Biological Function and Site II Ca\(^{2+}\)-induced Opening of the Regulatory Domain of Skeletal Troponin C Are Impaired by Invariant Site I or II Glu Mutations* 

To investigate the roles of site I and II invariant Glu residues 41 and 77 in the functional properties and calcium-induced structural opening of skeletal muscle troponin C (TnC) regulatory domain, we have replaced them by Ala in intact F29W TnC and in wild-type and F29W N domains (TnC residues 1–90). Reconstitution of intact E41A/F29W and E77A/F29W mutants into TnC-depleted muscle skinned fibers showed that Ca\(^{2+}\)-induced tension is greatly reduced compared with the F29W control. Circular dichroism measurements of wild-type N domain as a function of pCa demonstrated that ~90% of the total change in molar ellipticity at 222 nm ([\(\theta\)]\(_{222}\) nm) could be assigned to site II Ca\(^{2+}\) binding. With E41A, E77A, and cardiac TnC N domains this [\(\theta\)]\(_{222}\) nm change attributable to site II was reduced to \(<40\%\) of that seen with wild type, consistent with their structures remaining closed in +Ca\(^{2+}\). Furthermore, the Ca\(^{2+}\)-induced changes in fluorescence, near UV CD, and UV difference spectra observed with intact F29W are largely abolished with E41A/F29W and E77A/F29W TnCs. Taken together, the data indicate that the major structural change in N domain, including the closed to open transition, is triggered by site II Ca\(^{2+}\) binding, an interpretation relevant to the energetics of the skeletal muscle TnC and cardiac TnC systems.

The troponin complex, present in skeletal and cardiac muscle in association with tropomyosin and actin of the thin filaments, is primarily responsible for the Ca\(^{2+}\) regulation of contraction and relaxation in these tissues. Each of the three components of this complex, troponin C, I, and T (TnC, TnI, and TnT, respectively) has distinct functional properties. Conformational changes in TnC associated with Ca\(^{2+}\) binding to its regulatory N domain lead to a strengthening of its interaction with TnI and a weakening of the latter’s interaction with actin. Inhibition of the actomyosin ATPase activity is thereby released. TnT fulfills the role of anchoring the complex to tropomyosin and potentiating actomyosin ATPase activity (for reviews, see Refs. 1–8).

As the molecule responsible for triggering this complex series of events, TnC has been the subject of intensive investigation over many years. X-ray crystallographic (9–14) and NMR solution studies (15–21) have demonstrated the presence of two domains, N and C, joined in the crystals by a solvent-exposed α-helix, which is partially disrupted and flexible in solution (16, 18). Each of the N and C domains consists of two intimately associated EF-hand or helix-loop-helix metal binding motifs. Those of C domain (sites III and IV) have high affinity for Ca\(^{2+}\) (K\(_d\) \approx 10^{-7} M) and bind Mg\(^{2+}\) competitively (K\(_d\) \approx 10^{-3} M). Sites I and II are of lower affinity with K\(_d\) values of ~16 and ~1.7 μM, respectively (22, 23), and are specific for Ca\(^{2+}\) at physiological Mg\(^{2+}\) concentrations. Helices are designated A–H, corresponding to the four helix-loop-helix motifs of sites I–IV. In addition, an extension of the NH\(_2\) terminus forms the N helix; the central helix seen in the crystals connecting site II to site III is designated D/E. Based on a substantial body of evidence, the C domain sites III/IV are believed to be occupied by Ca\(^{2+}\)/Mg\(^{2+}\) throughout the contraction/relaxation cycle and to serve a structural role in anchoring TnC to the other troponin components. Association and dissociation of Ca\(^{2+}\) from sites I/II of N domain are considered to fulfill the regulatory function of TnC.

As originally postulated by Herzberg et al. (24) for avian skeletal TnC N domain, Ca\(^{2+}\) binding to sites I/II has been shown to promote a structural transition from a closed (apo) to open (2Ca\(^{2+}\)) conformation (13, 14, 17, 20). Involving significant changes in interhelical angles, helices B and C are seen to have altered their positions relative to those of N, A, and D. As a result, a number of previously fully and partially buried apolar residues are exposed at the protein surface to create a hydrophobic pocket or patch. The intact TnC (4Ca\(^{2+}\)) now possesses two nonpolar surface patches, one in each domain, with surrounding constellations of negatively charged residues. Evidence for these as interaction sites with regions of TnI has been reported (see Ref. 25 and references therein).

In each loop of sites I–IV of TnC, Ca\(^{2+}\) complexes with seven ligands in a pentagonal bipyramidal arrangement. By convention, the six amino acids contributing to these liganding groups are designated X, Y, Z, −Y, −X, and −Z or as residues 1, 3, 5, 7, 9, 12 in the 12-residue loop. Depending on the position

Received for publication, February 7, 2000, and in revised form, August 18, 2000

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* These studies were supported by the Medical Research Council of Canada; the Heart and Stroke Foundation of Canada; and Financiadora de Estudos e Projetos, Conselho Nacional de Desenvolvimento Científico e Tecnológico. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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† The abbreviations used are: TnC, TnI, and TnT, troponin C, I, and T, respectively; sTnC and cTnC, skeletal (chicken) and cardiac (human) intact wild-type TnCs, respectively; sTnC\(_{1–90}\) and cTnC\(_{1–90}\), F29W single mutant of sTnC, sTnC\(_{2–26}\) and cTnC\(_{2–26}\), E41A and F29W/E77A double mutants, respectively, of sTnC; sNTnC and cNTnC, isolated recombinant wild-type N domain fragments of sTnC (residues 1–90) and cTnC (residues 1–89), respectively; sNTnC\(_{2–26}\) and sNTnC\(_{24–61}\), and regulatory N domain lead to a strengthening of its interaction with TnI and a weakening of the latter’s interaction with actin. Inhibition of the actomyosin ATPase activity is thereby released. TnT fulfills the role of anchoring the complex to tropomyosin and potentiating actomyosin ATPase activity (for reviews, see Refs. 1–8).
Troponin C Site I or II Glu to Ala Mutations

within the loop and the amino acid occupying a particular position, Ca$^{2+}$ ligation may be to a protein main or side chain oxygen or to H$_2$O. In most EF-hands/loops, the highly conserved bidentate Glu residue at position 12 ($\alpha$) provides that in the wild-type protein initial Ca$^{2+}$ face area although Ca$^{2+}$ Ca$^{2+}$ wild-type N domain, site II is deficient in Ca$^{2+}$ and, based on this and other considerations, is believed to have the higher affinity of the two (13, 23, 26). Ca$^{2+}$-titrations monitored by NMR spectral changes revealed that chemical shifts occur throughout the N domain upon binding of each Ca$^{2+}$ (22, 23). An important structural feature involved in the tight coupling is a short antiparallel $\beta$-sheet comprised of a segment of each of the loops of sites I and II (for structural details, see Ref. 13). The Ca$^{2+}$-induced transition from closed to open structures can be understood in terms of two hinge or pivot regions located in the loop regions of the two sites. Opening and closing of the structure would thus involve the reorientation of helices $\beta$C at these pivot points relative to that of the N/A/D unit whose helical dispositions one to the other are virtually unchanged.

Cardiac TnC, while highly homologous, differs in several important respects from its skeletal muscle counterpart. Amino acid sequence differences are largely confined to the first 41 residues corresponding to the N helix and site I EF-hand. Site I is deficient in Ca$^{2+}$ binding due to an amino acid insertion and the substitution of Ca$^{2+}$ liganding residues in its binding loop (27). Calcium binding to site II is relatively unaffected, however, with a dissociation constant similar to that of site II of the skeletal protein (23, 28-30). Recently, the apo and Ca$^{2+}$-saturated NMR solution structures of human cardiac intact and N domain TnCs have been reported (18, 19). Surprisingly, Ca$^{2+}$ binding is not accompanied by an “opening” of the regulatory domain and concomitant exposure of a hydrophobic surface area although Ca$^{2+}$ binding to site II leads to spectral shifts throughout the entire N domain sequence (23). In related NMR studies, Gagné et al. (26) have shown that with a mutated form of skeletal TnC in which the invariant Glu at position 41 of site I is replaced by Ala, the “closed” to “open” transition is impeded. An earlier study (31) had shown that mutation of the equivalent Glu in rabbit sTnC to either Asp or Gln led to a reduction in Ca$^{2+}$ binding and functional activity in a reconstituted skinned fiber assay. Gagné et al. (20, 26) have suggested that in the wild-type protein initial Ca$^{2+}$ binding to preformed site II would involve only minor structural changes but set the stage for Ca$^{2+}$ binding to site I. The latter would involve large conformational changes including opening of the structure. The invariant Glu$^{41}$ at position 12 of site I was postulated to be the key residue in facilitating this structural transition.

In order to provide further insight into the molecular mechanism of this Ca$^{2+}$-sensitive regulatory switch, we have replaced the invariant Glu residue 41 or 77 by Ala in intact sTnC$^{29W}$ and in the recombinant N domains sNTnC and sNTnC$^{29W}$. The effects of mutating Glu$^{77}$ on the properties of sTnC have not to our knowledge been previously investigated. Both Glu to Ala mutations in intact TnC largely abolished Ca$^{2+}$-induced tension development in muscle skinned fibers. Of the total Ca$^{2+}$-induced ellipticity change ($\Delta [\theta]_{222}$ nm) in isolated wild-type N domain, $\sim 90\%$ could be attributed to site II Ca$^{2+}$ binding. With sNTnC$^{29W}$, sNTnC$^{77A}$ and cNTnC, this $\Delta [\theta]_{222}$ nm change associated with site II was reduced to $<40\%$ of that seen in sNTnC, a result consistent with impairment of the closed to open structural transition (26). In keeping with this interpretation, the Ca$^{2+}$-induced changes in fluorescence, near UV CD, and difference spectra observed with sTnC$^{29W}$ are largely abolished with sTnC$^{29W}$ and sTnC$^{77A}$. Our interpretation is that sites I/II in a TnC are highly interdependent, that the closed to open structural change is associated with Ca$^{2+}$ binding to site II, and that the integrity of both sites I/II invariant Glu residues is essential for this important conformational transition. These deductions have important implications for interpretations of the intramolecular mechanisms for the opening of the skeletal structure and for energetic considerations of the skeletal and cardiac systems.

MATERIALS AND METHODS

Construction and Expression of TnC Mutants—To produce sTnC$^{29W}$, the double mutant of intact TnC, site-directed mutagenesis of M13mp19CIL.Fx.sTnC$^{29W}$ was performed by the oligonucleotide-directed (32) double priming method (33) as described previously for sTnC$^{29W}$ (34), using the 17-mer E77A oligonucleotide 5′-CTCGAG-GGTTCTCGGG-3′ (with base change underlined) and the 19-mer oligonucleotide 5′-AGGGCATAAATTAAACCA-3′ corresponding to part of the CII unaligned region. The purified BstXI/SacI fragment of M13mp19CIL.Fx.sTnC$^{29W}$ (33) was digested, transformation into rubidium-competent Echerichia coli K12C1, and final plaque sequencing has all been described previously (35). To produce sTnC$^{77A}$, the EcoRI fragment encompassing the complete coding region of sTnC was isolated from pCLII.Fx.sTnC and subcloned into phagemid vector pTZ18R (36, 37) purchased from Amersham Pharmacia Biotech. Using the Kunkel method (38, 39), uracil-containing single-stranded DNA was obtained by two rounds of transfection of the phagemid into competent E. coli C128 (ung ‘du’ –) and subsequent infection by helper phage M13KO7. A 17-mer oligonucleotide 5′-CACCAAGGCTTGGCA-3′ (with base change underlined) was used to introduce the single mutation. After mutagenesis, ligation mix was transformed into competent DH5α and transformants were selected on ampicillin plates. Eight colonies were screened by the dot blotting method and the DNA from positive colonies transformed into competent JM109. Single-stranded DNA was isolated and sequenced by the chain termination method (40) to confirm the base change. Since suitable sites were not available for digestion by restriction enzymes, the intact double mutant sTnC$^{29W}$ was subsequently produced by carrying out a second round of mutagenesis using the DNA from sTnC$^{77A}$ as the template and the 17-mer oligonucleotide reported previously (34) for sTnC$^{29W}$ (34). The EcoRI fragment with the entire coding region of sTnC$^{29W}$ was cloned into the phospahetase-treated BstXI/SacI fragment of expression vector pCLII.Fx.sTnC$^{29W}$ (33) for mutagenization, transformation into rubidium-competent Echerichia coli K12C1, and final plaque sequencing has all been described previously (35). To produce sTnC$^{77A}$, the EcoRI fragment of expression vector pCLII.Fx.sTnC. The ligation mix was transformed into competent E. coli QY13 as described previously (34). Expression of recombinant chicken fusion TnC, cleavage with factor Xi, and column purification procedures have been described earlier (35).

Construct pET3a.sNTnC$^{77A}$ was produced by the overlap extension method of Horton et al. (41) as follows: 1) PCR product I (269 base pairs) was made using pET3a.sNTnC as template, a 5′ TnC outside 30-mer primer 5′-GAGATATACATATG-3′ corresponding to part of the skeletal and cardiac systems. The invariant Glu$^{41}$ at position 12 of site I was postulated to be the key residue in facilitating this structural transition.

In order to provide further insight into the molecular mechanism of this Ca$^{2+}$-sensitive regulatory switch, we have replaced the invariant Glu residue 41 or 77 by Ala in intact sTnC$^{29W}$ and in the recombinant N domains sNTnC and sNTnC$^{29W}$. The effects of mutating Glu$^{77}$ on the properties of sTnC have not to our knowledge been previously investigated. Both Glu to Ala mutations in intact TnC largely abolished Ca$^{2+}$-induced tension development in muscle skinned fibers. Of the total Ca$^{2+}$-induced ellipticity change ($\Delta [\theta]_{222}$ nm) in isolated wild-type N domain, $\sim 90\%$ could be attributed to site II Ca$^{2+}$ binding. With sNTnC$^{29A}$, sNTnC$^{77A}$ and cNTnC, this $\Delta [\theta]_{222}$ nm change associated with site II was reduced to $<40\%$ of that seen in sNTnC, a result consistent with impairment of the closed to open structural transition (26). In keeping with this interpretation, the Ca$^{2+}$-induced changes in fluorescence, near UV CD, and difference spectra observed with sTnC$^{29W}$ are largely abolished with sTnC$^{29W}$ and sTnC$^{77A}$. Our interpretation is that sites I/II in a TnC are highly interdependent, that the closed to open structural change is associated with Ca$^{2+}$ binding to site II, and that the integrity of both sites I/II invariant Glu residues is essential for this important conformational transition. These deductions have important implications for interpretations of the intramolecular mechanisms for the opening of the skeletal structure and for energetic considerations of the skeletal and cardiac systems.
into the pET vector using PCR technology with TC1 as template, and the two primers 37-mer 5′-CCCCGGGCCCCCTATGGACATCTTCCGCT- TACAAGGCT-3′ (with NdeI site underlined) and 30-mer 5′-GCTGTA- GATCCAGGCTAGCTATCTTACAC-3′ (with BamHI site underlined); 2) pET3a.cTnC (produced in step 1) was used as template in the next round of PCR to produce N domain construct pET3a.cNTnC by including in the PCR mixture the same 37-mer 5′-primer (with NdeI site) used initially and a 36-mer primer 5′-GCTTCTAGGGATCCCATCTGACTG- GTGCTGCTCCTCATGC-3′ with a stop codon (in boldface type) after Ser99, followed by a BamHI site (underlined). Using the NdeI/BamHI sites, this second PCR product was cloned into pET3a.

For the three N domain fragments sNTnC46IA and sNTnCF29W (residues 1–90), and cNTnC (residues 1–88), pET3a.sNTnC46IA, pET3a.sNTnCF29W, and pET3a.cNTnC were transformed into Ca2+ competent BL21(DE3)pLysS cells (Novagen) for inducible protein expression with isopropyl-1-thio-β-d-galactopyranoside (43). A 50 mM Tris, 0.1 mM NaCl, 2 mM MgCl2, 0.01% NaN3, pH 8.0, extract of dried acetone powder of pelleted cells from a 3-liter culture was separated on a DEAE A-25 column at pH 8.0 using a 0.2–0.8 mM NaCl gradient. Further purification was achieved on a C8 HPLC Synchron column (20 mM TEAP, 100 mM NaClO4, pH 6.7, 1–60% CH3CN gradient). Final purity was checked by SDS-PAGE, gel electrophoresis and amino acid analyses.

**Tension Measurements**—Chemically skinned fibers were prepared from rabbit psoas muscle and stored in the presence of glycerol at −20 °C (44, 45). Segments of single fibers were dissected and mounted for isometric tension measurements as described by Metzger et al. (46) in chambers of 0.5–1.0 ml equipped with temperature control and rapid stirring. Before and after reconstitution with different proteins, maximal Ca2+-activated tensions were measured at pCa (~log(Ca2+) 1) 4.4 under standard conditions (pH 7.0, 15 °C; free Mg2+ 1.0 mM) using a solution of 152 mM potassium propionate, 10 mM imidazole, 10 mM KH2PO4, 100 mM KCl, 1 mM EGTA, 1 mM dithiothreitol, pH 7.1, buffer solution contained 5 mM K2EGTA in place of CaEGTA. Each Ca2+ tension was preceded by a brief exposure to relaxing solution containing 0.1 mM EGTA.

For extraction and reconstitution, endogenous TnC was extracted at 15 °C using 10 mM EDTA, 10 mM imidazole, and 0.2 mM trifuoroacetic acid at pH 7.2. Tests for completeness of extraction were performed at pCa 4.4 after washing to remove trifluoroacetic (45). After prolonged extraction (15–30 min.), tensions were reduced to 0–2% of the original P0. These small residual tensions were subtracted from those obtained following reconstitution with the mutants.

As a control for reconstitution with mutants sTnCF29W/R41A and sTnCF29W/R41A, chicken skeletal muscle TnC was used. Tensions generated when fibers were first reconstituted with this protein ranged from 60 to 80% of P0. In a previous report, reconstitution with mutant sTnCF29W was shown to be equal to that obtained using recombinant sTnC protein, within experimental error (47). Control experiments have established that the same is true for chicken muscle TnC as described earlier (34, 35). For the competition experiments, fibers were exposed for 15 min to a mixture of 22 μM mutant recombinant and 1.1 μM chicken muscle protein after exposure to the mutant alone. At the end, fibers were extracted and reconstituted again with the control protein. On average, the tension resulting from the second reconstitution with control was 3% of the first, and we were able to repeat the procedure with the second mutant. Fibers were discarded when the control reconstitution fell below 50% of the P0. Proportion of myosin, calculated from the data of Table I, was taken as 100% and given in Table II. While the F29W mutation appears to have increased the 222-nm ellipticity, both Glu → Ala mutations on the ellipticities of the intact proteins, CD analyses at 222 nm in the presence and absence of Ca2+ were carried out on sTnC, sTnCF29W, and sTnCF29W/R41A, and sTnCF29W/E77A TnCs. sTnCF29W was included to serve as a control for the sTnC, sTnCF29W, maximum tension was 93% of those obtained with chicken muscle protein (Table I). Although neither of the two Glu → Ala mutants was very effective as a replacement for native TnC (see reconstitution a in Table II), the site I mutant was better (19%) than the site II mutant (5%). In some experiments, tension recovery for the mutants was tested with higher concentrations of Ca2+ (up to pCa 3.7), with similar results.

In order to ascertain whether or not the mutants were able to bind to the thin filament, competition experiments were performed. Following exposure to the mutant, fibers were incubated with a 20:1 mixture of the mutant and chicken muscle proteins. In most cases, some increase in tension occurred to 25% of the control for sTnCF29W/R41A and to 9% of the control for sTnCF29W/R41A; see reconstitution b in Table II), with further increments if the incubation was repeated (data not shown). However, much higher tensions were recorded when the fibers were subsequently stripped of bound TnC and incubated with control protein for the same length of time in the absence of mutant (90% of the control for sTnCF29W/R41A and 89% of the control for sTnCF29W/R41A; see reconstitution c in Table II).

**Far UV CD Studies**—To investigate the effects of the two Glu → Ala mutations on the ellipticities of the intact proteins, CD analyses at 222 nm in the presence and absence of Ca2+ were carried out on sTnC, sTnCF29W, and sTnCF29W/R41A, and sTnCF29W/E77A TnCs. sTnCF29W was included as a control for the two F29W Glu → Ala mutants, since the latter were also studied by fluorescence measurements (see below). The data, presented as the percentage change in [θ]222 nm relative to sTnC taken as 100% are shown in Table III. While the F29W mutation appears to have increased the 222-nm ellipticity, both Glu → Ala mutations showed highly significant decreases of −20%. Previous studies in this laboratory (49) have shown that the total Ca2+-induced ellipticity in intact TnC, −27% could be assigned to N domain and −73% to C domain. Rather similar large contributions by C domain were reported by Johnson and Potter (50). To eliminate the large background of
Ca\(^{2+}\)-induced ellipticity change in C domain, we have prepared the F29W, E41A, and E77A mutants in isolated N domain. This approach is validated by our previous demonstration that the properties of isolated N domain are the same as N domain in intact TnC (49). Because of the recent demonstration that cNTnC fails to undergo the Ca\(^{2+}\)-induced transformation from closed to open conformation seen in sTnC N domain (18, 19), we have also prepared and examined the Ca\(^{2+}\)-induced ellipticity change of cNTnC (residues 1–89). The data as given in Table IV demonstrate that, in comparison with sNTnC, sNTnCF29W shows an increase in negative ellipticity, whereas the change for each of the Glu → Ala mutants and for cNTnC is very significantly reduced. Since both sNTnCE41A and cNTnC fail to undergo the Ca\(^{2+}\)-induced structural opening (18, 19, 26), the data indicate this to be true also for sNTnCF29W. These observations are consistent with the view that a significant proportion of the change in \(\theta_{222\text{ nm}}\) elicited by Ca\(^{2+}\) binding to sites I and II can be attributed to the reorientation of helices B and C relative to the three helices of the NAD structural unit, an interpretation previously proposed by Gagné et al. (15).

Calcium titrations of the changes in \(\theta_{222\text{ nm}}\) for sNTnC, sNTnCF29W, sNTnCE41A, sNTnCF29W/E77A, and cNTnC are shown in Fig. 1, a and b, expressed as a percentage of the total change observed with sNTnC. The curves for all five of these proteins are seen to be biphasic, corresponding to stepwise Ca\(^{2+}\) binding to sites II/I or I/II. Derivation of \(-\log K_D\) values as measures of Ca\(^{2+}\) affinity for the two sites were obtained by curve fitting procedures as described previously (35) and are presented in Table V. For sNTnC, sNTnCF29W, sNTnCE41A, and cNTnC, the \(-\log K_D\) value for tighter binding has been assigned to site II in accordance with previous evidence (13, 22, 23). In the case of sNTnCF29W/E77A, the higher \(-\log K_D\) value (5.9) has been assigned to site I and the lower \(-\log K_D\) value (3.0) to site II. This latter assignment is based on the demonstrated dramatic effects of mutations of invariant Glu residues in positions 12 of other EF-hand Ca\(^{2+}\)-binding loops (51–55). In contrast to the two Glu → Ala mutants the effects of the F29W mutation are to alter to a modest degree the Ca\(^{2+}\) affinity of sites I and II. This is evident from a comparison of the far UV CD data for sNTnCF29W and sNTnC in Fig. 1 and Table V as determined in the present study. The biphasic nature of the curve for cNTnC deserves comment. While site I of cNTnC is often described as nonfunctional in its ability to bind Ca\(^{2+}\), undoubtedly true from a physiological perspective, the data of Fig. 1b indicate that at high Ca\(^{2+}\) concentrations (in the millimolar range; pCa4 to pCa2), there is a structural change (increasing negative ellipticity) associated with a \(-\log K_D\) value for Ca\(^{2+}\) binding of \(-3.0\). These observations are in good agreement with the quality fluorescence Ca\(^{2+}\)-titration data reported by Johnson et al. (30) for cNTnC derivatized with 2-(4-acetamidoanyl)mephathalene-6-sulfonic acid (see cNTnCIA in Table V). They also observed a biphasic curve with \(-\log K_D\) values of 5.8 and 2.7 (see Table V) corresponding to site II and site I Ca\(^{2+}\) binding, respectively.

A striking feature of these data in the case of sNTnC is that \(-90\%\) of the Ca\(^{2+}\)-induced total ellipticity change is associated with Ca\(^{2+}\) binding to site II (Fig. 1 and Table IV), similar to the 80% previously noted by Li et al. (22). For sNTnCE41A, sNTnCF29W/E77A, and cNTnC, it is this site II Ca\(^{2+}\)-induced ellipticity change that is markedly reduced to \(-30–40\%\) of that observed with sNTnC (see Fig. 1 and Table IV). Thus, the effects of the invariant Glu mutations in both sites I and II as well as of the defunct site I in cNTnC are remarkably similar, observations consistent with failure of the closed to open structural opening in all three proteins. In contrast, the effects of the F29W mutation in sNTnCF29W are to increase the magnitude of the Ca\(^{2+}\)-induced ellipticity change and, as noted above, to alter the Ca\(^{2+}\) affinity to a modest degree.

**Tryptophan Fluorescence Studies** —Previous studies have demonstrated that the F29W mutation in intact sTnC is a useful fluorescence probe for monitoring Ca\(^{2+}\)-induced structural transitions of its N domain (34) and that these were not influenced by the presence of C domain as intact sTnC as compared with isolated sNTnCF29W (49). In the present study, we have examined the properties of TnC variants in which E41A or E77A mutation has been introduced into intact F29W. The fluorescence emission spectra and quantum yields for sNTnCF29W and the two mutants sNTnCF29W/E41A and sNTnCF29W/E77A in both the absence and presence of Ca\(^{2+}\) are shown in Fig. 2 and Table VI, respectively. In the apo state, the emission spectra of sNTnCF29W/E77A and the control sNTnCF29W were similar (both with quantum yields of 0.12), while that of sNTnCF29W/E41A was \(-1.5\)-fold greater (quantum yield = 0.18). In response to Ca\(^{2+}\), both F29W/Glu → Ala mutants showed a slight quenching, with quantum yields decreasing by 0.01–0.02, in contrast to the 3-fold increase with sNTnCF29W from 0.12 (apo) to 0.33 (+Ca\(^{2+}\)). These results indicate that for both Glu → Ala mutants, the environment of Trp\(^{29}\) is now altered in the Ca\(^{2+}\)-saturated state.

Although the Ca\(^{2+}\)-induced quenching of fluorescence for both sNTnCF29W/E41A and sNTnCF29W/E77A was small in comparison with the large increase for sNTnCF29W, it was possible to monitor the fluorescence change as a function of increasing Ca\(^{2+}\) concentration. From the monophasic curves shown in Fig. 3, \(-\log K_D\) values of 4.71 ± 0.08 and 3.51 ± 0.09 were deduced for sNTnCF29W/E41A and sNTnCF29W/E77A, respectively. Because of the uncertainties arising from the influence of the F29W mutation on Ca\(^{2+}\) affinities of sites I/I, we have not attempted to assign these to one site or the other.

**Near UV CD and UV Difference Absorption Spectroscopic Studies** —The near UV CD spectra for the apo and Ca\(^{2+}\)-loaded states for intact sNTnCF29W, sNTnCF29W/E41A and sNTnCF29W/E77A are shown in Fig. 4. In the apo state, the control sNTnCF29W has positive CD bands in the region of 270–300 nm. With the addition of Ca\(^{2+}\), the amplitude of the bands is much reduced to negative values over the entire wavelength range. In contrast, both of the double mutants sNTnCF29W/E41A and sNTnCF29W/E77A in the apo state have slightly higher positive ellipticity values than sNTnCF29W, and these are almost unchanged upon the addition of Ca\(^{2+}\).

The UV difference absorption spectra of Ca\(^{2+}\)-saturated apo forms of intact sNTnCF29W, sNTnCF29W/E41A, and sNTnCF29W/E77A are shown in Fig. 5. As described previously (34), the region of the difference spectrum of sNTnCF29W above 275 nm can be considered as arising from a red shift in the Trp absorption bands; below this wavelength, the difference spectrum is largely dominated by the contribution of Phe residues.

In the case of sNTnCF29W/E77A, the contribution of both the Trp and Phe residues are absent, resulting in almost no difference

### Table II

| Protein                  | sNTnCF29W/E41A | sNTnCF29W/E77A |
|--------------------------|----------------|----------------|
| a) Mutant alone          | 19 ± 1 (4)     | 5 ± 2 (5)      |
| b) Mutant + control      | 25 ± 2 (4)     | 9 ± 3 (4)      |
| c) Control               | 90 ± 3 (4)     | 89 ± 2 (5)     |

Reconstitutions were performed sequentially (a to c) following an initial extraction and reconstitution with control protein (chicken skeletal muscle TnC, 1.1 μM). Extractions preceded reconstitutions a and c (see "Materials and Methods"). Recovery was normalized to the average of the two tensions (%) recorded for each fiber with control protein, before and after the mutant (22 μM). The range of the S.E. is shown, followed by the number of experiments in parentheses.
The present study has revealed several important new insights into the molecular mechanisms by which the binding and dissociation of Ca$^{2+}$ to and from sites I and II of TnC elicits the Ca$^{2+}$-regulated events of contraction and relaxation of striated muscle. These include 1) the demonstration that mutation to Ala of either of the invariant Glu residues in position 12 of the site I and II Ca$^{2+}$ binding loops leads to virtual elimination of the Ca$^{2+}$ sensitivity of the contractile response in skinned muscle fibers; 2) the observation that by far the major fraction (90%) of the total Ca$^{2+}$-induced ellipticity change of isolated N domain of wild-type sTnC is associated with binding to site I; 3) that this latter change is substantially reduced to 30–40% in each of the two Glu → Ala mutants and in cTnC; 4) the Ca$^{2+}$-induced fluorescence, near UV CD, and UV difference spectral changes observed with sTnC are largely eliminated in both Glu → Ala mutants of sTnC, sTnCF29W, and cTnC; 5) in comparison with sTnC, sTnC F29W has minimal effect on biological function as assessed in skinned fibers, but in solution its isolated N domain shows an increase in both Ca$^{2+}$-induced ellipticity change and altered affinity for Ca$^{2+}$ at sites I and II. In the following, we discuss the significance of these observations in terms of our current detailed structural knowledge of the apo and 2Ca$^{2+}$-states of sTnC as well as in terms of suggestions for the intramolecular mechanism by which Ca$^{2+}$ binding is coupled to the structural transition from a closed to open conformation.

Recent NMR studies have demonstrated that the transition from closed to open structures in sTnC (56), the major contributing factor is likely to be the movement of helices B and C relative to those of A and D from the NH$_2$-terminal end of the B helix (residues 39–41) (13, 15, 17) it was concluded that 40% in each of the two Glu

FIG. 1. Ca$^{2+}$ titrations of recombinant wild-type and mutant sNTnCs and cNTnC monitored by far UV CD measurements at 222 nm. a, sNTnC (∼63.2–87.3 μM); sNTnCF29W (76.3 μM); sNTnCE41A (88.3 μM); sNTnCF29W/E41A (88.3 μM); sNTnCE41A (88.3 μM). The averaged data points from 2–4 titration points are shown with S.D. values indicated. In both a and b, the data for the mutant sNTnCs and for cNTnC are expressed as a percentage of that observed for sTnCT.

Table III

| Intact TnC     | n° | [θ]$_{222}$ nm $^a$ | Δ              | Ca$^{2+}$-induced change$^d$ |
|----------------|----|--------------------|----------------|-----------------------------|
|                |    | Apo                | +Ca$^{2+}$     |                             |
| sTnC           | 3  | −10,200 ± 330      | −16,580 ± 550  | −6380 ± 220                 | 100                          |
| sTnC F29W      | 6  | −11,170 ± 1000     | −18,420 ± 1530 | −7250 ± 590                 | 114                          |
| sTnC E41A      | 2  | −10,165 ± 37       | −15,182 ± 262  | −5052 ± 230                 | 79                           |
| sTnC F29W/E41A | 3  | −10,290 ± 511      | −15,640 ± 315  | −5350 ± 200                 | 84                           |

$^a$ Number of determinations.
$^b$ Values are averages ± S.D.
$^c$ Values of Ca$^{2+}$ titration points are shown with S.D. values indicated. In both the data for the mutant sNTnCs and for cNTnC are expressed as a percentage of that observed for sTnCT.
$^d$ Values of Ca$^{2+}$-induced change expressed as a percentage of that observed with sTnCT.

Table IV

| Protein       | n° | [θ]$_{222}$ nm $^a$ | Δ              | Total$^d$ |
|---------------|----|--------------------|----------------|-----------|
| sNTnC         | 4  | −15,420 ± 250      | −18,960 ± 130  | 100       |
| sNTnC F29W    | 2  | −15,530 ± 120      | −20,110 ± 80   | 129       |
| sNTnC E41A    | 2  | −16,090 ± 60       | −18,700 ± 300  | 74        |
| sNTnC F29W/E41A| 2 | −16,140 ± 90       | −18,430 ± 260  | 65        |
| cNTnC         | 2  | −14,130 ± 210      | −16,270 ± 290  | 60        |

$^a$ Number of determinations.
$^b$ Values are averages ± S.D.
$^c$ Values of Ca$^{2+}$-induced Δ are expressed as a percentage of that observed for sNTnC.
$^d$ Based on data of Fig. 1, the percentage of total [θ]$_{222}$ nm for each protein attributable to site II is given.

**DISCUSSION**
Troponin C Site I or II Glu to Ala Mutations

TABLE V
Calcium affinities of regulatory sites I and II of skeletal and cardiac TnCs

| Protein      | Type of analysis | Site I  | Site II  | Source          |
|--------------|------------------|---------|----------|-----------------|
| sTnC         | Far UV CD        | -       | 5.64 ± 0.08 | Present work     |
| sTnCF29W     | Far UV CD        | -       | 5.74 ± 0.01 | Present work     |
| sTnCF29W     | Fluorescence     | -       | 5.73 ± 0.01 | Present work     |
| sNTnC        | Far UV CD        | 4.54 ± 0.10 | 5.92 ± 0  | Present work     |
| sNTnC        | Far UV CD        | 4.29 ± 0.01 | 5.51 ± 0.02 | Ref. 22         |
| sNTnC        | NMR              | 4.59–5.24 | 5.58–6.19 | Ref. 22         |
| sNTnCF29W    | Far UV CD        | 3.72 ± 0.09 | 6.19 ± 0  | Present work     |
| sNTnCE41A    | Far UV CD        | 2.70–3.00 | 4.70–5.00 | Ref. 23         |
| sNTnCE41A    | NMR              | 3.09 ± 0.04 | 5.88 ± 0.01 | Present work     |
| sNTnCE77A    | Fluorescence     | -       | 2.95 ± 0.05 | Present work     |
| cTnCIA       | Fluorescence     | 2.70    | 5.65     | Ref. 30         |
| cTnCIA       | Fluorescence     | 5.60    | 5.59     | Ref. 28         |
| sNTnC        | Far UV CD        | 5.91 ± 0.02 | 5.86 ± 0.01 | Present work     |

a With intact sTnC and sTnCF29W, the Ca$^{2+}$-induced change in $\theta_{222}$ nm attributable to site I is largely masked by the substantial change arising from C domain and site II. Thus $-\log K_D$ for site I cannot be measured.

b The Ca$^{2+}$ titration versus fluorescence curves of sTnCF29W and sNTnCF29W are monophasic with $-\log K_D$ values corresponding to those of site II. This fluorescence change can thus be correlated with the larger (~90%) change in $\theta_{222}$ nm seen in the N domain now attributable to site II.

c In the present work data were derived from Ca$^{2+}$ titrations of far UV CD analyses. Data sets from 2 to 6 titrations were analyzed separately using a curve-fitting program as described previously (35).

The similarities in effects of the two Glu to Ala mutations on the properties of the intact TnC and its N domain are remarkable. In addition to their failure to function as replacements for wild-type intact sTnC in the skinned fiber system, when incorporated into isolated N domain they both show very similar effects on the magnitude of the elicited ellipticity changes as measured by far UV CD (see Tables III and IV and Fig. 1). In addition, when the Glu → Ala mutations are incorporated into intact F29W, the Ca$^{2+}$-induced changes in fluorescence, near UV CD spectra, and UV difference spectra seen with the sTnCF29W control are virtually eliminated (see Figs. 2, 4, and 5). These similarities lead to the important conclusion that, like E41A, the E77A mutation leads to disrup-

Fig. 2. Fluorescence emission spectra of intact TnC mutants with or without Ca$^{2+}$. Excitation was at 282 nm. The data for the three mutants were normalized using a Trp quantum yield of 0.13 as described (34). Both excitation and emission slits were set at 5-nm bandwidth. Buffer conditions were the same as in Fig. 1. Protein concentrations were as follows: sTnCF29W, 5.1 μM (Ca$^{2+}$, lower —; +Ca$^{2+}$, upper —); sTnCF29W/E41A, 4.8 μM (Ca$^{2+}$, --; +Ca$^{2+}$, ---); sTnCF29W/E77A, 5.8 μM (Ca$^{2+}$, ---; +Ca$^{2+}$, ---).

Table VI
Quantum yields from fluorescence measurements with and without Ca$^{2+}$ for intact TnC mutants sTnCF29W, sNTnCF29W/E41A, and sNTnCF29W/E77A

| Protein         | Intact TnC | Without Ca$^{2+}$ | With Ca$^{2+}$ |
|-----------------|------------|------------------|---------------|
| sTnCF29W        | 0.12       | 0.33             |
| sTnCF29W/E41A   | 0.18       | 0.17             |
| sTnCF29W/E77A   | 0.12       | 0.10             |

Quantum yields were measured in the same buffer as in Fig. 1. Excitation was at 282 nm. A quantum yield of 0.13 was used for free Trp in the same buffer. Excitation and emission slits were both at 5-nm band width.

Fig. 3. Ca$^{2+}$ titrations of intact sTnCF29W, sNTnCF29W/E41A, and sNTnCF29W/E77A monitored by Trp fluorescence emission measurements at 330 nm. The averaged data points from two titrations for sTnCF29W (3.3 μM, ●) and sNTnCF29W/E41A (5.8 μM, □) and from five titrations for sNTnCF29W/E77A (8.2 μM, ▽) are shown, with S.D. indicated by bars. The calculated fitted curves (indicated by solid lines) were obtained as described previously (35). For purposes of comparison, all three proteins were normalized to their respective 100% change in fluorescence intensities. Buffer conditions were the same as in Fig. 1.
in the presence and absence of Ca\textsuperscript{2+}. This important conformational transition is explicable in terms of several differences in their structural features. These include in site II

## Troponin C Site I or II Glu to Ala Mutations

Evidence bearing on the question of cooperativity of Ca\textsuperscript{2+} binding to sites I and II of sTnC has been contradictory. In direct Ca\textsuperscript{2+} binding measurements using equilibrium dialysis (57) or a Ca\textsuperscript{2+} ion-selective electrode (58), no cooperativity between sites I and II was observed. On the other hand, steep curves of fluorescence and \(\theta_{222}\) nm as a function of pCa attributable to the titration of sites I/II were observed for intact and isolated N domains of sTnC\textsuperscript{F29W} and sTnC (34, 35). These corresponded to high Hill coefficients (\(n = 2\)) indicating a high degree of cooperativity. Subsequently, however, Ca\textsuperscript{2+} titration of the NMR spectral changes in \(^{15}\)N-labeled sTnC revealed that Ca\textsuperscript{2+} binding to sites II and I was a stepwise process with little or no cooperativity of Ca\textsuperscript{2+} binding indicated (22). It was also demonstrated that when the large changes in \(\theta_{222}\) nm attributable to C domain are eliminated by working with isolated N domain, a biphasic curve was observed corresponding to the filling of binding loops II and I (22), an observation confirmed in the present work. The suggestion that the steepness of its ellipticity and fluorescence versus pCa curves now attributable to site II Ca\textsuperscript{2+} binding is not a reliable index of cooperativity is reinforced by the present titration data for cTnC. The steepness of its \(\theta_{222}\) nm versus pCa curve over the pCa range 7.0–4.0 (see Fig. 1b; calculated Hill coefficient, \(n = 1.7\)) is inconsistent with the known Ca\textsuperscript{2+} binding properties of this protein. We have also extracted and analyzed the data of Fig. 1 of Johnson et al. (30) in which the change in fluorescence of cTnC\textsuperscript{E41A} was monitored as a function of pCa. The steepness of the first phase of this curve (attributed to site II binding) over the same pCa range corresponds to a Hill coefficient of \(n = 2\). Although we do not presently understand the molecular basis of these excessively steep curves, it is clear that they cannot be considered as reliable measures of cooperative Ca\textsuperscript{2+} binding to sites II/I of skeletal and cardiac TnCs.

These and other considerations (13, 23) lead to the conclusion that Ca\textsuperscript{2+} binding to sTnC occurs stepwise to sites II and I, respectively, and that the higher affinity of site II versus site I is explicable in terms of several differences in their structural features. These include in site II versus site I a larger number of Ca\textsuperscript{2+}-coordinating residues arranged in a more preformed geometry, higher net negative charge, more stabilizing hydrogen bonds, and a lesser degree of conformational flexibility (see Ref. 13). Pertinent to the present investigation are the side chain dispositions of the two invariant bidentate glutamic acid residues at positions 12 of the two sites. While Glu\textsuperscript{77} of site II differs little in its position in both apo and Ca\textsuperscript{2+} states, Glu\textsuperscript{41} of site I in the apo state is directed completely out of the binding site and forms a salt bridge with Lys\textsuperscript{40} (see Ref. 13 and references therein). Only site II has this latter residue at position 11 of the loop.

A further important deduction from the present observations is that the major change in \(\theta_{222}\) nm and therefore in the conformational transition is closely coupled to the binding of Ca\textsuperscript{2+} to site II. Curve fitting analyses of the biphasic pCa versus \(\theta_{222}\) nm data for sNTnC of Fig. 1 provided \(-\log K_D\) values of 5.92\(\pm\)0.10 for site II binding and 4.54\(\pm\)0.10 for reasons described above, these measures of Ca\textsuperscript{2+} affinity have been assigned to sites II and I, respectively, and are seen to be in good agreement with other previous estimates and the present work (see Table V for a compilation of these). It is the very significant \(\theta_{222}\) nm change associated with site II Ca\textsuperscript{2+} binding that is much reduced in both the sNTnC\textsuperscript{E41A} and sNTnC\textsuperscript{E77A} mutants.

Comparison of the percentage of \(\theta_{222}\) nm versus pCa curves for sNTnC\textsuperscript{E41A} and sTnC of Fig. 1 indicates that the Glu to Ala mutation at residue 41 reduces site I Ca\textsuperscript{2+} affinity from \(-\log K_D\approx4.5\) to \(\sim3\). The same mutation has a more modest to minimal effect on site II Ca\textsuperscript{2+} affinity with reported values of \(-4.8\) (NMR; Ref. 23) and \(-5.9\) (present study). A similar comparison of sTnC\textsuperscript{E77A} and sNTnC from the data of Fig. 1 provided site I and site II \(-\log K_D\) values of \(-5.9\) and \(-3\), respectively. Thus, while Ca\textsuperscript{2+} affinity to site II was dramatically reduced (\(-1000\) fold), to site I was apparently modestly

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**Fig. 4.** Near UV CD spectra of the three intact F29W mutants in the presence and absence of Ca\textsuperscript{2+}. Spectra were run in +Ca\textsuperscript{2+} conditions as described previously (35). Buffer conditions were the same as in Fig. 1. Protein concentrations were as follows: F29W, 83.9 \(\mu\)M (−Ca\textsuperscript{2+}, thin line; +Ca\textsuperscript{2+}, thick line); F29W/E41A, 112.0 \(\mu\)M (−Ca\textsuperscript{2+}, dashed and dotted line; +Ca\textsuperscript{2+}, dashed line); F29W/E77A, 121.8 \(\mu\)M (−Ca\textsuperscript{2+}, ,−−−; +Ca\textsuperscript{2+}, −−−).

**Fig. 5.** Ca\textsuperscript{2+}-induced UV difference absorption spectra of the three intact mutant F29W TnCs. Buffer conditions were the same as in Fig. 1; temperature was 21 °C; protein concentrations were as follows: sTnC\textsuperscript{F29W}, 113.8 \(\mu\)M (−); sTnC\textsuperscript{F29W/E41A}, 112.0 \(\mu\)M (−−−); sTnC\textsuperscript{F29W/E77A}, 121.8 \(\mu\)M (−−−).
increased (~10 fold). These large reductions in site I and site II Ca\(^{2+}\) affinities by mutation to Ala of the invariant Glu residues in each of these loops are consistent with the reported dramatic effects of mutation of invariant Glu to Gln, Lys, or Ala in the Ca\(^{2+}\) binding loops of calmodulin and calbindin (51–55). The increase in site I Ca\(^{2+}\) affinity by the mutation at the site II invariant Glu as in E77A was, however, surprising. At our present level of understanding of the interplay of the multiple factors affecting Ca\(^{2+}\) affinity to EF-hands and without a high resolution structural analysis of main and side chain dispositions for E77A, we presently have no explanation for this apparently anomalous observation.

Previous studies (22, 34, 47, 49, 59, 60–62) have shown that the F29W mutation (immediately adjacent to the 1 (or X) position of the site I EF-hand) in intact sTnC serves as a useful fluorescence probe for monitoring Ca\(^{2+}\) binding and associated structural changes of N domain. Comparison of Ca\(^{2+}\)-induced changes in [\(\theta\)\(_{222}\) nm] of sTnC\(^{C\text{P}29\text{W}}\) and sTnC proteins detected no significant differences in properties of the two, although, as pointed out (34), “it would be unrealistic not to anticipate some local disruption of the immediate environment when Phe is replaced by Trp.” Subsequently, comparison (22) of the far UV fluorescence probe for monitoring Ca\(^{2+}\) binding, we may conclude that the effects of mutation of invariant Glu to Ala of the invariant Glu residues in the other and of the two strands of short \(\beta\) structure (one from each of loops I and II) connecting them. Of the four main chain hydrogen bonds of this antiparallel \(\beta\) strand involving residues 35–39 of loop I and 71–75 of loop II, the distal two (1 and 4) are disrupted, and the remaining two between residues 37 and 73 are shortened and presumably strengthened. Other associated changes have been documented and include both hydrophobic and polar structural alterations (for details, see Ref. 13). The latter authors have suggested that Thr\(^{39}\) plays an important role in the transmission of the effects of site II calcium binding to the site I loop. In the less preformed apo state of loop I, residues 37–41 at the NH\(_2\)-terminal end of helix B are in a nonhelical conformation. This arises, at least in part, from the hydrogen bond between the main chain N of Thr\(^{39}\) and the main chain O of the \(\beta\) strand residue Gly\(^{71}\) of loop II. Disruption of this bond concomitant with calcium binding and compaction of loop II would permit the assumption of normal \(\alpha\)-helical conformation of Thr\(^{39}\) and neighboring residues. Associated and contributing changes would include the redirection of the distal region of helix B, its movement together with tightly associated C helix from closed to open conformation and the repositioning of the Glu\(^{11}\) side chain from its salt bridge with Lys\(^{49}\) to an orientation suitable for calcium liganding in site I.

This series of events ensuing from site II calcium binding is thus seen to involve a complex and interdependent rearrangement of intramolecular structural elements. The degree to which the mutation of an individual residue in this chain would affect the closed to open structural transition would depend on the importance of that residue in the series of events leading ultimately to the transition. Both of the invariant Glu residues in position 12 of the two loops would be expected to play critical roles in this, as shown in the present study. In a temporal sense, Glu\(^{77}\) can be considered as playing a key initial role in the compaction of loop II. Its replacement by Ala would largely eliminate Ca\(^{2+}\) binding and prevent this compaction. This in turn would negate the transmission of the signal through the \(\beta\)-strand region and subsequent structural rearrangements in site I. The closed to open structural transition would be blocked and functionality would be impaired as observed in the present investigation.

As demonstrated by Gagné et al. (26) and in the present...
study, the substitution of Glu 41 by Ala also impairs the closed to open structural transition as well as functionality (31) as assessed by tension measurements in the skinned muscle fiber system. This is so although the data presented in Fig. 1 indicate that for the wild-type sNTnC, calcium binding to site II is sufficient to elicit the closed to open structural transition. This could presumably only occur with the assumption of normal helical conformation of residues 37–41 at the NH2-terminal end of helix B, the latter’s redirection, and the repositioning of the Glu 41 side chain carboxyl from its ionic interaction with Lys40 to an orientation more compatible with calcium ligation. Subsequent calcium binding to site I, requiring higher calcium concentration, would be accompanied by more minor structural alterations, consistent with the small change in \( \theta_{222 \text{ am}} \) (see Fig. 1), including compaction of loop I (13) and associated changes throughout the N domain as detected by NMR (22). Since Glu41 is thus seen as an important element in this series of events leading to the opening of the structure, the effects of its replacement by Ala on this transition are understandable.

The effects of mutating other liganding residues in sites I and II of sTnC on its Ca\(^{2+} \) binding and functional properties have been previously reported (31, 63–68). These have been largely concerned with loop position 1 and/or 3 and include in loop I the mutations D30V and D30A (position 1) and D32G and D32A (position 3). Loop II mutations have included D66V, D66E, D66N, and D66A (position 1) and D68G, D68E, D68A, and D68N (position 3). Ca\(^{2+} \) binding presumably in the mutated loop in all of these was seriously compromised (with the exception of D68N), and functional activity was impaired to varying degrees as assessed by calcium regulation of actomyosin ATPase or skinned fiber tension measurements. It is presently not known whether the closed to open structural transition is impeded in these mutations.

Our proposal in the present study that the major structural transition from closed to open forms of sNTnC is associated with site II Ca\(^{2+} \) binding clearly has relevance to considerations of the energetics of the skeletal and cardiac systems. The free energy changes (\( G^\circ \)) associated with site II Ca\(^{2+} \) binding to the sNTnC and cNTnC proteins as calculated from their dissociation constants have been estimated to be \(-8.0 \pm 0.4 \) and \(-7.7 \pm 0.02 \) kcal mol\(^{-1} \), respectively (20, 23). While this small difference could conceivably account in part for the failure of the cardiac protein to undergo opening, a more important consideration is almost certainly the structural features of the cNTnC in comparison with those of the skeletal isoform. The most important of these are likely to be those in the site I region and include in the cardiac protein the Val28 insertion and the substitution of Leu29 and Ala31 for the two Asp residues at positions 1 and 3 of the skeletal isoform. Recent NMR relaxation measurements of sNTnC and cNTnC (69, 70) have provided important insights into this question. These have demonstrated that while sites II of the two isoforms in their apo states indicate little difference in their conformational entropies, site I of cNTnC shows a decrease of 0.9 \( \pm 0.3 \) kcal mol\(^{-1} \) in comparison with skeletal site I, indicating overall increased rigidity. Estimation of main chain order parameters (\( S^2 \)) and conformational entropies on a per residue basis indicate that residue positions 1–3 of site I in cNTnC contribute inordinately to this entropy difference. This increased rigidity is explicable in terms of the structural interactions and environments of Val28 and Leu29. Val28, having an \( S^2 \) value of 0.83 and indicating little flexibility, is described as being largely buried and having several hydrophobic contacts with Ala31 (position 3) and positions 6–8 of site I. Hydrophobic contacts are also evident between Leu29 and Ile36/Ser37 (\( \beta \)-sheet residues of site I). These stabilizing structural features would be expected to create an energy barrier to the conformational changes involving the straightening of helix B and the closed to open structural transition. Additional energy input provided by TnI peptide/protein binding would be required to force this transition (see below). From this perspective, the failure of cNTnC to undergo full opening upon site II Ca\(^{2+} \) binding, as we propose for sNTnC, can be understood not in terms of its inability to bind Ca\(^{2+} \) at site I but rather in terms of the hydrophobic nature of the amino acid alterations that lead to this loss of Ca\(^{2+} \) binding. Pertinent to these considerations are the experiments carried out by Putkey, Sweeney, and collaborators a decade ago (66–68) in which Ca\(^{2+} \) binding to site I of cNTnC was activated by deletion of Val28 and substitution of residues 29–32 (positions 1–4) of site I by their skeletal TnC counterparts. This construct was fully active as a replacement for cNTnC in slow skeletal muscle fiber tension measurements and as active as wild-type cNTnC in a fast skeletal muscle system. It would be of great interest to establish whether these site I substitutions are sufficient to promote site II Ca\(^{2+} \)-induced opening of the N domain structure.

As indicated above, NMR structural studies have shown that the Ca\(^{2+} \)-induced structural opening observed with sNTnC does not occur with the E41A mutant (26) or the cardiac protein (18, 19, 21). Most recently, however, opening of the structure in the presence of Ca\(^{2+} \) has been observed when either of these is complexed with peptides corresponding to one of the two inhibitory region(s) of the corresponding TnI (i.e. skeletal TnI residues 115–131 and cardiac residues 147–163) (71, 72). With both the skeletal and cardiac TnC N domain, these TnI peptides have been shown to interact in the region of their hydrophobic pockets (Refs. 25 and 73 and references therein). Based on the recent data of Dong et al. (74) working with the cardiac TnC-TnI complex, it seems likely that as with the TnI peptides, the respective intact TnIs would also induce the opening of the cardiac and E41A mutant proteins in the presence of Ca\(^{2+} \). However, in the case of the E41A mutant, and in contrast to cNTnC, the present data (Table II) show that this is insufficient to reverse the effects of the mutation on functional activity. In the case of mutant sNTnC(777A), it is presently unclear whether complexation with inhibitory peptide or intact TnI can bring about opening of the structure. In any case, functional activity as measured by tension measurements in skinned fibers is essentially eliminated with this mutant.

Finally, we wish to express the view that although we consider the evidence presented in this paper to be fully consistent with our interpretation that site II Ca\(^{2+} \)-binding is responsible for triggering the closed to open structural transition of sNTnC, it is possible that other experimental approaches in the future may show otherwise. In particular, it should in principle be feasible to address this issue by the application of appropriate NMR or other spectral measurements at different levels of N domain Ca\(^{2+} \) saturation.

Acknowledgments—We are indebted to Dr. C. M. Kay for providing access to the spectropolarimeter and fluorescence spectrophotometer and Kim Oikawa and Robert Luty for far and near UV CD analyses. We gratefully acknowledge Krystyna Golosinska, Pierre Dubord, Helena Edwards, and Roland Dargis for excellent technical assistance in preparation of proteins, Mike Nattriss for amino acid analyses, and Robert Boyko and Dr. B. D. Sykes for access to computer software used to determine the dissociation constants.

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