Solution Structure of Calcium-saturated Cardiac Troponin C Bound to Cardiac Troponin I*

Alex Dvoretsky, Ekram M. Abusamhadneh, Jack W. Howarth, and Paul R. Rosevear

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

Cardiac troponin C (TnC) is composed of two globular domains connected by a flexible linker. In solution, linker flexibility results in an ill defined orientation of the two globular domains relative to one another. We have previously shown a decrease in linker flexibility in response to cardiac troponin I (cTnI) binding. To investigate the relative orientation of calcium-saturated TnC domains when bound to cTnI, 1H-15N residual dipolar couplings were measured in two different alignment media. Similarity in alignment tensor orientation for the two TnC domains supports restriction of domain motion in the presence of cTnI. The relative spatial orientation of TnC domains bound to TnI was calculated from measured residual dipolar couplings and long-range distance restraints utilizing a rigid body molecular dynamics protocol. The relative domain orientation is such that hydrophobic pockets face each other, forming a latch to constrain separate helical segments of TnI. We have utilized this structure to successfully explain the observed functional consequences of linker region deletion mutants. Together, these studies suggest that, although linker plasticity is important, the ability of TnC to function in muscle contraction can be correlated with a preferred domain orientation and interdomain distance.

Muscle contraction is controlled by Ca$^{2+}$ gradients, with the troponin complex mediating the response to the Ca$^{2+}$ influx. The troponin complex consists of three proteins, troponin T, troponin I (TnI), and troponin C (TnC). TnT interacts with tropomyosin and has been implicated in transmission of the contraction signal along the thin filament. TnI, the inhibitory subunit, has extensive contacts with TnC and actin and is responsible for inhibition of the actin-myosin interaction. The cardiac isoform of TnI (cTnI) differs from the skeletal isoform (sTnI) primarily by the presence of a 20–32-residue-long extension at the amino terminus (1). This cardiac-specific extension contains two adjacent protein kinase A phosphorylation sites that play a role in modulating contraction in response to β-adrenergic stimulation.

TnC, the protein responsible for detection of the calcium signal and initiation of muscle contraction, consists of two globular domains separated by a flexible linker. In both skeletal (sTnC) and cardiac (cTnC) TnC (des-Met<sub>1</sub>,Ala<sub>2</sub>, C35S), the C-terminal domain contains two high affinity Ca$^{2+}$/Mg$^{2+}$-binding sites (III and IV) and anchors TnC in the complex. The N-terminal domain also contains two EF-hand motifs that have high specificity, but a lower affinity for Ca$^{2+}$ (2). In the cardiac isoform, Ca$^{2+}$-binding site I is inactivated by substitution of key metal-binding ligands and an amino acid insertion (3).

In the absence of bound Ca$^{2+}$, the regulatory domain is in the inactive or “closed” conformation. Binding of Ca$^{2+}$ to the sTnC regulatory domain induces an opening of the regulatory domain and increased exposure of a hydrophobic pocket, the proposed binding site for the regulatory region of TnI (4). In the cardiac isoform, the presence of both Ca$^{2+}$ and the cTnI regulatory region appears necessary to stabilize a more “open” conformation with increased exposure of the hydrophobic pocket (4, 5). The equilibrium between open and closed cardiac regulatory domain states can be modulated by protein kinase A phosphorylation of the cTnC-specific amino terminus or by binding of Ca$^{2+}$-sensitizing agents (6–9).

X-ray crystal structures of full-length TnC (10, 11) are available along with high resolution solution structures of isolated N- and C-terminal domains (4, 12). However, a structure of full-length Ca$^{2+}$-saturated TnC bound to full-length TnI is currently unavailable. A complete description of the solution structure for Ca$^{2+}$-saturated TnC bound to full-length TnI requires collection of both long-range distance and orientation restraints. In this study, orientation restraints were extracted from 1H-15N residual dipolar couplings (RDCs) measured by partially orienting Ca$^{2+}$-saturated [1H, 15N]TnC-cTnI in two different alignment media. RDCs in combination with cTnC domain structures (4, 12) and long-range distances (13–15) were used to elucidate the Ca$^{2+}$-saturated cTnC structure in the cTnC-cTnI complex.

EXPERIMENTAL PROCEDURES

Recombinant Proteins—[1H, 15N]cTnC-cTnI were expressed and purified, and the cTnC-cTnI complex was prepared as previously described (8). NMR samples of Ca$^{2+}$-saturated [1H, 15N]TnC-cTnI (0.4–0.6 mM) were prepared in buffer containing 10% 2H$_2$O, 20 mM Tris-$d_{1}$, (pH 6.8), 500 mM KCl, 10 mM CaCl$_2$, 5 mM dithiothreitol, 5 mM β-mercaptoethanol, 0.1 mM Pefabloc, and 0.1 mM leupeptin.

Alignment Media—Pf1 filamentous phage was prepared as described previously (16). Alignment of the medium was confirmed by observation of the quadrupole splitting of the 2H$_2$O signal. Typically, phage was titrated into the protein sample until a 2H$_2$O splitting of ~30 Hz was achieved.

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† These authors contributed equally to this work.

‡ To whom correspondence should be addressed: Dept. of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati, College of Medicine, 2015 MSB, 231 Albert Sabin Way, Cincinnati, OH 45267. Tel.: 513-558-3370; Fax: 513-558-8474; E-mail: paul.rosevear@uc.edu.

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The abbreviations used are: TnI, troponin I; cTnI, cardiac troponin I; sTnI, skeletal troponin I; TnC, troponin C; cTnC, cardiac troponin C; sTnC, skeletal troponin C; RDCs, residual dipolar couplings; cTnC-N, cTnC-(81–161) bound to cTnI-(33–80); cTnC-N, cTnC-(1–91) bound to cTnI-(148–163); CaM, calmodulin.
Troponin C Domain Orientation

Nonionic liquid-crystalline medium consisting of n-alkyloxy(ethyleneglycol)/n-alkyl alcohol (C₂₅E₆/n-hexanol) was prepared as described previously (13) at a final molar ratio of ~9.6. Typically, Ca²⁺-saturated cTnC-CtN complexes in 4.5% C₁₂E₅/n-hexanol lamellar phase were created by removal of amino acids corresponding to residues 90–96 and 86–92 from cTnC. The remaining linker residues were treated as rigid bodies separated by the known distance between consecutive residues assuming planar and trans-peptide bonds. Nine- and 12-amino acid deletions corresponding to residues 88–96 and 85–96 of cTnC, respectively, were also constructed.

To provide additional linker flexibility in the 12-amino acid deletion mutant, residues 81–83 were treated as rigid bodies in the rigid body minimization procedure.

For each linker region deletion mutant constructed, rigid body minimization was used to determine cTnC domain orientation and position as described for full-length cTnC. A constrained/restrained simulated annealing algorithm was subsequently used to refine suitable low energy structures as described for full-length cTnC.

RESULTS

Dipolar Couplings—Amide ¹H and ¹⁵N resonance assignments for Ca²⁺-saturated [²H,¹⁵N]cTnC-CtN in Pf1 filamentous phage or C₂₅E₆/n-hexanol were transferred from previously published assignments for Ca²⁺-saturated cTnC bound to cTnI (8). Comparison of Ca²⁺-saturated cTnC chemical shifts in oriented and non-oriented cTnC-CtN complexes showed minimal chemical shift perturbations due to the presence of the alignment media (data not shown). The absence of substantial chemical shift changes suggests that the cTnI-bound structure of cTnC is unaffected by addition of the alignment media. In the Pf1 filamentous phage liquid-crystalline medium, a total of 36 and 29 RDCs were measured in the N- and C-terminal domains of cTnC bound to cTnI, respectively. In the lamellar phase produced with C₂₅E₆/n-hexanol, 23 and 30 RDCs were measured in the N- and C-terminal domains of cTnC, respectively. In each alignment medium, a combination of resonance overlap, signal/noise, and line broadening prevented measurement of additional ¹H-¹⁵N RDCs. The magnitude of the principal axis and rhombicity of the alignment tensors were estimated from the distribution of the dipolar couplings (22). Values of 9.5 Hz for the magnitude of the principal axis of the alignment tensor and 30% rhombicity were estimated from RDCs measured in C₂₅E₆/n-hexanol lamellar phase. Similarly, values of 6.5 Hz and 25% for the magnitude and rhombicity, respectively, were estimated from RDCs measured in the Pf1 filamentous phage medium.

Estimated alignment tensor parameters were used with experimentally measured RDCs to determine the relative cTnC domain orientation in the presence of cTnI. To avoid the multiple minima associated with using RDCs for solution structure determination (23), an initial model was constructed for cTnC bound to cTnI from the domain solution structures using Ca²⁺-saturated cTnC-(1–89) (Protein Data Bank code 1MXL) (4) for the N-terminal domain and Ca²⁺-saturated cTnC-(81–161) (Protein Data Bank code 1IP5) (12) for the C-terminal domain. The RDC data, collected in both the C₂₅E₆/n-hexanol and Pf1 filamentous phage liquid-crystalline media, fit the cTnC N-terminal domain structure with an R-factor of 26%. Similarly, the RDC data collected in the C₂₅E₆/n-hexanol and Pf1 filamentous phage liquid-crystalline media fit the cTnC C-terminal domain structure with R-factors of 26 and 31%, respectively. The reasonable fit of experimentally measured RDCs in intact cTnC bound to cTnI with solution structures of the individual cTnC domains justified using the individual domain structures as starting points in the structure determination of cTnC bound to cTnI.

Structure Determination—Rigid body minimization was used to determine the relative domain orientation by simultaneously minimizing the N- and C-terminal domains to the
Experimentally measured RDCs recorded in both alignment media. In addition, the overall shape of cTnC was constrained using long-range distance restraints obtained from the radius of gyration derived from neutron scattering data (18) and an interdomain fluorescence resonance energy transfer distance (17). The inclusion of RDC data from two different alignment media does not completely eliminate the degeneracy in determining relative domain orientation. From seven initial starting structures, two different ensembles were obtained. In each ensemble, both sets of RDCs were simultaneously satisfied. In one ensemble, the linker region was significantly distorted, resulting in unfavorable backbone conformations as a consequence of simultaneously satisfying long-range distance restraints. Thus, structures within this ensemble were not further refined.

The remaining ensemble of cTnC domain-oriented structures was further refined, with a mock crystal symmetry molecule to prevent domain rotation, using a constrained/restrained simulated annealing algorithm (24). This permitted refinement of individual domain structures and minimization of the linker region against experimentally determined RDCs without significantly altering domain orientation. The cTnI-bound cTnC structure was refined to R-factors of 15 and 22% for RDCs collected in the lamellar phase and Pf1 filamentous phage, respectively (Fig. 1). The average domain-oriented solution structure for Ca$^{2+}$-saturated cTnC bound to cTnI is shown in Fig. 2. Fig. 2 depicts a structure in which the hydrophobic pockets in each domain face each other and act as a latch to constrain separate regions of the cTnI molecule. The quality of the average cTnC domain-oriented structure bound to cTnI was further examined using PROCHECK (25). Residues in both domains show good covalent geometries with well defined secondary structures, having 97% of residues in the most favorable and allowed regions of the Ramachandran plot.

Comparison with Existing TnC Structures—The structure of cTnC-C(81–161) bound to cTnI(33–80) (cTnC-C) (Protein Data Bank code 1FI5) (12) is available with a peptide corresponding to residues 33–80 of cTnI. Superposition of this structure with the C-terminal domain of cTnC in the intact cTnCcTnI complex yielded a backbone root mean square deviation of only 2.2 Å. Comparison of the structure of cTnC-C(1–89) bound to cTnI-(147–163) (Protein Data Bank code 1MXL) (4) with the N-terminal domain in cTnCcTnI yielded a backbone root mean square deviation of only 2.1 Å. The similarity of the cTnC-C and cTnC-N structures to their corresponding domains in the intact complex establishes the utility of using isolated domain structures as models for understanding the atomic details of divalent cation and TnI peptide interactions. Unfortunately, isolated domain studies cannot provide information on domain orientation, long-range protein-protein interactions, or the interdomain linker.

We also compared amide$^1$H and$^{15}$N chemical shifts for cTnC in the intact cTnCcTnI complex with those for cTnC-C (12) and cTnC-N (26). Differences in amide$^1$H chemical shifts are a sensitive probe of backbone conformational changes. The average chemical shift difference between cTnC-C and the C-terminal domain of cTnC in the intact cTnCcTnI complex was 0.02 ± 0.01 ppm. The chemical shift variation between cTnC-N and the N-terminal domain of cTnC in the cTnCcTnI complex was 0.12 ± 0.01 ppm. Although larger chemical shift differences were observed between N-terminal domain structures, these were modest and generally smaller than the chemical shift changes that occur upon binding cTnI-(148–163) and Ca$^{2+}$ to cTnC (26). Furthermore, the magnitude and direction of N-terminal domain chemical shift differences are consistent with minor perturbations of the closed-to-open regulatory domain equilibria. Because most cTnC N- and C-terminal domain amides showed the same resonances in the intact cTnCcTnI complex as observed in the individual isolated domains, the contact area between cTnC and cTnI must not be much larger than those observed in the cTnC(1–89)-cTnI(147–163) peptide (Protein Data Bank code 1FI5) (12) and the cTnC-N-cTnI peptide (Protein Data Bank code 1MXL) (4) structures. In addition,
significant interdomain interactions within the complex are not expected.

For each domain, solvent-exposed hydrophobic surface area was calculated and compared with that observed in the individual domain structures. The surface-accessible nonpolar area was determined utilizing the NMR-Refine module of Insight II (MSI). The calculated accessible nonpolar area for the N-terminal domain (residues 4–83) is 2614 Å² for intact cTnC-cTnI and 2320 Å² for cTnC-N (Protein Data Bank code 1MXL). For the C-terminal domain (residues 93–159), the calculated accessible nonpolar area is 2280 Å² for intact cTnC-cTnI and 2320 Å² for cTnC-C (Protein Data Bank code 1F15). Similarity in accessible nonpolar surface area further shows that individual cTnC domain conformations in cTnC-cTnI are similar to the aforementioned structures of the isolated N- and C-terminal domains bound to their respective cTnI binding peptides.

DISCUSSION

Uncertainty in domain orientation is a persistent problem in structure determination of multidomain proteins. This is especially true in the multidomain EF-hand protein family, exemplified by TnC and calmodulin (CaM), due to the presence of a long flexible linker connecting individual domains. Analysis of heteronuclear relaxation data established that binding of cTnI decreases conformational fluctuations within the cTnC interdomain linker (27). The observed decrease in linker flexibility indicates that, in the presence of cTnI, cTnC interdomain motion is restricted. In addition, paramagnetic effects on [methyl-13C]Met residues in cTnC from a nitroxide spin label attached at Cys84 suggested that binding of cTnI decreases interdomain motion, resulting in a more extended cTnC structure (28). In the absence of TnI, the interdomain linker is considerably more flexible, resulting in an ill-defined orientation between the two domains, with both domains aligning in the magnetic field to differing degrees.

Restriction of interdomain motion in the presence of bound cTnI suggested that RDCs measured in liquid-crystalline media could be used to determine the average domain orientation of Ca2+-saturated cTnC bound to cTnI. With experimentally measured RDCs in two different alignment media and previously published long-range distance restraints, we determined the relative domain orientation of Ca2+-saturated cTnC bound to cTnI. The RDC-determined solution structure for cTnC, having both hydrophobic pockets highlighted, with cTnI residues 33–80 interacting with the C-terminal domain hydrophobic pocket and cTnI residues 147–163 interacting with the N-terminal domain hydrophobic pocket is shown in Fig. 2. This structure represents the first full-length TnC solution structure defining the relationship between globular domains in the presence of full-length cTnI.

The relative domain orientation is such that hydrophobic pockets within each domain are positioned to “latch on” to different helical segments of cTnI. The relatively good fit of RDCs measured in Ca2+-saturated cTnC bound to cTnI with available domain structures (4, 12) together with the modest differences in chemical shifts between the intact complex and isolated domains support the assumption that high resolution TnC domain structures provide biologically relevant models for understanding atomic details of domain-cation and domain-target interactions.

Validation of domain structures permitted the use of long-range distance and orientation constraints to investigate how individual domains are functionally assembled to control muscle contraction. In addition, these studies suggest that cTnC may interact with relatively small regions of cTnI, being structurally unaffected by the remaining portions of the protein. A similar finding for CaM binding to CaM kinase I and peptide

![Fig. 3. Surface representations of EF-hand protein-target interactions.](http://www.jbc.org/)

Regions of cTnI outside the known peptide binding sequences have little effect on cTnC, consistent with disordered or flexible regions of polypeptide separating functional cTnI domains. Model peptide studies utilizing cTnI residues 129–149, containing the inhibitory region, suggest that the inhibitory region may not interact directly with cTnC (30). This finding is consistent with flexible linkers allowing TnI domains to move relative to one another.

To allow quantitative comparison of domain orientations between intact cTnC bound to cTnI with other Ca2+-binding protein-target complexes, the relative domain orientations are described by three parameters: bend, azimuth, and twist. When classified by the bend between the D- and E-helices, available TnC and CaM structures fall into two general families. The first family, as typified by the two Ca2+-loaded x-ray structure of TnC (Protein Data Bank code 1TOP), exhibits a slight bend along a particular azimuth with little or no twist. This structure has a helical linker and adopts an extended conformation with the hydrophobic pockets facing in opposition. The second family shows a larger bend with a portion of
the D/E-helical linker unwound (Fig. 3). The cTnI-bound cTnC structure falls into this second family, having a 70° bend in the linker region. Similarly, the crystal structure of sTnC bound to sTnI(1–47) has a 93° bend in the linker region (11). The azimuths for TnC bound to full-length TnI and TnI(1–47) are 30° and −162°, respectively, producing different relative orientations of the hydrophobic binding pockets (Fig. 3). Differences in the disposition of the hydrophobic pockets between the solution structure of cTnC bound to cTnI and the crystal structure of sTnC bound to cTnI(1–47) (11) may result from the influence of full-length cTnI or as a consequence of crystal-packing forces.

In the solution structure of cTnC, each Ca2+-saturated TnC domain is positioned to act as a latch constraining the separate helical regions of cTnI. The binding of Ca2+ and the regulatory region of cTnI to the N-terminal domain of cTnC is generally believed to be the key interaction in a series of conformational rearrangements necessary for relieving inhibition of the actomyosin ATPase and initiation of muscle contraction. Assuming a largely structural role for the cTnC C-terminal domain-cTnI N-terminal domain interaction, the role of the linker region may be to optimally position the N-terminal domain for interaction with the regulatory region of cTnI. The removal of Ca2+ from the regulatory domain would result in the unclutching of the cTnI regulatory region. Such a model suggests an essential role for linker plasticity in the ability of TnC to regulate muscle contraction in a Ca2+-dependent manner. Comparison of domain orientation in the absence of Ca2+ is not possible because cTnC would only be anchored to the N-terminal domain of cTnI via C-terminal domain interactions.

The functional and structural significance of the interdomain linker in TnC is poorly understood. Deletion of 7 residues was found not to impact regulatory function (31). In a similar study, systematic deletion of 3–12 linker residues showed that up to 7 residues could be deleted with little change in maximal force development (31, 32). However, with further deletions, inhibition increased until at 12 deletions the inhibition was complete (31, 32). In an attempt to provide a structural context to the observed functional consequences of linker region deletions, we have examined the influence of deleting 7, 9, and 12 residues from the linker region on cTnC length and domain orientation.

Two different 7-amino acid deletions in the linker region have been reported (31, 32). Seven-residue deletions in the TnC linker domain, corresponding to residues 90–96 and 86–92 in recombinant cTnC, were found to have little effect on maximal force development or the ability of sTnC to activate actomyosin ATPase (31–33). Computationally generated mutants, created by deletion of appropriate linker residues, were used as input structures in rigid body minimization and constrained/restrained simulated annealing protocols.

Computationally generated deletion mutants were evaluated for their ability to simultaneously satisfy both experimentally measured RDCs and long-range distance restraints. Acceptable low energy structures, satisfying all experimental constraints, were obtained for both computationally generated mutant cTnC proteins with 7-residue linker deletions. No significant differences in domain orientations were found between the 7-residue linker domain deletion mutants and wild-type cTnC.

Next, a computationally generated cTnC mutant with a 9-residue linker deletion, corresponding to residues 88–96, was subjected to rigid body minimization and constrained/restrained simulated annealing. Although structures satisfying the experimental constraints were obtained, significant strain within the linker domain was introduced. The strain was evidenced by the appearance of distorted covalent geometry in the linker region, including elongated covalent bonds and bond angles that deviate significantly from the ideal values. The PROCHECK G-factor (25), a composite measure of covalent geometry, was used to evaluate the consequences of each deletion. The G-factor for the solution structure of cTnC bound to cTnI is −0.21. For both 7-residue deletions, the G-factor remained essentially unchanged at −0.22. However, for the 9-residue deletion, the G-factor decreased to −0.63. This value is below the −0.5 threshold, indicating the development of significant deviations from ideal covalent geometry. Although TnC can accommodate a 9-residue linker deletion and not violate experimental constraints, the increase in intermolecular strain required to adapt a wild-type conformation is likely responsible for the significant decrease in maximal force generated (31). Finally, orientation and distance restraints were inconsistent with computationally generated TnC structures having 12 linker residues deleted. Contracture was completely inhibited by TnC containing a 12-residue linker deletion (31), in agreement with our modeling studies. Thus, the solution structure for cTnC bound to cTnI can successfully explain the observed functional consequences of linker region deletions. Although plasticity within the linker region may play an important role, the ability of TnC to function in muscle regulation can be correlated with a preferred domain orientation and interdomain distance. Thus, the linker region also functions as a spacer to maintain the N- and C-terminal domains at an appropriate distance that likely correlates with the separation of TnI target peptides 33–80 and 147–163 in cTnI.

Recent efforts to model the disposition of TnI relative to TnC have been hindered by the absence of structural information on TnI and long-range distance restraints (34, 35). In addition, the crystal structure of free TnC (10, 36) was assumed to provide an accurate model for the solution structure of TnC in the complex and was used to define the overall length and domain orientation. The experimentally determined solution structure for cTnC bound to TnI demonstrates that the domain orientation differs from that of the free protein in the crystal (Fig. 3). Although initial events in muscle contraction are well characterized at the domain level, propagation of the signal remains poorly understood largely due to the uncertainty of interdomain position. Our structure provides a scaffold for further studies aimed at mapping interactions within the troponin complex responsible for the initiation of muscle contraction.

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Alex Dvoretsky, Ekram M. Abusamhadneh, Jack W. Howarth and Paul R. Rosevear

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