Previously, we utilized $^{15}$N transverse relaxation rates to demonstrate significant mobility in the linker region and conformational exchange in the regulatory domain of Ca$^{2+}$-saturated cardiac troponin C bound to the isolated N-domain of cardiac troponin I (Gaponenko, V., Abusamhadneh, E., Abbott, M. B., Finley, N., Gasmi-Seabrook, G., Solaro, R. J., Rance, M., and Rosevear, P. R. (1999) J. Biol. Chem. 274, 16681–16684). Here we show a large increase in cardiac troponin C linker flexibility, corresponding to residues 85–93, when bound to intact cardiac troponin I. The addition of 2 M urea to the intact cardiac troponin I-troponin C complex significantly increased linker flexibility. Conformational changes in the regulatory domain of cardiac troponin C were monitored in complexes with troponin I-(1–211), troponin I-(1–211), troponin I-(1–80) and bisphosphorylated troponin I-(1–80). The cardiac specific N terminus, residues 1–32, and the C-domain, residues 81–211, of troponin I are both capable of inducing conformational changes in the troponin C regulatory domain. Phosphorylation of the cardiac specific N terminus reversed its effects on the regulatory domain. These studies provide the first evidence that the cardiac specific N terminus can modulate the function of troponin C by altering the conformational equilibrium of the regulatory domain.

Cardiac muscle contraction is regulated by Ca$^{2+}$-dependent interactions between members of the troponin complex and other thin filament proteins including actin and tropomyosin. The troponin complex is associated with tropomyosin and consists of troponin (Tn)$^\dagger$ I, the inhibitory subunit, troponin C (TnC), the Ca$^{2+}$-binding subunit, and troponin T (TnT), which makes primary protein-protein contacts with tropomyosin. TnC is required to confer Ca$^{2+}$ sensitivity on the actin-myosin interaction. The cardiac isoforms of TnC and TnI differ structurally and functionally from fast skeletal isoforms. Calcium binding to the regulatory domain of skeletal TnC results in movement of the B and C helices away from the N, A, and D helices producing a conformational “opening.” The newly exposed hydrophobic pocket allows additional interactions with TnI (1–5). Calcium binding site I in cTnC is naturally inactive and in the presence of Ca$^{2+}$ the major conformer of cTnC was found to maintain a “closed” regulatory domain conformation with minimal exposure of the hydrophobic pocket (5, 6). However, the Ca$^{2+}$-saturated regulatory domain of cTnC exhibits chemical exchange consistent with an equilibrium between open and closed conformations (6, 7). NMR studies on the isolated regulatory domain and fluorescence resonance energy transfer studies utilizing intact cTnC and a variety of synthetic cTnI peptides indicated that a structural opening of the cTnC regulatory domain similar to that observed in the skeletal isoform requires both Ca$^{2+}$ and cTnI-(147–163) (8, 9). The cardiac isoform of TnC is also unique in that it contains a cardiac specific extension of approximately 32 residues containing two adjacent Ser residues, 23 and 24, that are phosphorylated in vivo in response to β-adrenergic stimulation (10). Cardiac TnC and TnI are known to interact in an antiparallel manner such that the C-domain of TnC interacts with the N-domain of TnI (11). An N-terminal segment of cTnI, corresponding to residues 33–80, was found to be sufficient for interaction with the C-domain of cTnC in a manner identical to that observed for intact cTnI (11, 12). This region of cTnI is homologous to sTnI residues 1–47, which was shown to bind as an α-helix to the C-domain of sTnC by x-ray crystallography (13). In this complex, the D/E linker of sTnC was partially unwound and bent by 90° in contrast to the fully helical extended D/E linker seen in the x-ray crystal structures of sTnC (3, 13, 14).

Two low resolution models based on neutron scattering studies utilizing the intact skeletal TnIC complex have been published. In the first model, derived from data collected on the sTnIsTnC complex in the presence of 2–3 M urea, extended structures for both sTnC and sTnI were proposed (15, 16). Troponin I was found to wrap around TnC in a coiled manner, and cap each end of an extended TnC molecule (15, 16). Neutron scattering data, obtained in the absence of urea using the intact troponin complex, was more consistent with a two-domain model for TnI (17). The structure of TnI is predicted to

\[ \text{(TnC), the Ca}^{2+}\text{-binding subunit, and troponin T (TnT), which makes primary protein-protein contacts with tropomyosin. TnC is required to confer Ca}^{2+}\text{ sensitivity on the actin-myosin interaction. The cardiac isoforms of TnC and TnI differ structurally and functionally from fast skeletal isoforms. Calcium binding to the regulatory domain of skeletal TnC results in movement of the B and C helices away from the N, A, and D helices producing a conformational “opening.” The newly exposed hydrophobic pocket allows additional interactions with TnI (1–5). Calcium binding site I in cTnC is naturally inactive and in the presence of Ca}^{2+}\text{ the major conformer of cTnC was found to maintain a “closed” regulatory domain conformation with minimal exposure of the hydrophobic pocket (5, 6). However, the Ca}^{2+}\text{-saturated regulatory domain of cTnC exhibits chemical exchange consistent with an equilibrium between open and closed conformations (6, 7). NMR studies on the isolated regulatory domain and fluorescence resonance energy transfer studies utilizing intact cTnC and a variety of synthetic cTnI peptides indicated that a structural opening of the cTnC regulatory domain similar to that observed in the skeletal isoform requires both Ca}^{2+}\text{ and cTnI-(147–163) (8, 9). The cardiac isoform of TnC is also unique in that it contains a cardiac specific extension of approximately 32 residues containing two adjacent Ser residues, 23 and 24, that are phosphorylated in vivo in response to β-adrenergic stimulation (10). Cardiac TnC and TnI are known to interact in an antiparallel manner such that the C-domain of TnC interacts with the N-domain of TnI (11). An N-terminal segment of cTnI, corresponding to residues 33–80, was found to be sufficient for interaction with the C-domain of cTnC in a manner identical to that observed for intact cTnI (11, 12). This region of cTnI is homologous to sTnI residues 1–47, which was shown to bind as an α-helix to the C-domain of sTnC by x-ray crystallography (13). In this complex, the D/E linker of sTnC was partially unwound and bent by 90° in contrast to the fully helical extended D/E linker seen in the x-ray crystal structures of sTnC (3, 13, 14). Two low resolution models based on neutron scattering studies utilizing the intact skeletal TnIC complex have been published. In the first model, derived from data collected on the sTnIsTnC complex in the presence of 2–3 M urea, extended structures for both sTnC and sTnI were proposed (15, 16). Troponin I was found to wrap around TnC in a coiled manner, and cap each end of an extended TnC molecule (15, 16). Neutron scattering data, obtained in the absence of urea using the intact troponin complex, was more consistent with a two-domain model for TnI (17). The structure of TnI is predicted to

\[ \text{(TnC), the Ca}^{2+}\text{-binding subunit, and troponin T (TnT), which makes primary protein-protein contacts with tropomyosin. TnC is required to confer Ca}^{2+}\text{ sensitivity on the actin-myosin interaction. The cardiac isoforms of TnC and TnI differ structurally and functionally from fast skeletal isoforms. Calcium binding to the regulatory domain of skeletal TnC results in movement of the B and C helices away from the N, A, and D helices producing a conformational “opening.” The newly exposed hydrophobic pocket allows additional interactions with TnI (1–5). Calcium binding site I in cTnC is naturally inactive and in the presence of Ca}^{2+}\text{ the major conformer of cTnC was found to maintain a “closed” regulatory domain conformation with minimal exposure of the hydrophobic pocket (5, 6). However, the Ca}^{2+}\text{-saturated regulatory domain of cTnC exhibits chemical exchange consistent with an equilibrium between open and closed conformations (6, 7). NMR studies on the isolated regulatory domain and fluorescence resonance energy transfer studies utilizing intact cTnC and a variety of synthetic cTnI peptides indicated that a structural opening of the cTnC regulatory domain similar to that observed in the skeletal isoform requires both Ca}^{2+}\text{ and cTnI-(147–163) (8, 9). The cardiac isoform of TnC is also unique in that it contains a cardiac specific extension of approximately 32 residues containing two adjacent Ser residues, 23 and 24, that are phosphorylated in vivo in response to β-adrenergic stimulation (10). Cardiac TnC and TnI are known to interact in an antiparallel manner such that the C-domain of TnC interacts with the N-domain of TnI (11). An N-terminal segment of cTnI, corresponding to residues 33–80, was found to be sufficient for interaction with the C-domain of cTnC in a manner identical to that observed for intact cTnI (11, 12). This region of cTnI is homologous to sTnI residues 1–47, which was shown to bind as an α-helix to the C-domain of sTnC by x-ray crystallography (13). In this complex, the D/E linker of sTnC was partially unwound and bent by 90° in contrast to the fully helical extended D/E linker seen in the x-ray crystal structures of sTnC (3, 13, 14). Two low resolution models based on neutron scattering studies utilizing the intact skeletal TnIC complex have been published. In the first model, derived from data collected on the sTnIsTnC complex in the presence of 2–3 M urea, extended structures for both sTnC and sTnI were proposed (15, 16). Troponin I was found to wrap around TnC in a coiled manner, and cap each end of an extended TnC molecule (15, 16). Neutron scattering data, obtained in the absence of urea using the intact troponin complex, was more consistent with a two-domain model for TnI (17). The structure of TnI is predicted to

Overhauser enhancement spectroscopy; S$^2$, order parameters; cTnIC, cTnICtTnC; NOE, nuclear Overhauser effect.
Dynamics in Cardiac Troponin I-Troponin C Complexes

resemble two elliptical masses, one representing 70% and the other 30% of TnI. Calcium binding to αTnC was suggested to change the angle between TnI domains, whereas the radius of gyration for TnC was not affected (17). Qualitatively, although both sets of data are consistent with an extended αTnC structure, the mass distribution for TnI in the in situ complex differs significantly from that found in the TnIC complex in the presence of 2–3 mM urea (15–17). Discrepancies between the two models could result from either the presence of 2–3 mM urea or the additional TnT subunit.

High resolution solution NMR studies of TnC and TnI interactions have largely utilized isolated domains and synthetic peptides due to the large molecular mass (approximately 42 kDa) and limited solubility of the intact αTnC complex. Findings from these studies, by their nature, fail to reveal structural mechanisms that rely on interdomain interactions and must be verified in more sophisticated model systems. Low resolution structural techniques such as fluorescence and neutron scattering have provided important information on the intact complex. However, they do not contain sufficient detail to verify the findings of the high resolution single-domain experiments or completely elucidate the structural mechanisms of αTnC regulation and cTnI inhibitory function.

In an effort to correlate functional regions of cTnI with structural mechanisms in the cTnC system and bridge the gap between available high resolution structures of isolated domains and the low resolution data of the intact complex, a series of cTnC αcTnI complexes were created for multidimensional heteronuclear NMR analysis. Strategic deletion of functionally significant regions cTnI was utilized to investigate the structural interactions that underlie the switch mechanism within the cTnC complex. The dynamics of cTnC in the intact cTnC complex were monitored by 1H transverse relaxation rates and chemical shift mapping. Chemical shift data for cTnC bound to cTnI-(1–80), cTnI-(1–80)pp, cTnI-(1–80)DD, cTnI-(33–211), and cTnI-(1–211) were compared and used to monitor conformational equilibrium in the cTnC regulatory domain. Surprisingly, binding of both the cardiac specific N terminus and the C-domain of cTnI altered the cTnC regulatory domain conformation. The absence of the cardiac specific N terminus (cTnI-(33–211)), mutation of Ser23 and Ser24 to Asp (cTnI-(1–211)) and phosphorylation at Ser residues 23 and 24 (cTnI-(1–80)pp), shifts the conformational equilibrium toward that observed in free Ca2+-saturated cTnC. This provides the first evidence that the cardiac specific N terminus modulates the function of cTnC by altering the conformational equilibria within the regulatory domain. These results also demonstrate that a simple model based on Ca2+-dependent binding of cTnI residues 147–163 to the regulatory domain of cTnC is insufficient to account for the molecular details of cardiac troponin I-troponin C interactions.

MATERIALS AND METHODS

Proteins—[15N,2H]cTnC, cTnI-(1–80), cTnI-(1–80)DD, cTnI-(33–211), and cTnI-(1–211) were purified and complex formation carried out as described previously (7, 11, 18). Cardiac TnI-(1–80)pp was prepared as described previously (18). For NMR experiments, [15N,2H]cTnC, cTnI-(1–80), [15N,2H]cTnC-(33–211), and [15N]cTnC-(1–80)pp complexes were prepared at 1.0 mM concentration in 10% 2H2O, 20 mM Tris-d1, buffer (pH 6.8), 150 mM potassium chloride, 10 mM Ca2+, 10 mM β-mercaptoethanol, 10 mM dithiothreitol, 0.2 mM leupeptin, and 0.4 mM Pefabloc (7, 18). The [15N,2H]cTnC-(33–211) complex was prepared using 0.5 mM concentration (due to the limited solubility of these complexes) in 10% 2H2O, 20 mM Tris-d1, buffer (pH 6.8), 200 mM potassium chloride, 10 mM Ca2+, 10 mM dithiothreitol, and the protease inhibitor mixture Complete EDTA-free (Roche Molecular Biochemicals), or in the identical buffer containing 2 mM urea. No evidence of dimerization of cTnC or the various complexes was observed by light scattering or native gel analysis. Protein concentrations for cTnC were determined by UV and Bradford analysis. Cardiac TnI concentrations were determined by BCA assay (Pierce). Amino acid analysis was used to calibrate the colorimetric methods.

Spectroscopy—All experiments were carried out on Varian 600 or 800 MHz spectrometers. 1H-15N correlation experiments utilized pulse sequences based on either sensitivity-enhanced 1H-15N HSQC (19) or 1H-15N TROSY (20, 21). Three-dimensional NOESY-HSQC experiments using an 85-ms mixing time were generally used to confirm amide resonance assignments (22). 1H transverse relaxation experiments on cTnC-(1–211) complexes, having a molecular mass of approximately 42 kDa, utilized TROSY-based detection, allowing measurement of the molecular size for improved resolution. The TROSY-detected 15N R2 data were recorded using a conventional pulse sequence for 15N R2 measurements (23) that was modified by replacing the reverse polarization transfer element with a sensitivity-enhanced TROSY sequence (20, 21). Spectral widths in the t1 and t2 dimensions were 3.3 kHz and 12 kHz respectively. Spectra were collected with Carr-Purcell-Meiboom-Gill relaxation periods of 0, 8, 16, 24, 32, 40, 48, and 64 ms. Double points were collected with relaxation periods of 8, 16, 32, and 40 ms for error analysis. Data processing has been described previously (7).

Fluorescence Assay—Calcium binding to cTnC was measured as described previously (24). Binding measurements were made with the aid of the fluorescent probe, 2-(4'-iodoacetamidoanilino)-naphthalene-6-sulfonic acid (IAANS), which reports Ca2+ binding to the regulatory site. Cardiac troponin C (1.5 mg/ml) was labeled in 10 mM MOPS, 90 mM KCl, 2.6 mM CaCl2, 2 mM EGTA, 6 mM urea, pH 7.0 and a 5-fold molar excess of IAANS, prepared fresh as a stock solution in Me2SO. Labeling proceeded for 8–10 h at room temperature with constant shaking. The solution was then dialyzed against two changes of 4 liters of 10 mM MOPS, 90 mM KCl at pH 7.0. The molar ratio of the incorporated probe to cTnC was approximately 1.5. Labeled cTnC (cTnCIA) was complexed with cTnI as described previously (24). Fluorescence measurements were carried out in a Perkin-Elmer SS-5B luminescence spectrometer using an excitation wavelength of 330 nm. The peak emission wavelength (445–455 nm) was determined prior to Ca2+ titrations. Ca2+ titrations were carried out with and without 2 mM urea in 3 ml of a solution containing 1 µM of the cTnCIA complex, 20 mM MOPS, 90 mM KCl, 3 mM MgCl2, pH 7.0, 1 mM EGTA. A range of free Ca2+ concentrations was achieved by sequential addition of CaCl2, as calibrated using binding constants reported by Godt and Lindley (25). Changes in fluorescence intensities were normalized to the maximum change, and the data fitted to the Hill equation as described previously (24).

RESULTS

Previously, we have shown that the N-domain of cTnI containing the cardiac specific N terminus, cTnI-(1–211), interacts with both domains of cTnC and decreases chemical exchange in the regulatory domain (7). Binding of cTnI-(1–80), however, does not significantly affect flexibility in the D/E linker region. To further elucidate dynamic relationships within the cTnC complex, we examined the binding of cTnI-(1–80), cTnI-(1–80)pp, cTnI-(1–80)DD, (cTnC-(1–33–211), and cTnI-(1–211) to [15N,2H]cTnC. Chemical shifts for the amide resonances of cTnC bound to cTnI-(1–80), cTnI-(1–80)DD, and cTnI-(1–80)pp have been assigned previously (18). Assignments for amide resonances of [15N,2H]cTnC bound to either cTnI-(1–211) or cTnC-(33–211) were made by comparison of 1H-15N TROSY spectra with 1H-15N HSQC spectra of [15N,2H]cTnC-(1–80) (7, 18). When necessary, assignments for cTnC-(1–211) and cTnC-(33–211) were confirmed using H N TROSY-HSQC experiments using an 85-ms mixing time. The 800-MHz 1H-15N TROSY spectrum for [15N,2H]cTnC bound to cTnI-(1–211) with cross-peaks assigned is shown in Fig. 1. Multiplet conformations were observed for Ala31, Gly34, Gly42, Gly66, Gly68, Val72, Asp73, and Val79 in the 1H-15N TROSY spectra of the 42-kDa cTnC-(1–211) complex. For these residues, the most intense cross-peak representing the predominant conformation of cTnC was utilized for chemical shift comparison and to obtain rate information (Fig. 2). A number of resonances throughout...
the regulatory domain were broadened beyond detection or shifted upon binding either cTnI-(1–80) or cTnI-(33–211). Interestingly, cross-peaks for Ala31, Asp33, and Gly34 in inactive calcium binding site I, cross-peaks for Leu48 and Gly49 in the B/C loop region, and cross-peaks for Glu66, Asp67, and Thr71 in calcium binding site II were significantly perturbed only when cTnC was bound to cTnI-(1–80) or cTnI-(1–211).

To further explore the conformational exchange observed in the regulatory domain of cTnC, 15N transverse relaxation rates were measured in the intact [15N,2H]cTnC-zcTnI-(1–211) complex. 15N transverse relaxation rates, $R_2$, depend on the rotational correlation time of the molecule, internal motions, conformational exchange, dipole-dipole interactions, and chemical shift anisotropy. Internal motion and conformational exchange are indicated by 15N $R_2$ rates lower and higher than the average 15N $R_2$ value for the molecule, respectively. The previously published 15N $R_2$ values for free cTnC are included for comparison (7). 15N $R_2$ values for free Ca$^{2+}$-saturated cTnC demonstrate distinct averages for the N- and C-domains, indicating some independence in the tumbling of the two domains resulting in distinct rotational correlation times (7). Additionally, lower than average rates are observed in the D/E linker, consistent with rapid internal motions due to flexibility in this region (7). Higher than average rates within defunct site I were previously shown to result from conformational exchange by comparison of 15N transverse cross-correlation rates and 15N transverse relaxation rates (7).

The average 15N $R_2$ values for the N- and C-domains of cTnC in the cTnC-zcTnI-(1–211) are 34 ± 7 s$^{-1}$ and 35 ± 7 s$^{-1}$, respectively, suggesting a uniform rotational correlation time across the molecule (Fig. 2B). Larger errors for the $R_2$ values in the cTnC-zcTnI-(1–211) complex are a consequence of the large molecular mass (42 kDa) and lower solubility of the intact cTnIC complex. Despite larger errors for the intact complex, trends in rates through the molecule are discernable. Collection of 15N transverse cross-correlation rates was not practical due to the lower protein concentration of the complex, the larger molecular mass (42 kDa) of the complex, and the inherent insensitivity of the experiment. Further, collection of a complete set of relaxation parameters and calculation of order parameters ($S^2$) would be impractical given the physical limitations of the more biologically relevant systems utilized in this study. Determination of $S^2$ values in the absence of detailed tertiary structures is ambiguous due to the uncertainty of the diffusion tensors (26–28). Significantly, $R_2$ values for residues 85–93, which comprise the linker region, are nearly as large as those for the N- and C-domains, indicating that full-length cTnI considerably restricts motion of these residues in the intact complex (Fig. 2). 15N $R_2$ values for Asp25, Ile26, and Gly34 in defunct Ca$^{2+}$ binding site I, as well as Val75 and Asp77 in Ca$^{2+}$ binding site II were equal to or greater than the average rate in the intact cTnIC complex (Fig. 2). In contrast, the

---

**Fig. 1. TROSY spectrum of [15N,2H]cTnC-zcTnI-(1–211) at 800 MHz.** 1H-15N cross-peaks are labeled by residue number. Multiple cross-peaks were observed for residues Ala31, Glu32, Gly34, Gly42, Glu66, Gly68, Val72, Asp73, and Val79 at 2-fold lower contour levels. Cross-peaks for residues Ala31 and Asp73, shown in boxes at the appropriate chemical shifts, are included as insets as they are specifically discussed below. Gly70, also shown boxed at the appropriate position, is not visible in this TROSY spectrum, but is clearly visible in HSQC spectra.
and 157 were excluded from analysis due to peak overlap in the 1H-15N correlation spectra for residues 1, 2, 3, 6, 17, 20, 24, 41, 44, 47, 58, 62, 64, 65, 74, 78, 79, 81, 84, 87, 93, 98, 99, 102, and 150 were too broad to obtain accurate values in the presence of urea.

Relaxation parameters of cTnC in the intact complex were taken from Gaponenko et al. (7). A, resonance assignments in 1H-15N correlation spectra of the cTnC-cTnI-(1–211) complex could not be confirmed due to a lack of sequential NOEs in NOESY-HSQC spectra for residues 1, 2, 3, 6, 17, 20, 24, 41, 44, 47, 58, 62, 64, 65, 74, 78, 79, 81, 84, 87, 93, 98, 99, 102, and 150 were too broad to obtain accurate $R_2$ values in the absence of urea. B, cross-peaks for residues 25, 29, 30, 32, 40, 61, 66, 71, 74, 78, 79, 84, 93, 102, and 150 were too broad to obtain accurate $R_2$ values in the presence of urea. C, cross-peaks for residues 25, 29, 30, 32, 40, 61, 66, 71, 74, 78, 79, 84, 93, 102, and 150 were too broad to obtain accurate $R_2$ values in the presence of urea.

cTnC-cTnI-(1–80) complex has below average 15N $R_2$ values in site I as well as the linker region, indicating internal motion in both regions, as previously shown (7).

Relaxation parameters of cTnC in the intact complex were examined in the presence of urea to mimic the conditions used for neutron scattering studies of the skeletal complex (15, 16). In the presence of urea, the average 15N transverse relaxation rate increases to approximately 46 s$^{-1}$. The increase in average rate likely results from an increase in rotational correlation time due to either interactions of urea with amino acid side chains or partial disruption of cTnC-cTnI interactions. It has been shown that the residence times of urea molecules in solution sites near methyl groups of Val, Leu, and Ile are significant, possibly leading to a decrease in the hydrophobic effect within the protein and stabilizing solvent-exposed hydrophobic groups (29, 30). Urea, in the range of 2–4 M has been shown to increase the radius of gyration of other polypeptides (31). 15N transverse relaxation rates in the linker region are reduced to below 60% of the average rate and indicate increased mobility for these residues in the presence of urea. The observed change in linker flexibility may have important implications in analysis of data collected on the sTnI-sTnC complex in the presence of 2–3 M urea. In addition, 2 M urea affects Ca$^{2+}$ binding to the regulatory site of cTnC, as reported by fluorescence changes in IAANS (Fig. 3). A small change in the correlation between pCa and percentage of fluorescence intensity in the C-terminal region of cTnI, comprising residues 81–211, or as a consequence of an altered chemical exchange rate between solution conformations.

Conformational exchange in Ca$^{2+}$-saturated cTnC has been previously identified in defunct Ca$^{2+}$ binding site I (6, 7). In addition, multiple amide cross-peaks for a number of residues (Leu$^{29}$, Asp$^{32}$, and Gly$^{34}$) have been detected in the cTnC-cTnI-(1–80) complex (7). Initially, we analyzed interactions of cTnC with cTnI-(1–211) by comparing combined amide 1H and 15N chemical shift differences for cTnC bound to cTnI-(1–80) and cTnI-(1–211) (Fig. 4). Residues showing significant chemical shift perturbations were located in defunct Ca$^{2+}$ binding site I (residues 28–38), Ca$^{2+}$ binding site II (residues 65–76), and the linker region (residues 85–93) (Fig. 4). In general, chemical shift differences were considerably smaller than those observed for binding of the N-domain of cTnI to the C-domain of cTnC (17, 18). Observed chemical shift differences could result from either a direct interaction between cTnI and the C-terminal region of cTnC, comprising residues 81–211, or as a consequence of an altered chemical exchange rate between solution conformations.

Residues Ala$^{31}$, Glu$^{32}$, Gly$^{34}$, Glu$^{66}$, and Asp$^{73}$, were shown to exhibit some of the largest 1H and 15N chemical shift variations among complexes of intact cTnC bound to cTnI-(1–80), cTnI-(1–80)DD, cTnI-(1–80)pp, cTnI-(33–211), and cTnI-(1–211). In all cases, the presence of the cardiac specific N terminus and/or residues 81–211 of cTnI shifted the conformational
equilibrium toward a second conformation as judged by induced $^1$H and $^{15}$N chemical shift perturbations. Here we examine in detail the conformational transitions of two residues, Ala$^{31}$ and Asp$^{73}$, in Figs. 5 and 6, respectively. These residues were chosen since their $^1$H and $^{15}$N chemical shift values are unique and assignment of multiple resonances for each of these residues is unequivocal (Fig. 1).

Alanine 31 is located in defunct Ca$^{2+}$ binding loop I. A single cross-peak was found for Ala$^{31}$ in the $^1$H-$^{15}$N HSQC spectrum of cTnC bound to cTnI-(1–80)pp, cTnI-(1–80)DD or cTnI-(33–211) (Fig. 5, A, B, and D). In the presence of cTnI-(1–80) containing the cardiac specific N terminus (Fig. 5C), the resonance for Ala$^{31}$ was observed downfield from the cross-peak observed in either cTnI-(1–80)pp, cTnI-(1–80)DD, or cTnI-(33–211). Two cross-peaks for Ala$^{31}$ in cTnC bound to cTnI-(211) were found, suggesting the presence of multiple conformations in slow chemical exchange for this region of the regulatory domain (Fig. 5E). In the presence of 2 mM urea, two resonances for Ala$^{31}$ are observed with an additional downfield shift (Fig. 5F). Ligand-induced chemical shift changes result from perturbations of the magnetic environment of the resonance caused directly by the bound peptide ligand, or indirectly due to ligand-induced conformational changes, or alteration of chemical equilibria within the complex. These data demonstrate that the cardiac specific N terminus, as well as elements within cTnI-(81–211), can induce chemical shift changes consistent with a more open structure of the regulatory domain. Further, mutation of Ser$^{23}$ and Ser$^{24}$ to mimic phosphorylation or actual phosphorylation of Ser$^{23}$ and Ser$^{24}$ eliminates the effect of the cardiac specific N terminus on the conformation of the regulatory domain.

A similar pattern of chemical shifts is observed for Asp$^{73}$, located in the short $\beta$-sheet between Ca$^{2+}$-binding sites I and II, in Ca$^{2+}$-saturated cTnC complexes with cTnI (Fig. 6). A single cross-peak is found for Asp$^{73}$ in cTnC bound to cTnI-(1–80)pp or cTnI-(1–80)DD (Fig. 6, A and B). In the presence of cTnI-(1–80), having the non-phosphorylated cardiac N terminus, two downfield shifted cross-peaks are observed for Asp$^{73}$ (Fig. 6C). In the presence of cTnI-(33–211), lacking the cardiac N terminus and containing residues 81–211, multiple cross-peaks are observed for Asp$^{73}$ in the regulatory domain (Fig. 6D). Addition of the cardiac N terminus in the cTnC-cTnI-(1–211) complex shifts the Asp$^{73}$ cross-peaks downfield in both the $^1$H and $^{15}$N dimensions toward a second regulatory domain conformation as observed for cTnC bound to cTnI-(1–80) (Fig. 6, D and E). These resonances appear to be in intermediate to slow exchange between two or more conformations. Finally, addition of 2 mM urea to the cTnC-cTnI-(1–211) complex shifts the equilibrium for Asp$^{73}$ toward the second regulatory domain conformation (Fig. 6F).

These comparisons demonstrate that not only is the region 81–211 of cTnI capable of inducing conformational changes in the regulatory domain of cTnC, but the cardiac N terminus can also influence the cTnC regulatory domain conformation, either independently or in conjunction with the inhibitory motifs. Phosphorylation of Ser$^{23}$ and Ser$^{24}$ or mutation of Ser$^{23}$ and Ser$^{24}$ to mimic phosphorylation removes the effect of the cardiac specific N terminus on the cTnC regulatory domain conformation. The pattern of chemical shift changes for Ala$^{31}$ and Asp$^{73}$ (Figs. 5 and 6), as well as those for Glu$^{40}$, Gly$^{42}$, Gly$^{43}$, Glu$^{66}$, Val$^{72}$, and Val$^{79}$ (data not shown) closely resembles the pattern of chemical shift changes observed in the isolated N-domain of cTnC upon titration with the peptide corresponding to cTnI-(147–163) (8). In general, cTnC regulatory domain chemical shift perturbations attributable to the presence of residues 81–211 of cTnI were more prominent for residues in calcium binding site II, whereas chemical shift changes in site I were largely due to the presence of the unmodified cardiac specific N terminus of cTnI.

Many of the cross-peaks for residues that constitute the hinges of the cTnC regulatory domain, Glu$^{40}$ and Val$^{79}$ (8), are broadened beyond detection in $^1$H-$^{15}$N correlation spectra of all of the complexes studied here. Broadening of resonances from residues surrounding Glu$^{40}$, near the N terminus of helix B, and Val$^{79}$, near the C terminus of helix C, suggests these regions experience significant motions under a wide range of conditions. This would be consistent with a dynamic equilibrium of cTnC regulatory domain between open and closed conformations. Interestingly, a weak H$\alpha$ to H$\alpha$ NOE is observed in 85- and 300-ms mixing time NOESY-HSQC spectra between Ser$^{27}$ and Ile$^{61}$ in $^{15}$N,$^2$H-TnC bound to cTnI-(1–80). This NOE is not observed in NOESY-HSQC spectra of $^{15}$N,$^2$H-TnC bound to cTnI-(1–80)DD. The internuclear distance between amide protons of Ser$^{27}$ and Ile$^{61}$ in the calcium-saturated N-domain structure of cTnC (PDB identifier AP4) is 12.0 Å, whereas it is 8.3 Å in the structure of the N-domain of cTnC bound to cTnI-(147–163) (Protein Data Bank identifier 1MXL). An upper limit of 10 Å has been utilized for NOE-derived distance restraints in structure determination of highly perdeuterated proteins (32). These structures correspond to the closed and open cTnC regulatory domain conformations, describing the degree of hydrophobic exposure in the regulatory domain, respectively. Observation of the NOE between Ser$^{27}$, in the first $\beta$-strand near inactive Ca$^{2+}$-binding site I, and Ile$^{61}$, near the
C terminus of helix C, in [15N,2H]cTnC bound to cTnI-(1–80) is facilitated by 70% perdeuteration of cTnC, and is consistent with a more open conformation for this complex.

**DISCUSSION**

In molecular switch proteins consisting of multiple domains, an understanding of both interdomain and intradomain interactions is essential for elucidation of a detailed structural mechanism. However, structure determination in complexes with molecular mass larger than 25 kDa can be problematic, particularly in poorly soluble, largely α-helical proteins. Studies on isolated domains have provided high resolution structures and revealed domain level mechanisms, such as the opening of the globular domains of cTnC upon binding the appropriate segment of cTnI (8, 12). However, the study of single domain model systems prevents investigation of interdomain mechanisms within the troponin complex. Investigation of proposed mechanistic roles of the linker region of cTnC and the cardiac specific N terminus of cTnI require a model system based on the intact cTnC molecule.

Despite the rigid α-helical conformation of the D/E linker in x-ray crystal structures, considerable evidence suggests conformational flexibility in solution exists (1, 7, 33, 34). Mutational studies of the central helix in TnC have previously shown that both a required amount of flexibility and a critical length are necessary for optimal activity, suggesting that the linker region may function as a molecular ruler, maintaining a proper relationship between the N- and C- domains of cTnC (35–37). In the present study, we utilized 15N R\textsubscript{2} values to measure flexibility within the linker region of cTnC in the presence of full-length cTnI. There was a significant loss of flexibility for residues 85–93 in cTnC upon binding cTnI-(1–211), suggesting that residues 81–211 may interact with or immobilize the linker region in cTnC (Fig. 2). Alternatively, interactions between C-domain of cTnI and the regulatory domain of cTnC may fix the relationship between cTnC domains, resulting in reduced mobility in the linker region. Alterations in linker flexibility related to changes in interdomain relationships may play a crucial role in the conformational switch that signals muscle contraction (36–38).

Our data further demonstrate that urea significantly increases interdomain flexibility within cTnC (Fig. 2). The lack of large chemical shift differences and the small change in the p\textsubscript{Ca}²⁺-fluorescence relationship in IAANS-labeled cTnCcTnI complex in 2 M urea (Fig. 3) suggest minimal perturbation of the globular domains in cTnC bound to cTnI in the presence of urea. However, regulatory domain chemical shifts (Figs. 5 and 6) and the p\textsubscript{Ca}₅₀ shift (Fig. 3) are consistent with urea stabilizing a more open structure of the regulatory domain, possibly by solvating exposed hydrophobic surfaces (39). Taken together, the data suggest that urea may alter interactions between the regulatory domain of cTnC and the C-domain of cTnI.

The amide cross-peaks of Ala\textsuperscript{31} and Asp\textsuperscript{73} (Figs. 5 and 6), as well as those for Glu\textsuperscript{32}, Gly\textsuperscript{34}, Gly\textsuperscript{42}, Gly\textsuperscript{68}, Glu\textsuperscript{66}, Val\textsuperscript{72}, and Val\textsuperscript{79} (data not shown) of Ca\textsuperscript{2⁺}-saturated cTnC shift in the same direction and by approximately the same magnitude in the presence of intact cTnI-(1–211) as was observed when the isolated regulatory domain bound cTnI peptide corresponding to residues 147–163 was bound to cTnC-(1–89) (8). These chemical shift changes were shown to correlate with a conformational opening of the isolated regulatory domain (8). Fluorescence resonance energy transfer measurements also indicate that the Ca\textsuperscript{2⁺}-saturated regulatory domain of cTnC bound to cTnI-(1–211) assumes an open conformation (9). These correlations suggest that the second regulatory domain conformation observed here corresponds to the open state. This conclu-
sion is further supported by urea stabilization of the second or open state. It has been shown that urea can decrease the hydrophobic effect within the protein and may stabilize solvent-exposed hydrophobic surfaces (29, 30). Interestingly, addition of the well known cTnI inhibitory peptide, comprising residues 129–147, to the cTnC-cTnI-(1–80) complex had little effect on conformational exchange or the number of conformers detected in residues Ala31, Glu32, Gly34, Gly42, Glu66, Gly68, Val72, Asp73, and Val79 (40). However, addition of cTnI-(129–166) to the cTnC-cTnI-(1–80) complex resulted in regulatory domain chemical shifts similar to those observed in cTnC-(1–89) bound to cTnI-(147–163) (8).

Surprisingly, binding of cTnI-(1–211) to full-length cTnC did not induce a single open conformation in the regulatory domain as judged by multiple NMR detectable conformers for residues Ala31, Glu32, Gly34, Gly42, Glu66, Gly68, Val72, Asp73, and Val79 (40) located within this region (Figs. 5 and 6). Although chemical shift changes and the presence of multiple conformations do not necessarily indicate a large structural transition from a closed to open conformation, the observed multiple HN chemical shifts for residues in the regulatory domain do show a number of different magnetic environments for each amide resonance. These magnetically different states presumably result from conformational differences in the regulatory domain of cTnC when bound to cTnI. The structural uniqueness of these multiple isoforms is at present unclear. However, based on the available data for the isolated regulatory domain of cTnC binding to cTnI-(147–163) (8), and our $R_2$ values for the various cTnIC complexes (7), it is likely that the observed conformational isomerism represents exchange between a closed and a more open state for the regulatory domain of cTnC.

The possibility remains that inhibitory motifs within cTnI-(81–211) fail to open the regulatory domain to the degree observed in the skeletal system. Comparison of the structural opening induced by binding cTnI-(147–163) to cTnC-(1–89) with the Ca$^{2+}$ induced opening of sTnC has led Li et al. (8) to suggest that the regulatory domain in the cardiac complex is less open than observed for the skeletal system. Effects of urea on exchange in the regulatory domain support either further stabilization or an additional opening of the hydrophobic pocket by urea.

The effects of the cardiac specific N terminus of cTnI on the cTnC regulatory domain conformation have not previously been recognized. The unexpected finding that the cardiac specific N terminus of cTnI can also shift the conformational equilibrium of the cTnC regulatory domain suggests a novel and more complex mechanism for activation of cardiac muscle troponin. Based on the data presented in Figs. 5 and 6 and the observation of the open-state specific H$_N$ to H$_N$ NOE between Ser$^{37}$ and Ile$^{61}$ in the cTnC-cTnI-(1–80) complex, the cardiac specific N terminus may play a role in shifting the equilibrium toward a more open conformation. Phosphorylation or mutation to Asp of Ser23 and Ser24 in cTnI does not significantly affect the conformational states observed for the regulatory domain compared with binding cTnI-(33–211), demonstrating that the phosphorylated cardiac specific N terminus does not directly interact with the regulatory domain of cTnC. This was first proposed based on $R_2$ values for cTnC bound to cTnI-(1–80) and cTnI-(1–80)DD (7).

These results are consistent with a variety of in vitro and functional studies. Phosphorylation of cTnI reduces the fluorescence-detected Ca$^{2+}$ affinity of site II in the cTnIC complex (41). Replacement of cTnI with cTnI-(33–211) in skinned fiber bundles from rat induces a desensitization to activation by

---

2 M. B. Abbott, unpublished data.
Ca\textsuperscript{2+} (42). Desensitization to Ca\textsuperscript{2+} was also observed upon cAMP-dependent protein kinase phosphorylation of cTnI (1–211) (43). Finally, reconstitution with cTnI (1–211)DD, mimicking the phosphorylated state, led to a reduced Ca\textsuperscript{2+} sensitivity in skinned cardiac muscle fibers compared with reconstitution with wild type cTnI (44).

The available experimental evidence supports a novel mode of action for the cardiac specific N terminus during cardiac muscle contraction. Interaction of the cardiac specific N terminus appears to facilitate a “partial opening” of the regulatory muscle contraction. Interaction of the cardiac specific N terminus with the regulatory domain of Ca\textsuperscript{2+}-bound cTnC and cTnI and suggests a structural mechanism of cTnC. Phosphorylation of the cardiac specific N terminus eliminates the stabilizing effect on the regulatory domain resulting in reduced calcium sensitivity of the troponin switch. This novel mechanism utilizes the unique isoform differences in both cardiac TnC and TnI and suggests a structural mechanism for modulating the cTnC and cTnI interactions that regulate cross-bridge cycling. Chemical exchange in the regulatory domain of Ca\textsuperscript{2+}-saturated cTnC bound to cTnI indicates that a simple closed to open transition is insufficient to completely describe the conformational isomerism observed in the intact system.

In summary, we have demonstrated that binding of cTnI (1–211) to Ca\textsuperscript{2+}-saturated cTnC results in a significant decrease in linker region flexibility as well as a decrease in conformational exchange and a shift toward an open regulatory domain facilitating binding of the C-terminal domain of cTnI. We also describe a novel role for the cardiac N terminus of cTnI. We propose that a region of the cardiac specific N terminus of cTnI interacts with the regulatory domain and shifts the conformational equilibrium toward a more open form, possibly by stabilizing the defunct Ca\textsuperscript{2+} binding site I. Phosphorylation of the cardiac specific N terminus at Ser\textsuperscript{23} and Ser\textsuperscript{24} results in a loss of interaction between the regulatory domain and the phosphorylated N terminus, resulting in an additional entropic energy barrier to the activation of cardiac muscle contraction. This is completely consistent with available physiological data showing a decrease in Ca\textsuperscript{2+} affinity upon phosphorylation or deletion of the cardiac specific N terminus (45). Structure determination of cTnC in the presence of the cardiac specific N terminus and the inhibitory region of cTnI will better define the molecular basis for the observed conformational isomerism within the regulatory domain of cTnC.

REFERENCES
1. Herzberg, O., Moult, J., and James, M. N. (1986) J. Biol. Chem. 261, 2638–2644
2. Strynadka, N. C., Cherney, M., Sielicki, A. R., Li, M. X., Smillie, L. B., and James, M. N. (1997) J. Mol. Biol. 273, 238–255
3. Houdusse, A., Love, M. L., Dominguez, R., Grabarek, Z., and Cohen, C. (1997) Structure 5, 1695–1711
4. Gagne, S. M., Li, M. X., McKay, R. T., and Sykes, B. D. (1996) Biochem. Cell Biol. 76, 392–312
5. Sia, S. K., Li, M. X., Strynopoulos, L., Gagne, S. M., Liu, W., Putkey, J. A., and Sykes, B. D. (1997) J. Biol. Chem. 272, 18216–18221
6. Paakkonen, K., Annila, A., Sorsa, T., Pollesello, P., Tilgmann, C., Kilpelainen, I., Karisola, P., Umanen, I., and Drakenberg, T. (1998) J. Biol. Chem. 273, 15633–15638
7. Gaponenko, V., Abusamhadneh, E., Abbott, M. B., Finley, N., Gasmi-Seabrook, G., Solaro, R. J., Rance, M., and Rosevear, P. R. (1999) J. Biol. Chem. 274, 16681–16684
8. Li, M. X., Strynopoulos, L., and Sykes, B. D. (1999) Biochemistry 38, 8289–8298
9. Dong, W. J., Xing, J., Villain, M., Helling, M., Robinson, J. M., Chandra, M., Solaro, R. J., Umeda, P. K., and Cheung, H. C. (1999) J. Biol. Chem. 274, 31382–31390
10. Meir, A. J., Solaro, R. J., and Perry, S. V. (1980) Biochem. J. 185, 505–513
11. Krudy, G. A., Kleerekoper, Q., Guo, X., Howarth, J. W., Solaro, R. J., and Rosevear, P. R. (1994) J. Biol. Chem. 269, 23731–23735
12. Gasmi-Seabrook, G. M., Howarth, J. W., Finley, N., Abusamhadneh, E., Gaponenko, V., Brito, R. M., Solaro, R. J., and Rosevear, P. R. (1999) Biochemistry 38, 8315–8322
13. Vassylyev, D. G., Takeda, S., Nakatsuki, S., Maeda, K., and Maeda, Y. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 4847–4852
14. Herzberg, O., and James, M. N. (1985) Nature 313, 653–659
15. Olah, G. A., and Trewhella, J. (1994) Biochemistry 33, 12900–12906
16. Olah, G. A., Rokop, S. E., Wang, C. L., Blechner, S. L., and Trewhella, J. (1994) Biochemistry 33, 8223–8239
17. Stone, D. B., Timmins, P. A., Schneider, D. K., Krylova, I., Ramos, C. H., Reinach, F. C., and Mendelson, R. A. (1998) J. Mol. Biol. 281, 689–704
18. Finley, N., Abbott, M. B., Abusamhadneh, E., Gaponenko, V., Dong, W., Gasmi-Seabrook, G., Howarth, J. W., Rance, M., Solaro, R. J., Cheung, H. C., and Rosevear, P. R. (1999) FEBS Lett. 453, 107–112
19. Kay, L. E., Keifer, P., Saarinen, T. (1992) J. Am. Chem. Soc. 114, 10663–10665
20. Pervushin, K., Riek, R., Wider, G., and Wuthrich, K. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 12366–12371
21. Mandel, A. M., Akke, M., and Palmer, A. G., III (1995) J. Mol. Biol. 246, 144–163
22. Dotsch, V., Wider, G., Siegel, G., and Wuthrich, K. (1995) FEBS Lett. 366, 6–10
23. Hibbard, L. S., and Talinsky, A. (1978) Biochemistry 17, 5460–5468
24. Konno, T., Kanatari, Y. O., Kataoka, M., and Akasaka, K. (1997) Protein Sci. 6, 2242–2249
25. Mok, Y. K., Kay, C. M., Kay, L. E., and Forman-Kay, J. (1999) J. Mol. Biol. 289, 139–145
26. Herzberg, O., Moult, J., and James, M. N. (1986) Ciba Found. Symp. 122, 120–144
27. Krudy, G. A., Kleerekoper, Q., Guo, X., Howarth, J. W., Solaro, R. J., and Rosevear, P. R. (1995) Biochemistry 34, 13343–13352
28. Ding, X. L., Akella, A. B., Su, H., and Gulati, J. (1994) Protein Sci. 3, 2089–2096
29. Ramakrishnan, S., and Hitchcock-DeGregori, S. E. (1996) Biochemistry 35, 15151–15152
30. Ramakrishnan, S., and Hitchcock-DeGregori, S. E. (1995) Biochemistry 34, 16769–16796
31. Sheng, Z. L., Francois, J. M., Hitchcock-DeGregori, S. E., and Potter, J. D. (1991) J. Biol. Chem. 266, 5711–5715
32. Caflisch, A., and Karpus, M. (1999) Struct. Fold. Des. 7, 477–488
33. Abbott, M. B., Dvoretzky, A., Gaponenko, V., and Rosevear, P. R. (2000) FEBS Lett. 469, 168–172
34. Robertson, S. P., Johnson, J. D., Holroyde, M. J., Kranias, E. G., Potter, J. D., and Solaro, R. J. (1982) J. Biol. Chem. 257, 260–263
35. Chandra, M., Montgomery, D. E., and Solaro, R. J. (1999) J. Mol. Cell. Cardiol. 31, 867–880
36. Zan, H., Zhan, J., and Potter, J. D. (1995) J. Biol. Chem. 270, 36773–36780
37. Kohli, C., al-Hillawi, E., Day, F. M., and Ruegg, J. C. (1995) FEBS Lett. 377, 131–134
38. Solaro, R. J., and Baric, H. M. (1998) Circ. Res. 83, 471–486