Effects of Troponin I Phosphorylation on Conformational Exchange in the Regulatory Domain of Cardiac Troponin C*

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Conformational exchange has been demonstrated within the regulatory domain of calcium-saturated cardiac troponin C when bound to the NH2-terminal domain of cardiac troponin I-(1–80), and cardiac troponin I-(1–80)DD, having serine residues 23 and 24 mutated to aspartate to mimic the phosphorylated form of the protein. Binding of cardiac troponin I-(1–80) decreases conformational exchange for residues 29, 32, and 34. Comparison of average transverse cross correlation rates show that both the NH2- and COOH-terminal domains of cardiac troponin C tumble with similar correlation times when bound to cardiac troponin I-(1–80). In contrast, the NH2- and COOH-terminal domains in free cardiac troponin C and cardiac troponin C bound cardiac troponin I-(1–80)DD tumble independently. These results suggest that the nonphosphorylated cardiac specific NH2 terminus of cardiac troponin I interacts with the NH2-terminal domain of cardiac troponin C.

Troponin C (TnC)1 is the Ca2+- binding component of the troponin complex that is required to confer Ca2+-sensitivity on the actin-myosin interaction. The troponin complex consists of three proteins: troponin I (TnI), which inhibits the actomyosin Mg2+-ATPase; TnC, which removes TnI inhibition triggering the Mg2+-ATPase; and TnT, which makes primary protein contacts with tropomyosin. The cardiac isoforms of TnC and TnI are known to interact in an antiparallel manner such that the COOH-terminal domain of TnC interacts with the NH2-terminal domain of TnI (5).

Conformational changes induced by Ca2+-binding to the NH2-terminal domain of skeletal TnC (sTnC) have been followed by both x-ray (6) and solution NMR (7). For the free skeletal TnC protein, Ca2+-binding results in a conformational change from the “closed” form to an “open” form exposing a patch of hydrophobic residues for interaction with TnI (7). Surprisingly, the Ca2+-bound NH2-terminal domain of cTnC was found to maintain a closed conformation (8). The solution structure of the Ca2+-saturated regulatory domain of human cTnC, cTnC-(1–91), in 9% trifluoroethanol, also reveals a “closed” conformation with little exposed hydrophobic surface (9). In addition, 15N relaxation studies on human cTnC-(1–91) suggested conformational exchange for Val28, Thr38, Lys39, Ile61, and Val64. The authors suggest the origin of this conformational exchange results from exchange between the closed form and a low population of the open form. However, monomer-dimer exchange could not unequivocally be ruled out.

To elucidate the role of phosphorylation of the cardiac-specific amino terminus of cTnI on full-length cTnC and to unequivocally demonstrate the presence of conformational exchange in the regulatory domain of full-length cTnC, we have probed the dynamics of cTnC free and bound to cTnI-(1–80) and cTnI-(1–80)DD. We show that residues within inactive Ca2+-binding site I undergo chemical exchange consistent with an equilibrium between closed and opened forms both in the presence and absence of the NH2-terminal cTnI domain. Based on chemical shift perturbation mapping and transverse relaxation rates, the cardiac-specific NH2 terminus of cTnI-(1–80) appears to make additional interactions with the regulatory domain of cTnC, which results in slowing down the exchange rate in the defunct Ca2+-binding site I as evidenced by the presence of 1H-15N cross peaks representing two distinct conformations for Leu29, Gln32, and Gly34. These interactions were not observed in the free protein or in the complex with cTnI-(1–80)DD.

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1 The abbreviations used are: Tn, troponin; cTnC, recombinant cardiac troponin C (desMet1-Ala2, C35S); sTnC, skeletal TnC; cTnI, cardiac troponin I; PKA, cyclic AMP-dependent protein kinase A; NOESY-HSQC, nuclear Overhauser effect spectroscopy-heteronuclear single quantum coherence; CSA, chemical shift anisotropy.

through 40, is naturally inactive (1). Residues 65 through 76 form the regulatory calcium binding site II. The cardiac isoform of cTnI is unique in that it contains an additional NH2-terminal extension of approximately 32 residues. This extension contains two adjacent serine residues that can be phosphorylated by PKA. Phosphorylation has been demonstrated to modulate myofilament sensitivity to Ca2+ by reducing Ca2+ affinity for the NH2-terminal regulatory site of cTnC (2). The reduction in the sensitivity of myofilament force development to Ca2+ induced by phosphorylation of cTnI can be mimicked by exchange of the native unphosphorylated cTnI with cTnI-DD, in which Ser23 and Ser24 have been mutated to Asp (3). Cardiac TnI-DD is also able to mimic effects of phosphorylation on the steady-state and pre-steady-state binding of cTnI to cTnC (4). Cardiac TnC and TnI are known to interact in an antiparallel manner such that the COOH-terminal domain of TnC interacts with the NH2-terminal domain of TnI (5).

Conformational changes induced by Ca2+-binding to the NH2-terminal domain of skeletal TnC (sTnC) have been followed by both x-ray (6) and solution NMR (7). For the free skeletal TnC protein, Ca2+-binding results in a conformational change from the “closed” form to an “open” form exposing a patch of hydrophobic residues for interaction with TnI (7). Surprisingly, the Ca2+-bound NH2-terminal domain of cTnC was found to maintain a closed conformation (8). The solution structure of the Ca2+-saturated regulatory domain of human cTnC, cTnC-(1–91), in 9% trifluoroethanol, also reveals a “closed” conformation with little exposed hydrophobic surface (9). In addition, 15N relaxation studies on human cTnC-(1–91) suggested conformational exchange for Val28, Thr38, Lys39, Ile61, and Val64. The authors suggest the origin of this conformational exchange results from exchange between the closed form and a low population of the open form. However, monomer-dimer exchange could not unequivocally be ruled out.

To elucidate the role of phosphorylation of the cardiac-specific amino terminus of cTnI on full-length cTnC and to unequivocally demonstrate the presence of conformational exchange in the regulatory domain of full-length cTnC, we have probed the dynamics of cTnC free and bound to cTnI-(1–80) and cTnI-(1–80)DD. We show that residues within inactive Ca2+-binding site I undergo chemical exchange consistent with an equilibrium between closed and opened forms both in the presence and absence of the NH2-terminal cTnI domain. Based on chemical shift perturbation mapping and transverse relaxation rates, the cardiac-specific NH2 terminus of cTnI-(1–80) appears to make additional interactions with the regulatory domain of cTnC, which results in slowing down the exchange rate in the defunct Ca2+-binding site I as evidenced by the presence of 1H-15N cross peaks representing two distinct conformations for Leu29, Gln32, and Gly34. These interactions were not observed in the free protein or in the complex with cTnI-(1–80)DD.

MATERIALS AND METHODS

Proteins—The single cysteine form of cTnC (cTnC35S) was used in the study (10). 15N and 1H labeling was accomplished using minimal medium containing 90% 2H2O and 1 g/liter 15NH4Cl. Cardiac troponin C was purified as described previously (5). Cardiac troponin I-(1–80) and cTnI-(1–80)DD were expressed as inclusion bodies purified in 8 M urea.2 All proteins were judged to be homogeneous by SDS-polyacrylamide gel electrophoresis and staining with Coomassie Brilliant Blue.

2 M. B. Abbott, unpublished data.
Complex formation for cTnC-cTnI-(1–80) and cTnC-cTnI-(1–80)DD was carried out as described previously (5). Samples of 1.0 mM concentration were prepared in 10% D2O, 20 mM Tris-d1 buffer (pH = 6.8), 150 mM potassium chloride, 10 mM Ca2+, 10 mM β-mercaptoethanol, and 10 mM dithiothreitol. All samples contained 0.2 mM leupeptin and 0.4 mM peflabloc to prevent protein degradation.

**NMR Spectroscopy**—All experiments were carried out on Varian Inova 600 or 800 MHz spectrometers. Amide 1H and 15N resonances of cTnC were obtained by means of heteronuclear triple resonance NMR experiments.2 To confirm the assignments for cTnC bound to cTnI-(1–80) and cTnI-(1–80)DD, 1H-15N NOESY-HSQC experiments with mixing times of 85 and 200 ms were performed at 800 MHz. The 1H R2 and dipolar CSA interference experiments were performed on Ca2+-saturated [1H,15N]cTnC free and bound to either cTnI-(1–80) or cTnI-(1–80)DD. For all experiments, the spectral widths in t1 and t2 dimensions were 2.00 and 15.62 kHz, respectively. The 1H carrier was set to the 15N frequency of the water resonance at 4.77 ppm. The number of transients used in R2 and Iauto transverse cross-correlation experiments was 16, while Icross transverse cross-correlation experiments were performed with 48 transients. 15N dipolar CSA interference experiments were performed as described by Kroenke et al. (12). Relaxation delays used for the transverse cross-correlation rate (τNC) measurements were 0.0320, 0.0534, 0.0748, 0.0961, and 0.1065 s. The R2 experiments were performed with recovery delays of 0.006, 0.032, 0.064, 0.096, 0.128, 0.160, 0.192, and 0.224 s as described previously (14). Double points were collected with recovery delays of 0.032, 0.128, and 0.192 s to estimate the uncertainties.

**Data Processing—**NMR data was processed using Felix 97.2 (MSI). Free induction decays were apodized with a skewed sine bell 90° function. Generally, peak heights were measured using Felix routines. For the transverse cross-correlation experiments Icross/Iauto, intensity ratios were normalized for the number of transients used in each experiment. Curve-fits for transverse relaxation and cross-correlation rates were performed using the CURVEFIT software (13).

**RESULTS**

To compare interactions of cTnI-(1–80) and cTnI-(1–80)DD with cTnC, 1H and 15N chemical shift differences of bound cTnC were analyzed. Chemical shift differences for amide 1H and 15N resonances observed between the two complexes are presented in Fig. 1. These chemical shift differences can be used to map to binding site of cTnI-(1–80) on the regulatory domain of cTnC. In addition, analysis of chemical shift differences between free cTnC and cTnC bound to cTnC-cTnI-(1–80) or cTnC-cTnI-(1–80)DD reveals that both proteins bind predominantly to the COOH-terminal domain of cTnC causing a conformational change, as was observed for cTnI-(33–80) binding to cTnC free and bound are observed. It is also apparent that Ser37 in both free cTnC and cTnC bound to cTnI-(1–80)DD, and free cTnC are depicted in Fig. 2. Not surprisingly, low R2 values for flexible NH2 and COOH termini of cTnC free and bound are observed. It is also apparent that Met85 through Thr93 in the linker region of cTnC free and cTnI bring new peaks representing the predominant conformation of cTnC bound to cTnI-(1–80) were chosen.

1H-15N correlation NMR spectroscopy was used to study the interactions of cTnI with TnC (Fig. 1A) and TnI-D (Fig. 1B). Chemical shift differences between Ca2+-saturated cTnC-cTnI-(1–80) and cTnC-cTnI-(1–80)DD. The horizontal lines in A and B represent the average chemical shift differences plus 1 S.D., 0.02 ± 0.02 ppm and 0.18 ± 0.17 ppm, respectively. Filled diamonds mark residues for which resonance assignments in 1H-15N correlation spectrum could not be confirmed due to a lack of sequential NOEs in NOESY-HSQC spectra. These residues are 6, 8, 10, 15, 17, 20, 24, 41, 44, 47, 48, 51, 58, 62, 64, 65, 75, 83, 85, 95, 96, 97, 101, 103, 118, 126, 132, 136, 150, and 154. Residues 52 and 54 are Pro. Cross-peaks for residues 28, 36, 37, 38, 39, and 40 are broadened beyond detection in 1H-15N correlation spectra of cTnC-cTnI-(1–80), and residues 38 and 39 are broadened beyond detection in 1H-15N correlation spectra of cTnC-cTnI-(1–80)DD. For residues 29, 32, and 34, chemical shifts of the highest intensity peaks representing the predominant conformation of cTnC bound to cTnI-(1–80) were chosen.

3 N. Finley, unpublished data.
4 Gasmis-Scabrook, G. M. C., Howarth, J. W., Finley, N., Absamadne, E., Gaponenko, V., Brito, R. M. M., Solaro, R. J., and Rosevear, P. R. (1999) Biochemistry, in press.

![Fig. 1. Absolute value amide nitrogen (A) and amide proton (B) chemical shift differences between Ca2+-saturated cTnC-cTnI-(1–80) and cTnC-cTnI-(1–80)DD. The horizontal lines in A and B represent the average chemical shift differences plus 1 S.D., 0.02 ± 0.02 ppm and 0.18 ± 0.17 ppm, respectively. Filled diamonds mark residues for which resonance assignments in 1H-15N correlation spectrum could not be confirmed due to a lack of sequential NOEs in NOESY-HSQC spectra. These residues are 6, 8, 10, 15, 17, 20, 24, 41, 44, 47, 48, 51, 58, 62, 64, 65, 75, 83, 85, 95, 96, 97, 101, 103, 118, 126, 132, 136, 150, and 154. Residues 52 and 54 are Pro. Cross-peaks for residues 28, 36, 37, 38, 39, and 40 are broadened beyond detection in 1H-15N correlation spectra of cTnC-cTnI-(1–80), and residues 38 and 39 are broadened beyond detection in 1H-15N correlation spectra of cTnC-cTnI-(1–80)DD. For residues 29, 32, and 34, chemical shifts of the highest intensity peaks representing the predominant conformation of cTnC bound to cTnI-(1–80) were chosen.](https://example.com/Figure1.png)
In the case of Leu29 and Gly34, mutual sequential cross-peaks for each residue have NOEs to the same amide protons. Thus, we are unable to unequivocally assign both cross-peaks to Glu32. Cross-peaks for Leu29, Glu32, and Gly34 are well resolved, and it is unlikely that the new peaks belong to some other unassigned amide protons experiencing extremely large chemical shift perturbations and having identical chemical shifts. It seems possible that cTnI-(1–80) binds to the NH2-terminal domain of cTnC and slows down chemical exchange in defunct Ca2+-binding site I, presumably changing the dynamic equilibrium between open and closed conformations.

On average, the \( R_2 \) values for the COOH-terminal domain of the free protein are smaller than those values for the NH2-terminal domain (Fig. 2C). This can be attributed to the fact that the NH2-terminal domain of cTnC is slightly larger than the COOH-terminal domain. The average \( R_2 \) value for the COOH-terminal domain, comprising residues 104 through 135, was 11.0 ± 1.9 s\(^{-1}\), while the average \( R_2 \) value for the NH2 terminus, comprising residues 14 through 23 and residues 41 through 80, was 14.6 ± 1.6 s\(^{-1}\). The linker region, the NH2 and COOH termini, and inactive calcium binding site I were excluded from this analysis on the basis of either increased mobility or possible conformational exchange in these regions.

When cTnC is bound to cTnI-(1–80)DD the average \( R_2 \) value (20.0 ± 1.9 s\(^{-1}\)) for the COOH-terminal domain is larger than the average \( R_2 \) value for the NH2-terminal domain (16.0 ± 1.8 s\(^{-1}\)) as shown in Fig. 2B. This difference could be explained by the fact that the NH2 terminus of cTnI binds to the COOH terminus of cTnC. However, for the cTnC-cTnI-(1–80) complex both the NH2- and COOH-terminal domains have similar average \( R_2 \) values, (30.3 ± 2.9 s\(^{-1}\)) and (31.6 ± 2.1 s\(^{-1}\)), respectively (Fig. 2A). A possible explanation of this phenomenon is that the NH2 terminus of the nonphosphorylated form of cTnI also makes contacts with the NH2-terminal domain of cTnC.

The two domains of cTnC which are connected by a flexible linker tumble independently, with different rotational correlation times, in both the free protein and in a complex with cTnI-(1–80)DD. This results in different 15N \( R_2 \) values for the two domains of cTnC. Binding of cTnI-(1–80) to both the NH2- and COOH-terminal domains of cTnC reduces the possibility of independent tumbling of the two domains, which results in more uniform rotational correlation times across the molecule and subsequently in similar 15N transverse relaxation rates. Interaction of cTnI-(1–80) with both domains of Ca2+-saturated cTnC does not preclude flexibility in the linker region as observed in the complex (Fig. 2A).

In general, the transverse cross-correlation rates repeat the same trend as \( R_2 \) values. However, for residues Val28, Ile36, and Ser37 the cross-correlation values are not increased like the \( R_2 \) relaxation rates in this region. Thus, the results of these experiments prove that residues adjacent to the calcium binding site I undergo conformational exchange in free cTnC and when bound to cTnI-(1–80)DD. Conformational exchange in Val28, Ile36, and Ser37 is slower when cTnC is bound to cTnI-(1–80), since these residues have broadened beyond detection. In support of this, multiple resonances for Leu29, Glu32, and Gly34 of cTnC were observed in the complex with cTnI-(1–80) (Fig. 3). Careful analysis of NOESY-HSQC spectra revealed that all cross-peaks observed for each residue have NOEs to the same amide protons. In the case of Leu29 and Gly34, mutual sequential NOEs are observed to Gly34 (Fig. 3A) and Asp33 (Fig. 3B), respectively. These results allow us to suggest that the new cross-peaks represent a different conformation for the defunct Ca2+-binding site I of cTnC. The resonance for Glu32 also appears to consist of multiple peaks, but H2N-H2N NOEs for Glu32 could not be observed in the recorded NOESY-HSQC spectra. Thus, we are unable to unequivocally assign both cross-peaks to Glu32. Cross-peaks for Leu29, Glu32, and Gly34 are well resolved, and it is unlikely that the new peaks belong to some other unassigned amide protons experiencing extremely large chemical shift perturbations and having identical chemical shifts. It seems possible that cTnI-(1–80) binds to the NH2-terminal domain of cTnC and slows down chemical exchange in defunct Ca2+-binding site I, presumably changing the dynamic equilibrium between open and closed conformations.

The results of our experiments demonstrate that chemical exchange does occur in the defunct Ca2+-binding site I in both intact free cTnC and cTnC bound to the NH2-terminal domain of cTnI. Thus, the regulatory domain of cTnC may be in equilibrium between a closed and open form both free in solution and when it is bound to the phosphorylated and nonphosphorylated forms of the NH2-terminal domain of cTnI. This argument is supported by the presence of conformational exchange as evidenced by differences in the \( R_2 \) values and transverse cross-correlation rates for Val28, Ile36, and Ser37 in the inactive COOH-terminal domain of the free protein as shown in Fig. 2C.
Ca\(^{2+}\) binding site I. Taken together, our studies suggest that when cTnI is phosphorylated, the regulatory domain of cTnC is destabilized, resulting in a decrease in Ca\(^{2+}\) affinity. This would explain the enhanced relaxation observed when cTnI is phosphorylated by PKA in response to \(\beta\)-adrenergic stimulation of the heart (17). Binding of the nonphosphorylated form of cTnI does not prevent chemical exchange in inactive calcium binding site I of cTnC. We propose, based on chemical shift mapping, \(R_1\) values, and the presence of multiple cross-peaks for certain residues in defunct Ca\(^{2+}\) binding site I that nonphosphorylated cTnI interacts with both domains of cTnC and increases chemical exchange presumably changing the dynamic equilibrium between open and closed conformations (8, 18). The fact that the transverse relaxation rates for the NH\(_2\)-terminal domain of cTnI-(33–80)DD are not increased upon binding cTnI-(1–80)DD provides strong evidence that the phosphorylated mient of cTnI binds only to the COOH terminus of cTnC. This interpretation is in agreement with the fact that there are no significant amide proton and amide nitrogen chemical shift differences in the NH\(_2\)-terminal domain of cTnC upon binding of cTnI-(1–80)DD. The crystal structure of sTnC complexed with a fragment of sTnI that corresponds to cardiac TnI-(33–80) clearly shows that sTnI interacts with both the NH\(_2\) and COOH termini of TnC (19). Specifically, the A-helix of sTnC makes several contacts with the NH\(_2\)-terminal portion of sTnI-(1–47) (19). We have shown recently that the solution structure of cTnC-(81–161) bound to cTnI-(33–80) is similar to that of sTnC bound to sTnI-(1–47). General agreement between sTnC and cTnC bound TnI structures suggest a common binding motif for the Ca\(^{2+}\)/Mg\(^{2+}\)-dependent interaction site in the TnI/TnC complex. It now appears that in the absence of phosphorylation, the Ca\(^{2+}\)/Mg\(^{2+}\)-dependent interaction site of cTnI makes additional contacts with the regulatory domain of cTnC similar to those interactions observed in the crystal structure for sTnCs/CsTnI-(1–47) (19). Data presented here are all consistent with this hypothesis. It is intriguing to speculate that loss of these interactions are responsible for a decrease in calcium affinity in Ca\(^{2+}\) binding site II of cTnC when cTnI is phosphorylated. However, the exact sequence of events in this process is not understood and requires further investigation.

This study provides the first structural and dynamic insight on the mechanism by which PKA phosphorylation of cTnI, in response to \(\beta\)-adrenergic stimulation of the myocyte, produces a decrease in the calcium affinity of the regulatory site in cTnC. The proposed mechanism utilizes the unique cardiac isofrom differences found in both TnC and TnI. Knowledge of this mechanism provides the basis for molecular approaches aimed at modifying cardiac muscle contraction.

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