figure 6.

helix is crucial for regulatory function. If a major role of the central helix were merely to allow proper positioning of the N- and C-terminal domains with respect to the target, one might have anticipated that the random coil insertion mutant would be sufficiently flexible to allow normal regulatory function. We were surprised that TnCinrc is impaired in activating the thin filament. If flexibility and molecular length alone were important, one would expect TnCinrc to function more normally than TnCinαh which has the potential to form two additional turns of α-helix and would be less flexible than TnCinrc, as implicated by the larger Stokes' radius. Clearly, our results are inconsistent with the hypothesis that the central helix serves merely as a flexible linker; the structure of the central helix must also play a crucial role in the cooperative activation of the thin filament.

Our results indicate that the nature of the structure of the TnC central helix is crucial for the regulatory function. Increasing flexibility (as with TnCinrc) or rigidity (as with TnCinpp) severely affects the regulatory function of TnC. The Stokes' radius of TnCinpp indicates that it is more extended than the other mutants, and the polyproline insert should form a rigid connector between the Ca^{2+}-binding domains. At the same time, the elongation of the molecule without a major change in its structure did not significantly affect the function, as seen with TnCinαh. Reducing the flexibility of the central helix by changing Gly 92 to Ala, Leu or Pro or changing Lys 91 and 93 to Leu has no marked effect on TnC function (Reinach & Karlsson, 1988; Ding *et al.*, 1994), indicating that minor perturbations to the central helix are more tolerated.

We have been unable to define the molecular basis for the impaired regulatory function of the insertion mutants. Increasing, or decreasing, the length of the linker does not affect the global folding of the molecule or the Ca^{2+}-binding affinities of the N- and C-terminal sites in any major way. All insertion mutants bind to both TnI and TnT in a Ca^{2+} dependent manner, and form a ternary complex with TnI and TnT with the appropriate stoichiometry. It must be that interactions within the ternary complex are altered, as we have suggested for a N-terminal deletion mutant of TnC (Smith *et al.*, 1994). Although the insertion mutant TnCs in the regulated actomyosin complex bind Ca^{2+} with relatively normal affinity, they are impaired in the signal transduction required for full activation.

The regulatory function of TnC is more tolerant to shortening of the central helix. Deletion of seven residues has little effect on function (Dobrowolski *et al.*, 1991a,b; Sheng *et al.*, 1991; Babu *et al.*, 1993) and activation of the regulated actomyosin ATPase activity is relatively normal in TnCd11. However, inhibition of the regulated thin filament in the absence of Ca^{2+} and relief of TnI inhibition of the actomyosin ATPase in the absence of Ca^{2+} are impaired, as we have found for two other central helix deletion mutants (d ^{91}KGK^{93} and d^{87}KEDAKGK^{93}. Dobrowolski *et al.*, 1991a,b). Unlike wild type TnC and all other mutants we have studied, TnCd11 binds tightly to TnI in the absence of divalent cation.

Our observation that TnCd11 can activate the regulated actomyosin ATPase in the presence of Ca^{2+} is consistent with the results of 12 residue deletion mutant (Babu *et al.*, 1993). Their SDS−PAGE analysis of TnC-d12-reconstituted TnC-depleted fibers showed that the Ca^{2+}-loaded fibers contained two times the normal amount of TnC, just as we have observed in our reconstituted troponin complex with the TnCd11 mutant. The 2-fold excess of TnCd11 in the reconstituted complex and skinned fibers supports the proposal of Babu *et al.* (1993) that the two domains in TnCd11 are too close together for one molecule to bind both the Ca^{2+}-dependent and the Ca^{2+}-independent TnC binding sites on TnI. In their model, two mutant TnC molecules bind independently to the target sites and both are necessary for activation of the thin filament. Their model predicts that twice as much TnCd12 as wild type TnC would be needed for full activity. Even though our *in vitro* assay conditions are similar to their over-loading conditions in the presence of Ca^{2+}, we have not observed this to be the case. Possibly, the equilibria may be too complex to detect only a 2-fold difference. Since TnCd11 binds more strongly to TnI in the absence of Ca^{2+} than wild type, it is possible that this binding may be responsible for the higher activation of the actomyosin ATPase in the absence of Ca^{2+} and may explain how one mole of TnC/complex could allow full activation in the presence of Ca^{2+}. Babu *et al.* (1993) did not measure the TnC dependence of tension development in the presence of EGTA or Ca^{2+}.

In this work we have shown that mutations predicted to alter the flexibility of the central helix impair the ability of TnC to activate the thin filament. TnC is more tolerant of deletions in the central helix and to some extent increases in the length. Our observations suggest the structural and functional elements of the TnC central helix provide more than simply connectivity between the domains and may play a major role in Ca^{2+} signaling in the ternary complex.

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