Introduction of Negative Charge Mimicking Protein Kinase C Phosphorylation of Cardiac Troponin I

EFFECTS ON CARDIAC TROPOVIN C*

Natosha L. Finley‡ and Paul R. Rosevar§

From the Department of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati, College of Medicine, Cincinnati, Ohio 45267-0524

Protein kinase C phosphorylation of cardiac troponin, the Ca\(^{2+}\)-sensing switch in muscle contraction, is capable of modulating the response of cardiac muscle to a Ca\(^{2+}\) ion concentration. The N-domain of cardiac troponin I contains two protein kinase C phosphorylation sites. Although the physiological consequences of phosphorylation at Ser\(^{45}/\text{Ser}^{45}\) are known, the molecular mechanisms responsible for these functional changes have yet to be established. In this work, NMR was used to identify conformational and dynamic changes in cardiac troponin C upon binding a phosphomimetic troponin I, having Ser\(^{45}/\text{Ser}^{45}\) mutated to Asp. Chemical shift perturbation mapping indicated that residues in helix G were most affected. Smaller chemical shift changes were observed in residues located in the Ca\(^{2+}/\text{Mg}^{2+}\)-binding loops. Amide hydrogen/deuterium exchange rates in the C-lobe of troponin C were compared in complexes containing either the wild-type or phosphomimetic N-domain of troponin I. In the presence of a phosphomimetic domain, exchange rates in helix G increased, whereas a decrease in exchange rates for residues mapping to Ca\(^{2+}/\text{Mg}^{2+}\)-binding loops III and IV was observed. Increased exchange rates are consistent with destabilization of the Thr\(^{129}/\text{Asp}^{132}\) helix capping box previously characterized in helix G. The perturbation of helix G and metal binding loops III and IV suggests that phosphorylation alters metal ion affinity and inter-subunit interactions. Our studies support a novel mechanism for protein kinase C signal transduction, emphasizing the importance of C-lobe Ca\(^{2+}/\text{Mg}^{2+}\)-dependent troponin interactions.

Troponin and tropomyosin form the Ca\(^{2+}\)-sensitive switch that regulates striated muscle contraction. Troponin is a ternary assembly of proteins composed of the Ca\(^{2+}\)-binding subunit troponin C (TnC),\(^1\) the inhibitory subunit troponin I (TnI), and the tropomyosin-binding protein troponin T (TnT) that anchors troponin to the thin filament. Troponin C, a member of the EF-hand family of Ca\(^{2+}\)-binding proteins, contains two globular domains connected by a linker. Each domain of TnC contains two EF-hand or Ca\(^{2+}\)-binding motifs. The N-lobe contains two lower affinity Ca\(^{2+}\)-binding motifs, sites I and IV, which also bind Mg\(^{2+}\) with lower affinity. Interactions between the C-lobe of TnC and the N-domain of TnI form the Ca\(^{2+}/\text{Mg}^{2+}\)-dependent TnCIvTnI interaction site. In addition, the C-lobe of TnC also interacts tightly with the C terminus of cardiac troponin T (2). These interactions form the core of the troponin complex, tethering all three subunits throughout the contraction cycle. A variety of effectors can modulate the frequency and intensity of myocardial contraction by charge modification upon the phosphorylation of TnC and cardiac troponin T. In the heart, phosphorylation of TnC appears to be of particular importance in cardiac hypertrophy and failure (3–5). Cardiac TnC can be phosphorylated by protein kinase A at Ser\(^{23}\) and Ser\(^{24}\) (6). β-Adrenergic stimulation leads to protein kinase A phosphorylation at Ser\(^{23}\) and Ser\(^{24}\) of TnC, enhancing relaxation by decreasing the Ca\(^{2+}\) affinity at site II (7, 8). Cardiac TnC can also be phosphorylated by PKC at Ser\(^{43}\), Ser\(^{45}\), and Thr\(^{144}\) (9). Phosphorylation at Ser\(^{43}\) and Ser\(^{45}\) is known to decrease maximal actomyosin Mg\(^{2+}\)ATPase activity, Ca\(^{2+}\) sensitivity, and cross-bridge binding to the thin filament (4, 9–11). These biochemical changes lead to maladaptive growth and diminished contractility, culminating in end-stage heart failure (5). Although it is clear that PKC phosphorylation of cardiac troponin modulates contraction in response to hemodynamic stressors such as hypertension and myocardial infarction, the molecular mechanisms of this modulation remain unknown.

The effects of PKC phosphorylation at Ser\(^{43}\) and Ser\(^{45}\) of TnC can be mimicked, both in vitro and in vivo, by the introduction of negative charge at positions 43/45 (3, 4, 11). Serine

---

\(^1\) The abbreviations used are: TnC, troponin C (Ca\(^{2+}\)-binding); TnI, troponin I (inhibitory); cTnC, recombinant cardiac troponin C (desMet\(^1\)-Ala\(^3\), C35S); CcTnC, recombinant mouse cardiac troponin C with residues 32–80 with the N-terminal Met cleaved during expression and purification; Ser\(^{45}\) and Ser\(^{45}\) mutated to Asp; NcTnI, recombinant mouse TnI corresponding with lower affinity at site II (7, 8). Cardiac TnC can also be phosphorylated by PKC at Ser\(^{43}\), Ser\(^{45}\), and Thr\(^{144}\) (9). Phosphorylation at Ser\(^{43}\) and Ser\(^{45}\) is known to decrease maximal actomyosin Mg\(^{2+}\)ATPase activity, Ca\(^{2+}\) sensitivity, and cross-bridge binding to the thin filament (4, 9–11). These biochemical changes lead to maladaptive growth and diminished contractility, culminating in end-stage heart failure (5). Although it is clear that PKC phosphorylation of cardiac troponin modulates contraction in response to hemodynamic stressors such as hypertension and myocardial infarction, the molecular mechanisms of this modulation remain unknown.

* This work is supported in part by National Institutes of Health Grant AR44324 and United States Department of Defense Grant ARO MURI DAAD 19-02-1-0227 (to P. R. R.). 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.

The atomic coordinates and structure factors (code 1SCV) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

‡ Supported by National Institutes of Health Training Grant 5T32 HL07382.

§ To whom correspondence should be addressed: Dept. of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati, College of Medicine, 231 Bethesda Ave., Cincinnati, OH 45267. Tel.: 513-558-3370; Fax: 513-558-8474; E-mail: rosevar@proto.med.uc.edu.

This paper is available online at http://www.jbc.org

Received for publication, July 22, 2004, and in revised form, September 30, 2004

Published, JBC Papers in Press, October 14, 2004, DOI 10.1074/jbc.M408304200

Vol. 279, No. 52, Issue of December 24, pp. 54833–54840, 2004

Printed in U.S.A.

Published by the American Chemical Society

© 2004 American Chemical Society
residues 43/45 of cTnI are at the N terminus of an amphiphilic α-helix that binds in a hydrophobic cleft in the C-lobe of cTnC (2). Stability of the hydrophobic core in CcTnC is largely governed by metal binding at site III (12). The exchange of Ca$^{2+}$ for Mg$^{2+}$ in Ca$^{2+}$/Mg$^{2+}$-binding sites III and IV resulted in a partial closure of the hydrophobic binding cleft around site IV, allowing the possibility that Ca$^{2+}$/Mg$^{2+}$ exchange can modulate contraction via Ca$^{2+}$/Mg$^{2+}$-dependent cTnC/CcTnI interactions (13, 14). However, even absent excess Ca$^{2+}$, Mg$^{2+}$ did not readily displace Ca$^{2+}$ in the C-lobe of cTnC bound to NcTnI (14).

To define the structural consequences resulting from cTnI phosphorylation at Ser$^{43}$/Ser$^{45}$ by PKC, we have utilized solution NMR to identify residues in cTnC that are important in transmission of the phosphorylation signal. Backbone resonance assignments in Ca$^{2+}$/Mg$^{2+}$-loaded [13C,15N]CcTnC bound to NcTnI(S43D/S45D) were assigned and used for secondary structure determination. The phosphorylation mimetics did not significantly alter the secondary structural elements of the paired Ca$^{2+}$/Mg$^{2+}$-binding motifs in CcTnC. Residues in CcTnC important for transmission of the phosphorylation signal were identified using chemical shift perturbation mapping and amide proton exchange. The phosphorylation mimetics induced localized conformational/dynamic perturbations in the N terminus of helix G and Ca$^{2+}$/Mg$^{2+}$-binding loops III and IV. Specifically, the presence of a negative charge at Ser$^{43}$/Ser$^{45}$ of cTnI was found to destabilize the Thr$^{129}$–Asp$^{132}$ N-cap in helix G of CcTnC. Perturbation of N-cap interactions in helix G have been shown previously to decrease metal ion affinities at site III (15). These findings support a mechanism for PKC modulation of cardiac contractility wherein the introduction of negative charge at Ser$^{43}$/Ser$^{45}$ of cTnI results in altered C-lobe metal ion affinities and perturbation of Ca$^{2+}$/Mg$^{2+}$-dependent protein-protein interactions. Presumably, these changes are transmitted to other regulatory and switch regions within cardiac troponin. These changes establish a role for the Ca$^{2+}$/Mg$^{2+}$-dependent cTnC/CcTnC interaction in transmitting the phosphorylation signal and modulating Ca$^{2+}$ sensitivity.

MATERIALS AND METHODS

Recombinant Protein Expression and Purification—Isotopically enriched and unlabelled recombinant proteins were expressed, purified, and quantified as described (16, 17).

Complex Formation and Resonance Assignment—Samples of Ca$^{2+}$-saturated [1H,15N]CcTnC or [13C,15N]CcTnC were combined in equimolar amounts with either cTnI(S43D/S45D) or NcTnI(S43D/S45D) to form stable binary complexes as described previously (16). Binary complexes were judged 1:1 by native gel electrophoresis in the presence of 10 mM CaCl$_2$ and by sensitivity-enhanced 1H-15N HSQC spectra (16). Samples for NMR were –1 mM in Ca$^{2+}$-saturated protein in 20 mM Tris$_2$-HCl, pH 6.8, 100 mM KCl, 5 mM dithiothreitol, 10 mM CaCl$_2$, and 10% D$_2$O. Complete EDTA-free protease inhibitor mixture (Roche Applied Science) was used to prevent protein degradation during NMR analysis.

NMR Methodology—Experiments were collected at 40 °C using 400, 600, and 800 MHz Varian Inova spectrometers equipped with pulse-field gradient units and triple resonance probes. Acquisition parameters for heteronuclear multidimensional NMR experiments and chemical shift referencing details can be found in Gasmi-Seabrook et al. (17). Inter-residue NOEs obtained from 13N-edited nuclear Overhauser effect spectroscopy-HSQC experiments at mixing times of 70 and 150 ms were used to confirm consecutive assignments. 1H-15N transverse relaxation-optimized spectroscopy spectra of the intact binary complex [1H,15N]CcTnC-CcTnI(S43D/S45D) were acquired with 2048 points in the indirect dimension, 64 points in the indirect dimension, and 320 scans per increment. Felix 2000 was employed to process and analyze NMR data. Amide proton chemical shift differences between Ca$^{2+}$-loaded [1H,15N]CcTnC and [13C,15N]CcTnC were measured by subtrating exchange rates for each residue in CcTnC-NcTnI from the same residue in CcTnC-NcTnI(S43D/S45D).

RESULTS AND DISCUSSION

NMR Signal Assignment and Secondary Structure—Changing a phosphorylation site to a negatively charged residue such as Asp or Glu can often be used to mimic phosphorylation and facilitate biophysical studies. Incorporation of a negative charge at Ser$^{43}$/Ser$^{45}$ by mutation was shown to provide functional mimetics for PKC phosphorylation of cTnI (4, 18). A comparison between cTnI and cTnI(S43D/S45D) bound to Ca$^{2+}$-loaded [1H,15N]CcTnC was made. Chemical shift perturbation mapping was used to monitor conformational changes in cTnC induced by the introduction of a negative charge at Ser$^{43}$/Ser$^{45}$ of cTnI in the intact binary complex. Amide proton chemical shift differences between Ca$^{2+}$-loaded [1H,15N]CcTnC-cTnI(S43D/S45D) and [1H,15N]CcTnC-cTnI are shown in Fig. 1A. The majority of chemical shift perturbations induced by the mutation of Ser$^{43}$/Ser$^{45}$ to Asp were localized to the C-lobe of cTnC, with residues in helix G experiencing the largest chemical shift changes (Fig. 1A). Small amide proton chemical shift perturbations were observed for Gly$^{69}$, Gly$^{70}$, and Ser$^{69}$ in the N-lobe of cTnC (Fig. 1A). These residues are located in the regulatory Ca$^{2+}$-binding site (site II). Titration of cTnC with a cTnI peptide phosphorylated at Ser$^{43}$/Ser$^{46}$ was also found to induce small amide proton chemical shift perturbations in N-lobe residues corresponding to Gly$^{63}$, Val$^{72}$, and Val$^{79}$ (19). Chemical shift perturbations may reflect charge-induced changes in local electrostatic interactions or changes in protein structure. It is unlikely that N-lobe chemical shift perturbations result from local electrostatic interactions, because these residues are >15 Å from the sites of cTnI phosphorylation in the core cardiac troponin structure (2).

To examine in more detail conformational perturbations induced in the C-lobe of cTnC by PKC phosphorylation at Ser$^{43}$/Ser$^{45}$ of cTnI, we have studied a model phosphomimetic complex, CcTnC-NcTnI(S43D/S45D). NMR resonance assignments and solution structures for both Mg$^{2+}$- and Ca$^{2+}$-loaded CcTnC in the CcTnC-NcTnI complex are available (13). Comparison of C-lobe chemical shift perturbations in CcTnC-CcTnI(S43D/S45D) and CcTnC-NcTnI(S43D/S45D) demonstrate that CcTnC-NcTnI(S43D/S45D) provides a suitable model for PKC phosphorylation-induced structural changes in the Ca$^{2+}$/Mg$^{2+}$-dependent cTnC interaction site (Fig. 1). This finding is consistent with the core cardiac troponin x-ray structure showing that NcTnI primarily makes contacts with the C-lobe of cTnC (2). Phosphorylation sites Ser$^{43}$/Ser$^{45}$ in cTnI are located at the N terminus of helix H1 in cTnI, corresponding to residues 43–79 (2). Residues 43–65 of cTnI bind to the hydrophobic cleft in CcTnC (2).

Two dimensional 1H-15N HSQC NMR spectroscopy was used to monitor individual amide resonances of residues in the C-lobe of cTnC upon complex formation with NcTnI(S43D/S45D). Only a single set of bound CcTnC amide resonances were observed as expected for high affinity binding (K$_D$ < 1 μM) in the slow exchange regime. These results are consistent with isothermal titration microcalorimetry showing that bisphosphorylation at Ser$^{43}$/Ser$^{45}$ of cTnI (1–64) had little effect on.$^*$
cTnC affinity ($K_a \sim 1 \times 10^{-7} \mu M$) (20).

Backbone chemical shift assignments for Ca$^{2+}$-loaded $^{13}$C,$^{15}$N-CcTnC bound to NcTnI(S43D/S45D) were obtained using standard triple resonance assignment strategies (13, 17). The resonance assignment strategy relied primarily on $(H\beta)$C$_\alpha$C$_\beta$(CO)NNH, HNC$_\alpha$C$_\beta$, HNC$_\alpha\beta$, and HNCO triple resonance experiments. Assignments were obtained for 75 of the 81 CcTnC amino acid residues. Backbone chemical shifts for the C$_\alpha$, C$_\beta$, C$_\gamma$, and H$_\beta$ resonances were used to determine chemical shift index values for each residue in Ca$^{2+}$-loaded CcTnC bound to NcTnI(S43D/S45D) (21). The chemical shift-index determined secondary structure showed a characteristic paired EF-hand motif with four helices spanning residues 93–103 (E), 114–123 (F), 130–139 (G), and 150–158 (H), as well as two short β-strands extending from residues 111–113 and 147–149 (Fig. 1B). The overall secondary structure is analogous to that previously determined for Ca$^{2+}$- and Mg$^{2+}$-loaded CcTnC bound to NcTnI (13). The similarity in secondary structure for CcTnC bound to NcTnI and NcTnI(S43D/S45D) justified the use of available C-lobe cTnC structures for interpreting changes in chemical shifts and H/D exchange rates upon the introduction of a negative charge at the two PKC phosphorylation sites (2, 13).

**Chemical Shift Mapping**—Chemical shifts are sensitive to the local environment and can be used to monitor subtle structural changes. We have shown previously that amide chemical shifts for cTnC are extremely sensitive to the small conformational changes that occur upon cAMP-dependent protein kinase A phosphorylation of cTnI (22, 23). Chemical shifts for the $^1$H$_\alpha$, $^{15}$N$_\alpha$, $^{13}$C$_\alpha$, and $^{13}$C$_\beta$ resonances in CcTnC-NcTnI (17) were
used for a residue-by-residue comparison with the chemical shifts obtained in CcTnC-NcTnI(S43D/S45D). Perturbations in the combined $^1$H/$^{15}$N chemical shifts are plotted on the structure of Ca$^{2+}$-loaded CcTnC bound to NcTnI (Fig. 2).

The largest $^1$H/$^{15}$N chemical shift perturbations were observed in the N terminus of helix G, Ile$^{128}$ and Thr$^{129}$, and in helix E, Glu$^{95}$ (Fig. 1B). These residues cluster around the N-cTnI-binding site (2). Threonine 129 is the N-cap residue in the N-terminal helix capping box of helix G in both Ca$^{2+}$- and Mg$^{2+}$-loaded CcTnC-NcTnI complexes (13, 17). In both complexes, the side-chain hydroxyl of Thr$^{129}$ hydrogen bonds to the amide of Asp$^{132}$, and the Asp side-chain, in turn, forms a hydrogen bond with the amide of Thr$^{129}$ (13, 17). The upfield
PKC Phosphorylation of Cardiac Troponin I

Amide proton chemical shift for Thr^{129} in the phosphomimetic complex is consistent with destabilization of the hydrogen bond between the amide of Thr^{129} (N-cap residue) and the carboxylate side-chain of Asp^{132} (N-cap residue). The \(^3\)N_{C\alpha}-C\beta\) coupling constant for Thr^{129} decreased slightly from 9 Hz (17) to 8.3 Hz in the phosphomimetic complex, consistent with a decrease in the average \(\phi\) backbone torsion angle. Changes in the amide proton chemical shift and the \(^3\)N_{C\alpha}-C\beta\) coupling constant for Thr^{129} are consistent with destabilization of the N-terminal helix G capping box. In addition, upfield amide nitrogen chemical shifts are also observed for Ile^{128} and Thr^{129} in the phosphomimetic complex (data not shown). Hydrophobic interactions between the N\(^\prime\) residue (Ile^{128}) and the N4 residue (Ile^{133}) stabilize the N-cap box (24). Such stabilizing interactions would be expected to deshield the amide nitrogen resonances of Ile^{128} and Thr^{129}. However, destabilization of the hydrogen-bonding network in the Thr^{129}→Asp^{132} N-cap box would reverse this effect, shifting the amide nitrogen resonances upfield as observed in the phosphomimetic complex.

Chemical shift perturbations were also observed in \(^1\)HCO and \(^1\)HCS\beta\) resonances of CcTnC residues upon the introduction of a negative charge at Ser\(^43\)/Ser\(^45\) in cTnI. Residues showing the largest \(^1\)HCO/\(^1\)HCS\beta\) chemical shift changes are located in helix E (Leu-97, Phe-101, and Phe\(^\prime\)104), helix F (Phe\(^\prime\)117 and Leu\(^\prime\)121), and helix H (Phe\(^\prime\)153, Leu\(^\prime\)154, and Phe\(^\prime\)156) (Fig. 2B). Hydrophobic and polar residues identified by chemical shift indexing form contiguous surfaces that define the binding interface between CcTnC and NcTnI within the Ca\(^2\)/Mg\(^2\)-dependent cTnC-NcTnI interaction site (Fig. 2C). Residues 43–65 of cTnI form an amphiphilic \(\alpha\)-helix that binds to the C-lobe hydrophobic crevice via multiple polar and Van der Waals interactions (2), similar to the interactions observed in the skeletal sTnC-sTnI (1–47) complex (25).

**Hydrogen/Deuterium Exchange—**Changes in local dynamic behavior have also been utilized to identify residues that undergo conformational change accompanying protein binding. Hydrogen/deuterium exchange allows characterization of changes in global thermodynamic stability and local conformational motion with exchange time constants on the order of minutes to days. Whereas chemical shifts report on the magnetic environment of nuclei, H/D exchange kinetics provides information about backbone dynamics, conformation, and proton-solvent interactions. Comparison of H/D exchange rates provides a mechanism to assess the effects of the PKC phosphorylation mimetics on conformational fluctuations in CcTnC. To this end, amide proton H/D exchange rates in \[^{15}\)N\)CcTnC bound to either NcTnI or NcTnI(S43D/S45D) were measured at 25 °C. At temperatures >25 °C, many of the exchange rates were too fast to reliably measure, whereas temperatures <25 °C resulted in the broadening of \(^1\)H-\(^1\)^\(^5\)N correlations. Hydrogen/deuterium exchange rates could be monitored for 59 of the 75 assigned \(^1\)H-\(^1\)^\(^5\)N correlations in the HSQC spectrum of CcTnC. The remaining residues were excluded from analysis because of weak signal intensity and/or resonance overlap. The amide proton exchange kinetics could be classified into five categories, namely rapidly exchanging (within the first 20 min), fast (\(k_{\text{ex}} > 8.5 \times 10^{-2} \text{ min}^{-1}\)), moderately fast (8.5 \(\times 10^{-3} \text{ min}^{-1} < k_{\text{ex}} < 8.5 \times 10^{-2} \text{ min}^{-1}\)), slow (2.5 \(\times 10^{-3} \text{ min}^{-1} < k_{\text{ex}} < 8.5 \times 10^{-3} \text{ min}^{-1}\)), and very slow (\(k_{\text{ex}} < 2.5 \times 10^{-3} \text{ min}^{-1}\)) (Table I). Rapidly exchanging amide correlations, disappering within the first 20 min of exchange in both complexes, were assigned to residues Asp\(^87\), Asp\(^88\), Ser\(^89\), Lys\(^90\), Gly\(^91\), Lys\(^92\), Thr\(^93\), Gly\(^94\), Ser\(^98\), Asp\(^99\), Arg\(^102\), Met\(^120\), Thr\(^124\), Gly\(^125\), Gly\(^126\), Thr\(^127\), Gly\(^130\), Asn\(^144\), Glu\(^152\), and Gly\(^158\). Most of these correspond to residues that are located in unstructured or mobile loop regions (13). The time course of H/D exchange for 28 residues could be followed in both complexes and used to calculate exchange rates (Table I). The introduction of a negative charge mimicking PKC phosphorylation in NcTnI was found to both increase and decrease the amide proton H/D exchange rate for each residue in CcTnC-NcTnI from the measured H/D exchange rate for the same residue in CcTnC-NcTnI(S43D/S45D). A negative value indicates H/D exchange decreased in the phosphomimetic complex. A positive value indicates that H/D exchange increased in the phosphomimetic complex.

| Residue | NcTnI-H/D exchange | NcTnI(S43D/S45D)-H/D exchange | Δk \(10^{-2} \text{ min}^{-1}\) |
|---------|---------------------|-------------------------------|-------------------------------|
| Glu\(^92\) | 3.0 ± 0.6           | 7.2 ± 1.2                     | 4.2 ± 1.3                     |
| Met\(^103\) | 1.2 ± 0.2           | 5.4 ± 0.2                     | -4.2 ± 1.2                    |
| Phe\(^104\) | 68.4 ± 18.6        | 82.2 ± 0.6                    | 13.8 ± 18.6                   |
| Asn\(^107\) | 19.8 ± 1.8         | 7.7 ± 0.4                     | -12.6 ± 1.8                   |
| Ala\(^108\) | 20.4 ± 0.6         | 46.2 ± 0.6                    | -23.4 ± 0.8                   |
| Gly\(^110\) | 6.0 ± 1.2           | 2.4 ± 0.5                     | -3.6 ± 1.3                    |
| Tyr\(^111\) | 105.0 ± 11.4       | 141.0 ± 22.0                  | 36.0 ± 25.5                   |
| Asp\(^115\) | 135.0 ± 29.4       | 53.4 ± 7.8                    | -81.6 ± 30.4                  |
| Gly\(^118\) | 56.4 ± 1.8         | 42.9 ± 0.6                    | -13.8 ± 1.8                   |
| Gly\(^122\) | 15.0 ± 0.6         | 126.0 ± 0.3                   | -23.4 ± 0.7                   |
| Thr\(^129\) | 6.6 ± 8.4          | 41.4 ± 3.0                    | 34.8 ± 8.9                    |
| Asp\(^132\) | 9.0 ± 0.5           | 16.2 ± 0.6                    | 7.2 ± 0.7                     |
| Ile\(^133\) | 4.2 ± 0.3           | 15.6 ± 0.6                    | 11.4 ± 0.7                    |
| Glu\(^134\) | 3.6 ± 0.3           | 6.0 ± 1.2                     | 2.4 ± 1.2                     |
| Gly\(^135\) | 4.8 ± 2.2           | 8.4 ± 0.5                     | 3.6 ± 0.5                     |
| Met\(^137\) | 9.6 ± 2.4           | 34.8 ± 4.8                    | 25.2 ± 5.4                    |
| Lys\(^138\) | 2.4 ± 1.8           | 3.6 ± 0.6                     | 1.2 ± 1.9                     |
| Asp\(^139\) | 34.2 ± 3.0         | 34.8 ± 2.4                    | 0.6 ± 3.8                     |
| Gly\(^140\) | 15.0 ± 1.8         | 6.0 ± 0.5                     | -8.4 ± 1.9                    |
| Asp\(^141\) | 1.8 ± 0.6           | 3.0 ± 0.0                     | 1.2 ± 0.6                     |
| Lys\(^142\) | 0.6 ± 0.1           | 0.1 ± 0.2                     | -0.5 ± 0.6                    |
| Asn\(^143\) | 18.0 ± 0.0         | 7.2 ± 0.2                     | -10.8 ± 0.6                   |
| Gly\(^146\) | 10.8 ± 0.6         | 4.8 ± 0.2                     | -6.0 ± 0.6                    |
| Arg\(^147\) | 1.8 ± 0.5           | 1.8 ± 0.6                     | 0.0 ± 0.6                     |
| Glu\(^149\) | 4.2 ± 2.4           | 2.4 ± 0.2                     | -1.8 ± 0.4                    |
| Met\(^157\) | 6.0 ± 0.4           | 2.4 ± 0.3                     | -3.6 ± 0.5                    |
| Lys\(^158\) | 33.0 ± 3.6         | 14.4 ± 0.4                    | -18.6 ± 0.7                   |
| Glu\(^161\) | 41.4 ± 5.0         | 7.1 ± 2.4                     | 30.0 ± 3.8                    |
occupy position 3 within Ca\(^{2+}\)/Mg\(^{2+}\) binding loops III and IV, respectively. The amide proton of the residue at position 3 is known to hydrogen bond with the side-chain carboxylate of Asp at position 12 (26). The decrease in amide proton H/D exchange is consistent with increased hydrogen bonding interactions between the amide protons of Asn\(^{107}\) and Asn\(^{113}\) and the carboxylate groups of Glu\(^{116}\) and Glu\(^{152}\), respectively. Decreased amide H/D exchange in the PKC phosphomimetic complex is also observed for Ala\(^{108}\), Gly\(^{110}\), Asp\(^{113}\), and Glu\(^{115}\) in site III and for Gly\(^{146}\) in site IV (Table I and Fig. 4). The side chain of Asp\(^{113}\), located at the ninth position in Ca\(^{2+}\)/Mg\(^{2+}\) binding loop III, directly coordinates the bound metal ion. Amide protons of Gly\(^{110}\) and Gly\(^{146}\), located at the sixth position in Ca\(^{2+}\)/Mg\(^{2+}\) binding loop III and IV, respectively, hydrogen bond to the carboxylate side chain of the conserved Asp residues at position 1 (12). Decreased H/D exchange for residues within Ca\(^{2+}\)/Mg\(^{2+}\) binding loops III and IV is consistent with the stabilization of intra-loop hydrogen bonding interactions and the compaction of the metal binding loops in the phosphomimetic complex. These changes suggest that phosphorylation of NcTnI alters the conformation of Ca\(^{2+}\)/Mg\(^{2+}\)-binding sites III and IV, possibly resulting in altered metal ion affinity and Ca\(^{2+}\)/Mg\(^{2+}\) exchange.

**Summary**—The structural consequences of PKC phosphorylation at Ser\(^{43}/\)Ser\(^{45}\) of cTnI on cTnC have been examined using chemical shift mapping and H/D exchange. To facilitate structural studies, PKC phosphorylation mimetics of cTnI having Ser\(^{43}/\)Ser\(^{45}\) mutated to Asp were utilized. The overall picture obtained from chemical shift mapping shows structural perturbations predominately localized to the C-lobe of cTnC, with smaller N-lobe perturbations around Ca\(^{2+}\)-binding site II (Fig. 1). Distances between N-lobe Ca\(^{2+}\)-binding sites and Ser\(^{43}/\)Ser\(^{45}\) of cTnI in the core cardiac troponin structure (2) suggest that chemical shift perturbations in Ca\(^{2+}\)-binding site II are the result of long range effects as opposed to direct binding interactions.

Recently, measurement of fluorescence resonance energy transfer distance distributions from a single cTnC donor/acceptor pair in cTnC-cTnI and cTnC-cTnI(S43E/S45E, T144E) showed that the introduction of a negative charge in cTnI alters N-lobe conformational equilibria (3). Previously we showed that amide chemical shifts could be utilized to monitor conformation equilibria between open and closed N-lobe substates (16). We see no evidence that the introduction of a negative charge at Ser\(^{43}/\)Ser\(^{45}\) of cTnI significantly alters conformational equilibria in the N-lobe of cTnC (Fig. 1). It is likely that the N-lobe conformational change detected by fluorescence resonance energy transfer analysis (3) results from the additional negative charge at Thr\(^{144}\) of cTnI. This probability is consistent with N-lobe conformational changes detected by NMR chemical shift analysis of cTnC binding to a cTnI regulatory peptide.
phosphorylated at Thr\textsuperscript{144} (19). Taken together, these results help clarify the molecular consequences of PKC phosphorylation at Ser\textsuperscript{43, 45}, and Thr\textsuperscript{144} in cTnI on Ca\textsuperscript{2+}-bound cTnC. Phosphorylation of Ser\textsuperscript{43, 45} in cTnI induces conformational perturbations in the C-lobe of cTnC containing Ca\textsuperscript{2+}/Mg\textsuperscript{2+}-binding sites III and IV, whereas phosphorylation at Thr\textsuperscript{144} directly alters conformational equilibria in the N-lobe of cTnC containing Ca\textsuperscript{2+}-binding site II.

A combination of chemical shift mapping and H/D exchange was used to examine conformational perturbations in the Ca\textsuperscript{2+}/Mg\textsuperscript{2+}-dependent cTnC-cTnI interaction site induced by the introduction of a negative charge at Ser\textsuperscript{43, 45} of cTnI. Chemical shift mapping identified structural perturbations in CcTnC residues lining the NcTnI hydrophobic binding cleft (Fig. 2). Amide hydrogen/deuterium exchange results are consistent with the destabilization of N-terminal helix G-capping interactions and the conformational perturbation of Ca\textsuperscript{2+}/Mg\textsuperscript{2+}-binding sites III and IV (Fig. 4). Whereas metal binding at site III is primarily responsible for stabilizing the hydrophobic core (12), helix-capping interactions are known to increase domain stability and accelerate folding (28). Destabilization of N-cap interactions in helices C and G by mutation have been shown to decrease Ca\textsuperscript{2+}-binding affinity in TnC (15, 27). Thus, weakening of the hydrogen-bonding lattice in helix G would be expected to alter metal ion affinity and Ca\textsuperscript{2+}/Mg\textsuperscript{2+} exchange at site IV. Microcalorimetry data suggest that Ca\textsuperscript{2+}/Mg\textsuperscript{2+}-dependent protein-protein interactions are 8-fold stronger in the presence of Ca\textsuperscript{2+} than in the presence of Mg\textsuperscript{2+} (29). Substitution of Mg\textsuperscript{2+} for Ca\textsuperscript{2+} in CcTnC bound to NcTnI is characterized by condensation of the C-terminal portion of the metal binding loops and partial closure of the cTnI hydrophobic binding cleft around site IV (13). The close association between helix stability and metal ion affinity provides an attractive model for modulating Ca\textsuperscript{2+}/Mg\textsuperscript{2+} exchange and Ca\textsuperscript{2+}/Mg\textsuperscript{2+}-dependent protein-protein interactions by PKC phosphorylation.

A negative charge introduced at Ser\textsuperscript{43, 45} of cTnI, either by mutation or phosphorylation, is expected to stabilize the NeTnI helix through favorable electrostatic interactions between neighboring polar side chains and the helix backbone (30). Residues 43–65 in cTnI form a β-helix that binds to the C-lobe of cTnC through multiple polar and van der Waals interactions (2). Computational analyses of cTnI having Glu substituted at Ser\textsuperscript{43, 45} suggest that the incorporation of a negative charge extends the N terminus of the cTnI helix to residue 40 (4). Solution analysis of the secondary structure of NcTnI bound to CcTnC, based on experimentally determined chemical shift index values, suggests that the NeTnI helix begins at residue 46. The mutation of Ser\textsuperscript{43} to Asp stabilizes and extends the N terminus by three residues, from residue 46 in NeTnI to residue 43 in NeTnI (W43). Structural changes in CcTnC and NeTnI induced by the phosphorylation of Ser\textsuperscript{43, 45} support a mechanism for PKC modulation of cardiac contractility wherein the introduction of a negative charge results in altered C-lobe metal ion affinities and perturbation of Ca\textsuperscript{2+}/Mg\textsuperscript{2+}-dependent protein-protein interactions. Structural changes in NeTnI and CcTnC, forming the Ca\textsuperscript{2+}/Mg\textsuperscript{2+}-dependent protein-protein interaction site, can then be transmitted to other regulatory or switch regions in troponin. These studies emphasize the importance of Ca\textsuperscript{2+}/Mg\textsuperscript{2+}-dependent cTnC-cTnI protein-protein interactions in the modulation of cardiac contractility.

REFERENCES
1. van Eerd, J. P., and Takahashi, K. (1975) Biochem. Biophys. Res. Commun. 64, 122–127
2. Takeda, S., Yamashita, A., Maeda, K., and Maeda, Y. (2003) Nature 424, 35–41
3. Kobayashi, T., Dong, W.-J., Burkart, E. M., Cheung, H. C., and Solaro, R. J. (2004) Biochemistry 43, 5996–6004
4. Sumandea, M. P., Burkart, E. M., Kobayashi, T., de Tombe, P. P., and Solaro, R. J. (2004) Ann. N. Y. Acad. Sci. 1015, 39–52
5. Montgomery, D. E., Wolska, B. M., Pyle, W. G., Roman, B. B., Dowell, J. C., Buttrick, P. M., Koretsky, A. P., Del Nido, P., and Solaro, R. J. (2002) Am. J. Physiol. 282, H1297–H1305
6. Mittmann, K., Jaquet, K., and Heilmeier, L. M., Jr. (1990) FEBS Lett. 273, 41–45
7. Zhang, R., Zhao, J., and Potter, J. D. (1985) J. Biol. Chem. 260, 30773–30780
8. Gandra, M., Dong, W.-J., D. P., Cheung, H. C., and Solaro, R. J. (1997) Biochemistry 36, 13305–13311
9. Noland, T. A., Jr., Guo, X., Royner, R. L., Jideama, N. M., Averyhart-Fullard, S., Solano, R. R., and Kain, J. P. (1998) J. Biol. Chem. 273, 25445–25454
10. Huang, L., Wolska, B. M., Montgomery, D. E., Burkart, E. M., Buttrick, P. M., and Solaro, R. J. (2003) Am. J. Physiol. 280, C1114–C1120
11. Burkart, E. M., Sumandea, M. P., Kobayashi, T., Nili, M., Martin, A. F., Homber, E., and Solaro, R. J. (2003) J. Biol. Chem. 278, 11265–11275
12. Brito, R. M., Krudy, G. A., Negele, J. C., Putkey, J. A., and Rosevear, P. R. (1993) J. Biol. Chem. 268, 20966–20973
13. Finley, N. L., Howard, J. W., and Rosevear, P. R. (2004) Biochemistry 43, 11371–11379
14. Finley, N., Dvoretzky, A., and Rosevear, P. R. (2000) J. Mol. Cell. Cardiol. 32, 1439–1446
15. Trigo-Gonzalez, G., Awang, G., Racher, K., Nened, K., and Bortfod, P. (1993) Biochemistry 32, 9826–9831
16. Abbett, M. B., Gaponenko, V., Abusamhadneh, E., Finley, N., Li, G., Dvoretzky, A., Rance, M., Solano, R. J., and Rosevear, P. R. (2000) J. Biol. Chem. 275, 20610–20617
17. Gaponenko, V., Brito, R. M., Solano, R. J., and Rosevear, P. R. (1999) Biochemistry 38, 8313–8322
18. Burkart, E. M., Arteaga, G. M., Sumandea, M. P., Prabhukar, N., Wiezorek, H., and Solano, R. J. (2004) J. Mol. Cell. Cardiol. 39, 1285–1293
19. Li, M.-X., Wang, X., Lindhout, D. A., Buecemi, N., Van Eyk, J. E., and Sykes, B. D. (2003) Biochemistry 42, 14460–14468
20. Ward, D. G., Brewer, S. M., Gallon, C. E., Gao, Y., Levine, B. A., and Trayer, J. P. (2004) Biochemistry 43, 5772–5781
21. Wishart, D. S., and Sykes, B. D. (1994) J. Biomol. NMR 4, 171–180
22. Finley, N., Abbett, M. B., Abusamhadeh, E., Gaponenko, V., Dvoretzky, A., Misirah, H., Gaponenko, V., Howell, J. W., Rance, M., Solano, R. J., Cheung, H. C., and Rosevear, P. R. (1999) FEBS Lett. 453, 107–112
23. Abbett, M. B., Dong, W.-J., Dvoretzky, A., DaGue, B., Caprioli, R. M., Cheung, N. L. Finley, unpublished results.
PKC Phosphorylation of Cardiac Troponin I

H. C., and Rosevear, P. R. (2001) Biochemistry 40, 5992–6001
24. Harper, E. T., and Rose, G. D. (1993) Biochemistry 32, 7605–7609
25. Vassylyev, D. G., Takada, S., Wakatsuki, S., Maeda, K., and Maeda, Y. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 4847–4852
26. Strynadka, N. I., and James, M. N. (1989) Annu. Rev. Biochem. 58, 951–998
27. Leblanc, L., Bennet, A., and Borgford, T. (2000) Arch. Biochem. Biophys. 384, 296–304
28. Kapp, G. T., Richardson, J. S., and Oas, T. G. (2004) Biochemistry 43, 3814–3823
29. Calvert, M. J., Ward, D. G., Trayer, H. R., and Trayer, I. P. (2000) J. Biol. Chem. 275, 32508–32515
30. Smart, J. L., and McCammon, J. A. (1999) Biopolymers 49, 225–233
