Mutations in the N- and D-Helices of the N-Domain of Troponin C Affect the C-Domain and Regulatory Function

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ABSTRACT Troponin C contains a 14-residue α-helix at the amino terminus, the N-helix, that calmodulin lacks. Deletion of the first 11–14 residues of troponin C alters function. In the present investigation a mutant lacking residues 1–7 of the N-helix has normal conformation, Ca\textsuperscript{2+} binding, and regulatory function. Thus, residues 8–14 of the N-helix are generally sufficient for troponin C function. In the x-ray structures of troponin C there is a salt bridge between Arg 11 in the N-helix and Glu 76 in the D-helix. Destroying the salt bridge by individually mutating the residues to Cys has no effect on function. However, mutation of both residues to Cys reduces troponin C’s affinity for the troponin complex on the thin filament, reduces the stability of the N-domain in the absence of divalent cations, increases the Ca\textsuperscript{2+} affinity and reduces the cooperativity of the Ca\textsuperscript{2+}-Mg\textsuperscript{2+} sites in the C-domain, and alters the conformational change that takes place upon Ca\textsuperscript{2+} binding (but not Mg\textsuperscript{2+} binding) to the C-domain. Cross-linking with bis-(maleimidomethyl)ether partially restores function. The Ca\textsuperscript{2+}-specific sites in the N-domain, those closest to the sites of the mutations, are unaffected in the assays employed. These results show that the N-helix is a critical structural element for interaction with and activation of the thin filament. Moreover, mutations in the N-helix affect the C-terminal domain, consistent with recent structural studies showing that the N-helix and C-terminal domain are physically close.

INTRODUCTION

Troponin C (TnC) is a Ca\textsuperscript{2+}-binding protein that in a complex with troponin I (TnI) and troponin T (TnT) on the actin-tropomyosin thin filament is required for Ca\textsuperscript{2+}-dependent regulation of striated muscle contraction (for review, see Herzberg and James, 1995; Tobacman, 1996; Squire and Morris, 1998).

Troponin C has four Ca\textsuperscript{2+}-binding sites. Two sites in the carboxyl-terminal domain are occupied by either Ca\textsuperscript{2+} or Mg\textsuperscript{2+}. Occupancy of the amino-terminal sites by Ca\textsuperscript{2+} is required for activation of contraction. The availability of high-resolution crystal structures of TnC has allowed comparison of two states where the amino-terminal regulatory sites are unoccupied and occupied (Herzberg and James, 1988; Satyshur et al., 1988, 1994; Gagné et al., 1995; Slupsky and Sykes, 1995; Houdusse et al., 1997; Strynadka et al., 1997). Herzberg et al. (1986) proposed a model for the molecular switch in which a hydrophobic patch is exposed upon Ca\textsuperscript{2+} binding as a consequence of movement of the B and C helices away from the N, A, and D helices, based on the early TnC and calmodulin structures (Babu et al., 1988). Experimental evidence and recent nuclear magnetic resonance (NMR) and x-ray structures have supported and extended this model (Gagné et al., 1995; Slupsky and Sykes, 1995; Houdusse et al., 1997; Strynadka et al., 1997; reviewed in Farah and Reinach, 1995). However, the conformational change is not a universal requirement for activation as cardiac TnC, which lacks a functional site I, does not undergo an extensive opening (Sia et al., 1997).

Although TnCs and calmodulin have an overall similar structure, two differences are a 14-residue α-helix at the extreme amino terminus of TnC (the N-helix), which is absent in calmodulin, and one additional turn in the central helix of TnC. The N-helix is a ubiquitous feature of TnC. The N-helix sequences of avian and mammalian fast TnCs are closely related except the avian forms are three residues longer at the amino terminus. In contrast, the sequences of fast and slow/cardiac TnC N-helices are quite different (Putkey et al., 1987; Collins, 1991). The N-helix interacts with the A and D helices via hydrophobic and electrostatic interactions (Herzberg and James, 1988; Satyshur et al., 1988; Houdusse et al., 1997; Strynadka et al., 1997).

There is evidence that the N-helix has a specific role in TnC-dependent regulatory function even though its association with the A- and D-helices is maintained upon Ca\textsuperscript{2+} binding (Gagné et al., 1995; Slupsky and Sykes, 1995; Houdusse et al., 1997; Strynadka et al., 1997). Deletion of the N-helix (12–14 residues, based on the avian fast TnC sequence) from fast or slow/cardiac TnC results in a TnC that gives only ~50% Ca\textsuperscript{2+}-dependent activation of the thin filament in a reconstituted actomyosin assay, as well as in TnC-depleted myofibrils or muscle fibers (Gulati et al., 1993; Liu et al., 1994; Smith et al., 1994). Troponin C lacking the first 11 residues partially activates the regulated actomyosin S1 ATPase but fully activates TnC-depleted fibers (Chandra et al., 1994). Deletion of 11–14 residues reduces the affinity of the low-affinity Ca\textsuperscript{2+}-binding sites as well as the stability of the amino-terminal domain (Chandra et al., 1994).
et al., 1994; Liu et al., 1994; Smith et al., 1994; Fredricksen and Swenson, 1996).

In the present study we have shown that residues 8–14 of the N-helix are sufficient for function. Single-site mutations that destroy the salt bridge between Arg 11 of the N-helix and Glu 76 in the D-helix have no observable effect on function. Mutation of both of them, however, affects the affinity and cooperativity of the C-domain Ca\(^{2+}\)-binding sites and its affinity for TnIT in a regulated actin filament. Portions of this work have been published in a preliminary form (Smith, 1995).

**MATERIALS AND METHODS**

**DNA constructs**

A synthetic cDNA for chicken fast skeletal muscle TnC was used in this study (Xu and Hitchcock-DeGregori, 1988). Two codons in the original synthetic cDNA sequence were changed using site-directed mutagenesis to match the amino acid sequence of chicken pectoral muscle TnC (E99A, D100N) (Golosinska et al., 1991).

The mutants were made using oligonucleotide-directed mutagenesis (Zoller and Smith, 1983; Kunkel, 1985) using a Bio-Rad Muta-Gene in vitro mutagenesis kit, (Bio-Rad Laboratories, Hercules, CA) with modifications (Xu and Hitchcock-DeGregori, 1988). The nucleotides encoding amino acid residues 1–7 of the N-helix of TnC were deleted. The oligonucleotide sequence (3’ GATCTCCTAGTGACGACTTCGACAGC5’; C101L(R11,E76), 3’-CATGGTGACGTAGAAGCCGAGACACATAC5’; C101L(R11,E76), 3’-CTCCTGAGACGTGAAAGCCTAGAAGC5’). The triple mutant (R11C, E76C, C101L) was made by combining all three oligonucleotides in a single reaction. *Escherichia coli* DH5α cells were transformed with a portion of the synthesis reaction, single plaques were isolated, and the DNAs were screened for mutants using DNA sequencing. After plaque purification, the complete cDNA sequences were determined using the dideoxynucleotide chain termination method with [α-32P]dATP (Sanger et al., 1977).

Recombinant DNA methods such as transformations, agarose and polyacrylamide gel electrophoresis, small- and large-scale preparation of plasmids, restriction fragment and single-stranded DNA preparations, isolation of fragments from agarose gels, phosphorylation, dephosphorylation, ligation, and enzyme digestions were carried out following the protocols of Sambrook et al. (1989) or as suggested by the supplier.

**Protein expression and purification**

The cDNAs were cloned into pET3d at NcoI and BanHI sites and expressed in *E. coli* BL21 (DE3) pLyS8 (Studier et al., 1990). Expression of TnC was induced by adding isopropyl-thio-b-D-galactopyranoside to 0.4 mM when cultures reached an OD\(_{600}\) of 0.6. After 4 additional hours of growth, the bacterial cells were harvested by centrifugation at 12,000 rpm for 10 min. The expression of TnC was determined by analyzing samples on 8% polyacrylamide gels containing 6 M urea, 2 mM EDTA (Head and Perry, 1974).

Mutant TnCs and wild-type TnC were purified by phenyl-Sepharose column chromatography and reverse phase high-pressure liquid chromatography as previously described (Xu and Hitchcock-DeGregori, 1988; Dobrowolski et al., 1991; Smith et al., 1994). The buffer was modified to be 10 mM imidazole, pH 6.5, 0.1 mM CaCl\(_2\), 1 mM dithiothreitol (DTT). The elution profiles of the mutant TnCs were similar to wild-type TnC. The purity of the proteins was evaluated using SDS-polyacrylamide gel electrophoresis and UV absorption spectroscopy. The yield of mutant TnCs was ~15–20 mg/L of culture.

**Circular dichroism spectroscopy**

Circular dichroism measurements were performed on an Aviv model 62 DS spectropolarimeter fitted with a thermal regulated cell holder in 1- or 2-mm rectangular quartz cuvettes. The temperature was controlled using a Haake model CH constant temperature bath fitted with Haake model F3 temperature controller as previously described (Smith et al., 1994).

**Thermal denaturation**

The temperature-dependent unfolding was monitored at 222 nm. The temperature was increased from 2°C to 90°C in 0.2°C intervals with a 0.1-min equilibration and 5-s collection time. Far UV spectra (200–250 nm) were taken at 2°C and 90°C and after refolding to 2°C (data not shown). The spectra before unfolding and after refolding were the same.

**Calcium titration**

For Ca\(^{2+}\) titrations, proteins were dialyzed for 48–72 h in 50 mM Tris/ HCl, pH 7.5, 100 mM KCl, 1.0 mM DTT, 0.9 mM EGTA, and 0.9 mM nitritotriacetic acid (NTA). To measure the Ca\(^{2+}\) dependence of the circular dichroism, increasing amounts of a 9.01 mM CaCl\(_2\) stock (Banco, Anderson Laboratories, Fort Worth, TX) were added. The pH was maintained by adding an equimolar amount of KOH after each Ca\(^{2+}\) addition. The free Ca\(^{2+}\) concentration was calculated as previously described (Dobrowolski et al., 1991; Smith et al., 1994) based on binding constants published by Sillen and Martel (1964) and calculated according to Perrin and Sayce (1967).

After each Ca\(^{2+}\) addition, a 1-min scan was taken at 250 and 222 nm. To correct for displacement of the cell, the ellipticity at 250 nm was subtracted from that at 222 nm. After correction for dilution, the data were normalized on a 0 to 1 scale by calculating the fraction of the total conformational change as a function of Ca\(^{2+}\). The experimental data were analyzed using SigmaPlot (Jandel Scientific, San Rafael, CA), a curve-fitting and statistical analysis program, using the equation as previously described (Smith et al., 1994).

**Magnesium titration**

Wild-type and mutant TnCs were dialyzed for 24 h in 100 mM KCl, 50 mM Tris/HCl, pH 7.5, 1 mM EDTA. The EDTA was removed by dialysis for 72 h in the same buffer containing 2 mM EGTA. Aliquots of a 25 mM MgCl\(_2\) stock were added until no further change in ellipticity was observed. Data collection and analysis were carried out as for the Ca\(^{2+}\) titrations.

**ATPase measurements**

Reconstituted thin filaments were prepared by co-sedimentation of actin, tropomyosin, and TnT in a 7:1.5:3.5 molar ratio. Troponin I and TnT (in 6 M urea) were combined in a 1:1 molar ratio and dialyzed in 0.25 M NaCl, 10 mM imidazole, pH 7.0, 1 mM DTT. The stoichiometry of TnI and TnT in the soluble complex was approximately 1:1 based on densitometry of a Coomassie-blue-stained SDS gel of the complex. The concentration of the complex was determined using an extinction coefficient (A(1% at 280 nm) = 4.2) and then mixed with actin and tropomyosin (see Fig. 1) in 0.15 M NaCl, 10 mM imidazole, pH 7.0, 1 mM DTT (final concentrations). The mixture was sedimented in a Beckman TL-100 ultracentrifuge at 60,000 rpm and 4°C for 30 min. The pellet was resuspended in the same buffer to give an approximate concentration of 24 μM actin.

The assays were carried out in a total volume of 75 μL, in the conditions described in the figure legends, in a thermostatted Molecules Devices Thermomix microtiter plate reader (Menlo Park, CA). The reaction was...
started by the addition of Mg-ATP to a final concentration of 5 mM and terminated after 15 min by addition of 13.4% SDS, 0.12 M EDTA. Time courses of the ATPases were carried out to determine that phosphate liberation was linear over the time of the experiment. The inorganic phosphate was determined colorimetrically according to White (1982) in microtiter plates that were read with a 650-nm filter. The data were fit to the Hill equation using SigmaPlot (version 2.0).

The Ca$^{2+}$ dependence of the actomyosin ATPase was carried out at a saturating TnC concentration, as described in the ATPase assay except for the presence of 0.45 mM Ca-EGTA. The ratio of Ca$^{2+}$ to EGTA to obtain the desired free Ca$^{2+}$ was determined using titration curves based on binding constants published by Sillen and Martel (1964) and calculated according to Perrin and Sayce (1967).

**Chemical modification**

**Cross-linking**

The triple mutant was fully reduced in 20 mM DTT, 6 M urea, 4 mM EDTA, 10 mM HEPES, pH 7.0, 20 mM NaCl. Reduction was carried out at 25°C for 2 h. All buffers were degassed with helium before use. The proteins were dialyzed overnight at 4°C in the same buffer without DTT and urea, to allow renaturation to occur slowly. A 10 mM stock of bis-(maleimidomethyl)ether (BMME) dissolved in N,N′-dimethylformamide (DMF) was used for cross-linking (Parks et al., 1994). A 10:1 molar ratio of BMME to protein was mixed for 1.5 h at 25°C. The reaction was terminated by the addition of 30 mM DTT.

**SH blocking**

Sulphydryls were blocked with N-ethylmaleimide (NEM) at 25°C for 4 h at a 20:1 molar ratio. A 10 mM stock of NEM was prepared in DMF. The reaction was stopped by adding 30 mM DTT. After termination of reactions (cross-linking and blocking), proteins were extensively dialyzed in 40 mM NH$_4$HCO$_3$ to remove DMF and unreacted materials. Proteins were then lyophilized, resuspended in 2 mM HEPES, pH 7.0, 20 mM NaCl, and dialyzed overnight.

Cross-linked and blocked products were analyzed on 6 M urea, 4 mM EDTA, 10% polyacrylamide gel electrophoresis gels. Cross-linking and SH blocking procedures are as described by Persechini et al. (1988) with modifications.

**Molecular modeling**

Molecular modeling was performed on an Aries (Indy) or Jupiter (Indigo2) Silicon Graphics system (SGI, Mountain View, CA) using the molecular modeling programs Sybyl version 6.0 (Tripos Associates, St. Louis, MO) and InSight II (MSI/Biosym, San Diego, CA). The coordinates of TnC were taken from Brookhaven Protein Data Bank (Herzberg and James, 1988).

**Preparation of contractile proteins**

Contractile proteins, other than recombinant TnC, were prepared from chicken pectoral muscle using methods that have been published. Skeletal actin was purified from an acetone powder (Hitchcock-DeGregori et al., 1985). Troponin was prepared according to Potter (1982) with the addition of protease inhibitors during extraction; troponin components were separated according to Hitchcock et al. (1981). Myosin was prepared as described by Margossian and Lowey (1982).

Protein concentrations were determined using the following extinction coefficients A (1% at 280 nm): actin, 11.0; troponomyosin, 3.0; myosin, 5.3. The concentration of TnC was determined colorimetrically using a biuret assay with bovine serum albumin as a standard (Goa, 1954).

**RESULTS**

**Determination of the minimal N-helix required for troponin C function**

All TnCs have an N-helix, although the length and sequence differ among isoforms and species (Collins, 1991). Deletion of 11 or more residues results in significant loss of TnC function (Gulati et al., 1993; Chandra et al., 1994; Liu et al., 1994; Smith et al., 1994). In the TnC structure, the first four residues are poorly resolved, and residues 8–14 account for most of the hydrophobic and electrostatic interactions between the N-helix and the A- and D-helices that flank the regulatory Ca$^{2+}$-binding sites I and II. To learn whether residues 8–14 are sufficient, we deleted the first seven residues of TnC using oligonucleotide-directed mutagenesis (Δ7-TnC).

All assays showed that Δ7-TnC had close to wild-type function. Regulatory function was assayed in a reconstituted actomyosin system where TnC was added to a thin filament containing actin, tropomyosin, and TnIT. The ability to relieve TnIT inhibition of the actomyosin Mg-ATPase in the presence of Ca$^{2+}$, but not EGTA, was assayed. In the presence of Ca$^{2+}$, the level of activation by Δ7-TnC was close to that of wild type (80–85%; Fig. 1 A) whereas mutants with deletions of 11 or 14 residues give only 50% activation (Chandra et al., 1994; Smith et al., 1994). Similarly, the Ca$^{2+}$ dependence of the actomyosin ATPase was close to that of wild type (Fig. 1 B).

The increase in ellipticity at 222 nm upon divalent cation binding to TnC has been used to measure indirectly Ca$^{2+}$ binding to the high- and low-affinity sites (e.g., Hinke et al., 1978; Golosinska et al., 1991; Gusev et al., 1991; Pearlstone et al., 1992; Smith et al., 1994). The profile of Δ7-TnC was similar to that of wild type; the ellipticity change upon Ca$^{2+}$ binding to both the high- and low-affinity sites, as well as the affinities, were similar to those in wild-type TnC (Fig. 1 C; see Table 2). Finally, the thermal stability of the α-helical conformation of Δ7-TnC was similar to that of wild-type TnC (Table 1). As the presence of residues 8–14 restored the functional deficiencies reported in mutants missing the first 14 residues, we conclude that residues 8–14 are sufficient for TnC function.

**Effect of destroying the Arg 11-Glu 76 salt bridge**

The N-helix is stabilized by hydrophobic and electrostatic interactions between residues Ala 10, Arg 11, Ala 12, Phe 13, and Leu 14 and residues in the A- and D-helices that flank sites I and II (Herzberg and James, 1988; Satyshur et al., 1988; Gagné et al., 1995; Slupsky and Sykes, 1995; Houdusse et al., 1997; Strynadka et al., 1997). Prominent among these is a salt bridge between Arg 11 and Glu 76 in the amino-terminal domain of TnC. The salt bridge is maintained, although Arg 11 does exhibit increased solvent...
assay was carried out for 15 min at 28°C. Specific activity is expressed as mM NaCl, 10 mM imidazole, pH 7.0, 0.5 mM MgCl₂, 5 mM MgATP, and 0.1 mM CaCl₂ (mol P_i/mg myosin/min.)

FIGURE 1 (A) Regulation of the actomyosin ATPase by Wt-TnC and Δ7-TnC. Increasing concentrations of Wt-TnC or Δ7-TnC were added to a reconstituted thin filament preparation containing actin, tropomyosin, TnI, and TnT (see Materials and Methods). The final concentrations were KCl, 50 mM Tris-HCl, pH 7.5, 0.9 mM EGTA, 0.9 mM NTA, 25°C in a 2 mm cuvette) were titrated with increasing Ca²⁺, Wt-TnC; h, Δ7-TnC. The ATPases were carried out as described in Fig. 1A, except at a saturating concentration of TnC (1 μM) and with 0.45 mM CaEGTA. The free Ca²⁺ concentration was controlled using a CaEGTA buffer system as described in Materials and Methods. The Kᵢ values are: Wt-TnC, 3.0 × 10⁻⁷ M; Δ7-TnC, 4.0 × 10⁻⁷ M. ○, Wt-TnC; □, Δ7-TnC. (C) Ca²⁺ titration of the change in ellipticity at 222 nm. Wild-type TnC and Δ7-TnC (0.1 mg/ml, in 100 mM KCl, 50 mM Tris-HCl, pH 7.5, 0.9 mM EGTA, 0.9 mM NTA, 25°C in a 2 mm cuvette) were titrated with increasing Ca²⁺ as described in Materials and Methods. The curves are representative of at least two experiments and are a representative data set from three to four experiments. The destabilization in EDTA was not a consequence of improper folding as all mutants unfolded (2–90°C) and refolded (90–2°C) reversibly.

TABLE 1 Thermal stability of TnC mutants

<table>
<thead>
<tr>
<th>Protein</th>
<th>Tₘ (°C)</th>
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<tr>
<td></td>
<td>Ca²⁺</td>
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<tr>
<td>Wild-type TnC</td>
<td>90</td>
</tr>
<tr>
<td>Δ7-TnC</td>
<td>90</td>
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<tr>
<td>Red[Δ11C, E76C, C101L]</td>
<td>90</td>
</tr>
<tr>
<td>BMME[Δ11C, E76C, C101L]</td>
<td>90</td>
</tr>
<tr>
<td>Ox[Δ11C, E76C, C101L]</td>
<td>90</td>
</tr>
<tr>
<td>NEM[Δ11C, E76C, C101L]</td>
<td>90</td>
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<tr>
<td>R11C-TnC</td>
<td>90</td>
</tr>
<tr>
<td>E76C-TnC</td>
<td>90</td>
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<tr>
<td>C101L-TnC</td>
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The Tₘ values listed for the TnC cysteine mutants were determined from the thermal denaturations analyzed by circular dichroism. In the presence of EDTA, BMME[Δ11C, E76C, C101L] had two major peaks. The values are a representative data set from three to four experiments. The destabilization in EDTA was not a consequence of improper folding as all mutants unfolded (2–90°C) and refolded (90–2°C) reversibly.

Together the results indicate that the conformational stability of either domain of TnC, the Ca²⁺ affinity of the sites in the amino- and carboxyl-terminal domains, and the interaction of TnC with TnI or TnT do not depend on the Arg 11-Glu 76 salt bridge. Glu 76 is adjacent to Glu 77, a residue that is critical for Ca²⁺ coordination in site II (Houdusse et al., 1997; Strynadka et al., 1998). The prediction that the loss of Arg 11 (or the salt bridge) would destabilize loop 2 and decrease Ca²⁺ affinity was not borne out by our assays, although deletion of the entire N-helix does reduce the Ca²⁺ affinity of the N-domain (Chandra et al., 1994; Smith et al., 1994). Our results are in agreement with Gulati et al. (1995) who reported that an R11A mutant could restore full tension regulation in TnC-depleted fibers.

Effect of an intramolecular cross-link between the N- and D-helices

The interaction between the N- and D-helices is stabilized by hydrophobic residues, as well as the Arg 11-Glu 76 salt bridge. To further investigate the relationship between the

The interaction between the N- and D-helices is stabilized by hydrophobic residues, as well as the Arg 11-Glu 76 salt bridge. To further investigate the relationship between the
N- and D-helices, we carried out the converse experiment to removal of the salt bridge described above; we stabilized the interaction by introducing a cross-link by creating a double mutant in which Arg 11 and Glu 76 were both mutated to Cys. This approach has been previously employed to restrict intradomain motions in TnC (Grabarek et al., 1990; Gusev et al., 1991).

To prevent interdomain cross-linking, the endogenous Cys 101 was changed to Leu, the residue found in slow/ cardiac TnC. The C101L mutation has been extensively used for analysis of TnC Cys mutants (e.g., Kobayashi et al., 1994; Luo et al., 1998) and has been found not to affect TnC regulatory function when combined with other N-domain mutations (e.g., Grabarek et al., 1990). We have extensively analyzed TnC-C101L using the assays as for other mutants. It is indistinguishable from wild-type TnC except that its \( T_m \) in EDTA is \(-10^\circ\)C lower (Table 1), consistent with Fredricksen and Swenson’s finding (1996) that the D/E-helix increases the stability of the N-domain. The Ca\(^{2+}\) titration of the ellipticity of TnC-C101L was indistinguishable from wild type, as were the other single-site mutants, making it an acceptable control for Cys mutations involving cross-linking within TnC or between TnC and other Tn subunits.

Analysis of [R11C, E76C, C101L] was carried out on reduced TnC, NEM-blocked TnC, and TnC cross-linked by BMME as well as S-S oxidation. The thermal stability of the four forms of [R11C, E76C, C101L] was unaltered except in EDTA where the \( T_m \) was \(-20^\circ\)C lower than wild type (Table 1). Cross-linking with BMME slightly stabilized the protein. Mutation of Cys 101 to Leu accounts for part of the difference from wild-type TnC. The TnCs were fully folded in the conditions of our assays.

Intramolecular cross-linking of troponin C mutants

The extent of intramolecular cross-linking was evaluated on the basis of differential mobility in urea-polyacrylamide gels (Fig. 2). The [R11C, E76C, C101L] TnC was completely cross-linked by disulfide oxidation or by BMME after 2 min or longer, independent of Ca\(^{2+}\). In a molecular model of the BMME-modified mutant the distance between the two reactive groups is 2.9 Å, a distance that is consistent with the observed complete cross-linking and similar to that of the Arg 11-Glu 76 salt bridge in the structure (2.7 Å).

**Calcium-dependent conformational changes of troponin C mutants analyzed using circular dichroism**

Most interesting is that although the modifications are in the amino-terminal domain, the major effect of the mutations in [R11C, E76C, C101L] was on Ca\(^{2+}\) binding to the carboxyl-terminal domain. In the reduced form there was higher affinity and lower cooperativity of the high-affinity, carboxyl-terminal sites and a smaller increase in ellipticity at 222 nm upon Ca\(^{2+}\) binding to those sites (Fig. 3A; Table 2). Disulfide cross-linking and NEM modification of the cysteines had little effect. Cross-linking with BMME, however, reduced the affinity of the high-affinity sites, compared with the reduced protein, and restored the cooperativity of binding. It also appeared to decrease the affinity of the regulatory sites. The data for the reduced, oxidized, and NEM-modified protein never showed clear demarcations between the low- and high-affinity sites, making it difficult to determine accurately the conformational change due to binding to the high-affinity sites. For the same reason it was difficult to determine the \( K_d \) of the low-affinity sites. Gusev et al. (1991) reported that introduction of two cysteines at other sites in the N-domain (combined with a C101L mutation) slightly increased the Ca\(^{2+}\) affinity of the C-domain sites without influencing the cooperativity, but did not affect the N-domain. Additional evidence for interaction between the two domains resulting from the flexibility of TnC comes from the observation that \(-50\%\) interdomain cross-linking was observed in the single-site mutants, Arg11Cys, and Glu76Cys, which retain the endogenous Cys101 (Fig. 1, lanes 8 and 9).

The effect of the mutations on the high-affinity sites was specific to Ca\(^{2+}\) binding. The increase in ellipticity at 222 nm upon Mg\(^{2+}\) binding to the high-affinity sites in the absence of Ca\(^{2+}\) was comparable to wild-type both in affinity and ellipticity (Fig. 3B). It is interesting that the
The mutant [R11C, E76C, C101L] fully relieved TnIT inhibition of the actomyosin ATPase in the presence of Ca\(^{2+}\) but not in its absence (Fig. 3 C). However, the amount of TnC required for half-maximal activity, an indication of the affinity of the TnC for the regulated thin filament, depended on the state of modification of the cysteines (reduced, 0.55 \(\mu\)M; NEM-blocked and BMME-cross-linked, 0.35 \(\mu\)M; wild type, 0.22 \(\mu\)M). The Ca\(^{2+}\) dependence of the actomyosin ATPase of [R11C, E76C, C101L] was similar to that of wild type, independent of the modification of the cysteines (\(K_d\) for wild-type WtTnC \(= 2.5 \times 10^{-7}\) M, for [R11C, E76C, C101L] \(= 2.5 \times 10^{-7}\) M, for BMME[R11C, E76C, C101L] \(= 1.8 \times 10^{-7}\) M, and for NEM[R11C, E76C, C101L] \(= 2.5 \times 10^{-7}\) M).

**DISCUSSION**

In the present work we have investigated the requirements of the 14-residue N-helix, a TnC-specific structure, for function and gained insight into the significance of this TnC-specific structure. We suggest that the N-helix may not be crucial for the Ca\(^{2+}\)-dependent switch, a function common to all Ca\(^{2+}\)-regulatory proteins, but that it is required for transmission of the signal to TnI via its interaction with TnI and probably TnT. We have shown that deletion of residues 1–7 has little effect on function whereas deletions of 11 or more residues are deleterious (Gulati et al., 1993; Chandra et al., 1994; Liu et al., 1994; Smith et al., 1994; Fredrickson and Carlson, 1996). Together the results define residues 8–14, which account for most of the hydrophobic and electrostatic interactions between the N-helix and A- and D-helices, as being generally sufficient for function.

Destruction of the salt bridge between Arg 11 in the N-helix and Glu 76 in the D-helix by individually mutating selective effect of the mutations on Ca\(^{2+}\) but not Mg\(^{2+}\) binding was also observed when the entire N-helix was deleted (Smith et al., 1994). There is considerable spectroscopic evidence showing that the conformation of the carboxyl-terminal domain with Mg\(^{2+}\) and Ca\(^{2+}\) occupying sites III and IV are different, but there is no high-resolution structural information with Mg\(^{2+}\) bound (Seamon et al., 1977; Levine et al., 1978; Trigo-Gonzalez et al., 1992; Francois et al., 1997).

**Regulation of the actomyosin ATPase activity**

The mutant [R11C, E76C, C101L] fully relieved TnIT inhibition of the actomyosin ATPase in the presence of Ca\(^{2+}\) but not in its absence (Fig. 3 C). However, the amount of TnC required for half-maximal activity, an indication of the affinity of the TnC for the regulated thin filament, depended on the state of modification of the cysteines (reduced, 0.55 \(\mu\)M; NEM-blocked and BMME-cross-linked, 0.35 \(\mu\)M; wild type, 0.22 \(\mu\)M). The Ca\(^{2+}\) dependence of the actomyosin ATPase of [R11C, E76C, C101L] was similar to that of wild type, independent of the modification of the cysteines (\(K_d\) for wild-type WtTnC \(= 2.5 \times 10^{-7}\) M, for [R11C, E76C, C101L] \(= 2.5 \times 10^{-7}\) M, for BMME[R11C, E76C, C101L] \(= 1.8 \times 10^{-7}\) M, and for NEM[R11C, E76C, C101L] \(= 2.5 \times 10^{-7}\) M).

**DISCUSSION**

In the present work we have investigated the requirements of the 14-residue N-helix, a TnC-specific structure, for function and gained insight into the significance of this TnC-specific structure. We suggest that the N-helix may not be crucial for the Ca\(^{2+}\)-dependent switch, a function common to all Ca\(^{2+}\)-regulatory proteins, but that it is required for transmission of the signal to TnI via its interaction with TnI and probably TnT. We have shown that deletion of residues 1–7 has little effect on function whereas deletions of 11 or more residues are deleterious (Gulati et al., 1993; Chandra et al., 1994; Liu et al., 1994; Smith et al., 1994; Fredrickson and Carlson, 1996). Together the results define residues 8–14, which account for most of the hydrophobic and electrostatic interactions between the N-helix and A- and D-helices, as being generally sufficient for function.

Destruction of the salt bridge between Arg 11 in the N-helix and Glu 76 in the D-helix by individually mutating selective effect of the mutations on Ca\(^{2+}\) but not Mg\(^{2+}\) binding was also observed when the entire N-helix was deleted (Smith et al., 1994). There is considerable spectroscopic evidence showing that the conformation of the carboxyl-terminal domain with Mg\(^{2+}\) and Ca\(^{2+}\) occupying sites III and IV are different, but there is no high-resolution structural information with Mg\(^{2+}\) bound (Seamon et al., 1977; Levine et al., 1978; Trigo-Gonzalez et al., 1992; Francois et al., 1997).
these residues to Cys has no observable effect, showing that this interaction, present whether the N-domain Ca\(^{2+}\)-binding sites are occupied or vacant, is not required for function.

The results are consistent with Fredricksen and Swenson’s conclusion (1996) that stabilization of the N-domain by the N-helix depends primarily on hydrophobic interactions.

When both Arg 11 and Glu 76 are changed to cysteine, in combination with mutation of the endogenous Cys 101 to Leu, TnC structure and function are significantly altered but not in the same way as deletion of residues 1–14 (Smith et al., 1994). Unlike the \(\Delta 14\)-TnC mutant, which destabilized the protein in all occupancy states, the stability of the [R11C, E76C, C101L] mutant was affected only in EDTA. The ellipticity present in EDTA has been attributed to the N-domain (for reviews see Leavis and Gergely, 1984; Grabarek et al., 1992; Fredricksen and Swenson, 1996). The replacement of two long, flexible, charged side chains with small polar residues may be more destabilizing than just one and may require atoms to move toward vacated space in the structure. Modeling suggests that introduction of bulky hydrophobic groups with the maleimide rings fills the space. This increases the affinity for the regulated thin filament but does not fully restore wild-type function. Formation of a cross-link between residues 11 and 76 to mimic the intradomain salt bridge also does not restore function.

The observed interdomain cross-links of residues 11 and 76 to Cys 101 in the single mutants (Fig. 2), and many other reports in the literature, give evidence for flexibility of the TnC central helix and for domain interaction. The effect of the double mutation in the N-domain to increase the affinity and reduce the cooperativity of the carboxyl-terminal Ca\(^{2+}\)-binding sites and the reduced change in ellipticity when they are occupied also provide evidence for domain interaction. In the recent x-ray structure of TnC complexed with an amino-terminal TnI peptide, the central helix is unwound and bent by 90°, and there are salt bridges between the N- and C-domains, including one between Ser 9 and Lys 140 (corresponding to residues 12 and 143 in avian TnC; Vassylyev et al., 1998). The N-helix is the closest structure in the N-domain to the C-domain in the structure. Modification of both residues 11 and 76, and introduction of a cross-link, may cause strain and affect the interaction between the domains.

Although the activation and Ca\(^{2+}\) dependence of the actomyosin Mg-ATPase were similar to wild type, the affinity of [R11C, E76C, C101L] for the TnIT-tropomyosin-actin filament is reduced, suggesting reduced affinity for TnI or TnT. There is growing evidence for the direct involvement of the N-helix in TnC regulatory function, consistent with reports that the carboxyl-terminal domain of TnI binds to the amino-terminal domain of TnC (Farah et al., 1994; Tripet et al., 1997). Kobayashi et al. (1994) showed that a Ser12Cys mutant (residue 15 in avian TnC) was extensively cross-linked to residues 132–141 of TnI. Resonance energy transfer experiments between TnI labeled at Cys 133 and TnC showed that the TnI probe was closest to TnC Cys 12, compared with several other sites on TnC, and the distance decreased upon Ca\(^{2+}\) binding (Luo et al., 1998). These studies place Cys 133 of TnI on the N-, A-, and D-helix side of TnC, opposite the hydrophobic cleft that is exposed upon Ca\(^{2+}\) binding (Luo et al., 1998). The N-helix has not been implicated as a primary TnI interaction site in the structure of the N-domain of TnC with an amino-terminal TnI peptide (Vassylyev et al., 1998), the TnI peptide is closest to the N-, A-, and D-helix side of TnC, including one between Ser 9 and Lys 140 (corresponding to residues 12 and 143 in avian TnC; Vassylyev et al., 1998). The N-helix has a critical role in TnC-specific regulatory function in modifying the Ca\(^{2+}\) affinity of the C-domain and in transmitting the signal of the Ca\(^{2+}\) switch to the target proteins TnI and TnT.

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