Structure of Cardiac Muscle Troponin C Unexpectedly Reveals a Closed Regulatory Domain*

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The regulation of cardiac muscle contraction must differ from that of skeletal muscles to effect different physiological and contractile properties. Cardiac troponin C (TnC), the key regulator of cardiac muscle contraction, possesses different functional and Ca$^{2+}$-binding properties compared with skeletal TnC and features a Ca$^{2+}$-binding site I, which is naturally inactive. The structure of cardiac TnC in the Ca$^{2+}$-saturated state has been determined by nuclear magnetic resonance spectroscopy. The regulatory domain exists in a “closed” conformation even in the Ca$^{2+}$-bound (the “on”) state, in contrast to all predicted models and differing significantly from the calcium-induced structure observed in skeletal TnC. This structure in the Ca$^{2+}$-bound state, and its subsequent interaction with tropinin I (TnI), are crucial in determining the specific regulatory mechanism for cardiac muscle contraction. Further, it will allow for an understanding of the action of calcium-sensitizing drugs, which bind to cardiac TnC and are known to enhance the ability of cardiac TnC to activate cardiac muscle contraction.

Transient increases in cytosolic Ca$^{2+}$ levels in the cardiac muscle cell must be recognized by the thin filament to regulate cardiac muscle contraction. This critical function is accomplished by cardiac TnC1 (161 residues), a member of the EF-hand family of Ca$^{2+}$-binding proteins, which relays the Ca$^{2+}$ signal via a conformational change to the rest of the tropinin-tropomysin complex, and ultimately signals the activation of the myosin-actin ATPase reaction. Although the sequence of cardiac TnC is 70% identical to that of skeletal TnC, there are significant differences in the first 40 residues, the most crucial being the inactivation of Ca$^{2+}$-binding site I due to an insertion (Val29) and substitutions of key ligands relative to skeletal TnC (Leu30 and Ala31 in cardiac TnC instead of Asp30 and Asp32 in skeletal TnC) (1). Despite the many functional, binding, and modeling studies performed on cardiac TnC (2), the absence of direct structural data makes the Ca$^{2+}$-induced conformational change in cardiac TnC unclear. The structures of TnC in the skeletal system, on the other hand, have been solved both in the 2-Ca$^{2+}$ (3, 4) and 4-Ca$^{2+}$ states (5), showing TnC to be a dumbbell-shaped molecule with separate N- and C-terminal domains connected by a central linker. Upon Ca$^{2+}$ binding, the regulatory N-domain of skeletal TnC switches from a “closed” to an “open” conformation, thereby exposing a patch of hydrophobic residues, which is thought to interact with skeletal TnI (6). In this report, we show that, in contrast to predicted models (7–9), the analogous conformational change does not occur in cardiac TnC, and that this is the direct structural consequence of inactivating Ca$^{2+}$-binding site I. In addition, a structural understanding of cardiac TnC has potential therapeutic value in the understanding of the mechanism of cardiac TnC-binding drugs known as “calcium-sensitizing drugs” (8, 10).

For the purposes of this study, the two Cys residues at positions 35 and 84 of wild type cardiac TnC have been mutated to Ser residues. This prevents the formation of intra- and intermolecular disulfide bonds, which confer Ca$^{2+}$-independent activity to cardiac TnC when assayed in skeletal muscle myofibrils (11). It has been shown that the conversion of these Cys residues to Ser residues has no effect on the ability of cardiac TnC to recover ATPase activity in TnC-extracted fast skeletal and cardiac myofibrils, and has little effect on Ca$^{2+}$ binding to the second regulatory state II of cardiac TnC (11). Thus, it is unlikely that the introduction of these two conservative mutations would result in gross conformational changes in the secondary or tertiary structure of cardiac TnC.

For NMR analysis, the protein was uniformly labeled with $^{13}$C and/or $^{15}$N by expression in Escherichia coli. Triple-resonance NMR experiments were used for assigning the resonances and subsequently to derive distance and dihedral angle restraints. 35 structures were then calculated using the simulated annealing protocol (12). Structural statistics for the 30 lowest energy structures (Table I) show that the N- and C-domains are very well defined separately, with the central linker shown to be flexible by relaxation measurements.

MATERIALS AND METHODS

Sample Preparation—To produce high level expression of chicken cardiac TnC with the mutations C55S and C84S (denoted cTnC(A-Cys)), the expression plasmid pTnC(A-Cys)P1 (11), which uses the P1 promoter, was digested with NcoI and HindIII, and the small fragment containing the full amino acid coding region was ligated into the NcoI/HindIII sites of the plasmid pET-23d (Novagen), which has a T7 promoter. Plasmids were maintained, and cTnC(A-Cys) was expressed in Escherichia coli [strain BL21(DE3)] withfolder-expressed quantities of cTnC(A-Cys).

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4 The atomic coordinates and structure factors (code 1AJ4) have been deposited in the Protein Data Bank, Brookhaven National Laboratory, Upton, NY. Coordinates of the 30 calculated structures for the N-domain (code 2CTN) and the C-domain (code 3CTN) have also been deposited.

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6 The abbreviations used are: TnC, troponin C; TnI, troponin I; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhancement spectroscopy; TOCSY, total correlation spectroscopy; MOPS, 4-morpholinepropanesulfonic acid.

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2 S. K. Sia, M. X. Li, L. Spyracopoulos, S. M. Gagné, W. Liu, J. A. Putkey, and B. D. Sykes, unpublished data.
the BL21(DE3)pLysS host strain E. coli after induction with isopropyl-1-thio-β-D-galactopyranoside. Isotope enrichment media consisted of M9 minimal media, in which the NH4Cl and/or the D-glucose was replaced with one of the following: 1) 15NH4Cl (1 g/liter) for uniform 15N labeling; 2) D-glucose-U-13C6 (2 g/liter) for uniform 13C labeling; 3) 15NH4Cl (1 g/liter) and D-glucose-U-13C6 (2 g/liter) for uniform 13C and 15N labeling; or 4) D-glucose-U-13C6 (0.5 g/liter) and D-glucose (1.5 g/liter) for partial 13C labeling. All isotopes were purchased from Cambridge Isotope Laboratories. After induction, bacteria were collected and lysed, and a soluble protein fraction was prepared as described previously (13). This was applied directly to a Macro-Q (Bio-Rad) anion exchange column and eluted with a 0–500 mM KCl gradient containing 0.5 mM EGTA. Appropriate fractions were pooled, made 3 M in (NH4)2SO4, and centrifuged to remove precipitates, after which the soluble fraction was dialyzed against 50 mM MOPS, 0.5 mM CaCl2 at pH 7.0. The dialyzed sample was then applied to a semiprep HPLC DEAE 5-PW anion exchange column (Millipore) and eluted with a 0–500 mM KCl gradient with 0.5 mM CaCl2. Purified cTnC(A-Cys) was pooled and dialyzed against 10 mM (NH4)HCO3 at pH 8.0, and lyophilized.

Structure Determination—All spectra were collected at 30 °C on a Varian Unity Plus 500-MHz spectrometer or a Varian Unity Plus 600-MHz spectrometer, both equipped with pulsed field gradient accessories. The NMR samples contained 2–4 mM cTnC(A-Cys) in 16 mM CaCl2, 10 mM KCl, 10 mM imidazole at pH 6.7, in either 90% H2O, 10% D2O or 99.9% D2O. The high affinities of cardiac TnC for calcium (with dissociation constants on the order of 10^-26 M for the N-domain and 10^-28 M for the C-domain) ensure that the protein is Ca2+ saturated at 16 mM CaCl2 (11).

Assignment of the main-chain NH, N, Ca, and Cb resonances were made based on the three-dimensional experiments HNCACB and CB-CA(CO)NH (14). Three-dimensional 15N-edited total correlation spectroscopy (TOCSY) (15) and three-dimensional HCCH-TOCSY (16) experiments were used to assign the chemical shifts for the side-chain atoms. A high resolution two-dimensional 1H-1H nuclear Overhauser spectroscopy (NOESY) experiment and assignments made by a previous study (17) were used to confirm the assignments for resonances of aromatic protons. Interproton distances were derived from three-dimensional 15N- and 15N/13C-edited NOESY experiments (18), both collected at 50 ms of mixing time. In converting NOE intensities into distance restraints, the NOE intensities were calibrated for each residue using its intraresidue d_N(i,i) and sequential d_N(i,i-1) NOEs as reference intensities (6).
Structure of Cardiac Troponin C

Table I  Structural statistics

|                | N-domain (2–89) | C-domain (90–161) |
|----------------|-----------------|-------------------|
| r.m.s.d. from the average structure (Å) | 0.0012 ± 0.0001 | 0.0012 ± 0.0001 |
| Backbone atoms | 1239            | 1060              |
| All heavy atoms | 0.97 ± 0.09     | 0.94 ± 0.10       |
| NOE restraints  |                 |                   |
| Total           | 1239            | 1060              |
| Intra-residue   | 477             | 421               |
| Sequential (i–j–i = 1) | 285          | 267               |
| Medium-range (2 ≤ | 315             | 222               |
| Long-range (i–j–i ≥ 5) | 162         | 170               |
| Distance restraints to Ca2+-ions | 6          | 12               |
| Dihedral restraints |               |                   |
| Total           | 104             | 87                |
| φ               | 49              | 41                |
| ψ               | 37              | 26                |
| χ1             | 18              | 20                |
| φ, ψ in core or allowed regions | 98%          | 99%               |
| Energies       |                 |                   |
| E_total         | 105 ± 4         | 84 ± 2            |
| E_NOE           | 2 ± 1           | 4 ± 1             |
| E_dihedral      | 0.03 ± 0.03     | 0.03 ± 0.05       |
| r.m.s.d. from idealized geometry |               |                   |
| bond lengths (Å) | 0.0012 ± 0.0001 | 0.0012 ± 0.0001 |
| bond angle (°)  | 0.47 ± 0.01     | 0.44 ± 0.01       |
| improper (°)    | 0.35 ± 0.01     | 0.33 ± 0.01       |
| Restriction violations |               |                   |
| distance > 0.1 Å but < 0.2 Å | 52 (1.7 structure) | 50 (1.7 structure) |
| dihedral > 1°   | 0 (0 structure) | 0 (0 structure)   |
| φ, ψ in core or allowed regions | 98%          | 99%               |

Note that calcium ions are not directly observed by NMR spectroscopy. No restraints involving the calcium ions were used in the initial stages of the structure calculations, and were added only in the final stages of refinement (see Materials and Methods). The final force constants were K_NOE = 50 kcal mol⁻¹ and K_dihedral = 200 kcal mol⁻¹ rad⁻². φ, ψ core and allowed regions were as determined by the program PROCHECK (36). There are no distance violations over 0.2 Å for the N-domain, and there is one distance violation over 0.2 Å for 30 structures for the C-domain.

The above structural differences between cardiac and skeletal TnC can be explained by what is in fact the most striking functional difference between the two proteins: namely that Ca²⁺-bound cardiac TnC is significantly more compact than the N-domain of Ca²⁺-saturated skeletal TnC (28–30). In skeletal TnC, there exists a kink at Glu 40 and D). Thus, the B-helix remains closed due to favorable

calmodulin-target peptide complex (26), resembles a dumbbell in shape, consisting of two separate domains connected by a flexible central linker (residues 86–94 in cardiac TnC). However, despite the general structural similarities to homologous Ca²⁺-binding proteins, the regulatory N-domain of Ca²⁺-saturated cardiac TnC is significantly more compact than the N-domain of Ca²⁺-saturated skeletal TnC (5, 6), exposing approximately 800 Å² less total accessible surface area (residues 5–84) than its skeletal counterpart (residues 7–85). In particular, the B-helix of defunct site I exists in the “closed” conformation, exhibiting an A-B interhelical angle of 142° (with the A- and B-helices corresponding to the two helices of the helix-loop-helix motif in Ca²⁺-binding proteins; Table II). The closed conformation is evidenced by 21 NOE connectives observed between the A- and B-helices (Fig. 2), most of which would not be observed if the B-helix were in an “open” conformation as in skeletal TnC (Fig. 3A). A compact regulatory domain is also consistent with a previous cysteine-reactivity study on wild type cardiac TnC (27).

The difference in the conformation of the B-helix is reflected most clearly in the main chain conformation of residue Glu 40 in cardiac TnC (equivalent to Glu 51 in skeletal TnC). Glu 41 of skeletal TnC has been proposed to be pivotal in the mechanism of the coupling between Ca²⁺ binding and the Ca²⁺-induced conformational change in Ca²⁺-binding proteins (28, 29). Indeed, the apo form of skeletal TnC (3) features a kink in the B-helix at Glu 41 which straightens out upon Ca²⁺ binding to sites I and II (28). In cardiac TnC, however, there exists a kink in the B-helix at Glu 40, even in the Ca²⁺-bound state (Ca²⁺ ions bound at sites II, III, and IV). The non-helical nature at Glu 40 is supported by a J_HNHa value of 7.8 Hz, an absence of an upfield-shift of its Hα resonance (4.37 ppm), and an absence of a downfield shift in its Cα resonance (56.8 ppm), all of which indicate non-helical conformations (30). On the other hand, both adjacent residues Lys 39 and Leu 41 exhibit 3JHNH values of less than 5.5 Hz, as well as appropriate shifts in their Hα and Cα resonances which indicate an α-helical conformation.

The above structural differences between cardiac and skeletal TnC can be explained by what is in fact the most striking functional difference between the two proteins: namely that site I in cardiac TnC is inactive due to an insertion and key substitutions of key ligands. In cardiac TnC, there is no Ca²⁺ at site I to pull the Glu 40 side-chain carboxylate group over to the entropic loss associated with exposing buried residues (which occurs in the “opening” of the B- and C-helices relative to helices N, A, and D). Thus, the B-helix remains closed due to favorable

| Table II  Interhelical angles of various EF-hands |
|----------|--------|--------|--------|--------|
|          | A-B    | C-D    | E-F    | G-H    |
| Calcium-binding protein |     |       |       |       |
| Cardiac TnC(1 Ca²⁺/2 Ca²⁺) | 138 ± 3 | 108 ± 4 | 115 ± 4 | 121 ± 4 |
| Skeletal TnC(2 Ca²⁺/2 Ca²⁺) | 81.5 ± 7 | 78 ± 7 | 99 ± 6 | 104 ± 7 |
| Calmodulin(2 Ca²⁺/2 Ca²⁺) | 138 | 145 | 105 | 111 |

The parentheses indicate first the state of the N-domain (i.e., A-B and C-D helix-loop-helices), followed by the state of the C-domain (i.e., E-F and G-H helix-loop-helices). Note that in cardiac TnC(1 Ca²⁺/2 Ca²⁺) as determined in the present study, defunct site I (i.e. A-B helix-loop-helix) is free of Ca²⁺1, while sites II, III, and IV are Ca²⁺-bound. Protein Data Bank accession codes are: 1TNW for the NMR structure of skeletal TnC(2 Ca²⁺/2 Ca²⁺); 5TNC for the crystal structure of skeletal TnC(apo/2 Ca²⁺), and 4CLN for the crystal structure of calmodulin(2 Ca²⁺/2 Ca²⁺).

a A large angle defines a “closed” conformation, whereas a small angle defines an “open” conformation. The axis for an α-helix is defined by two points, the two points being the average coordinates of the first and last 11 backbone atoms of the α-helix.
packing forces with the A-helix and D-helix (Figs. 1A and 3A; Table II, A-B interhelical angle). On the other hand, with Ca$^{2+}$ bound at site II, the C-helix is in fact in an open conformation, but does not open up to the extent seen in skeletal TnC (Figs. 1A and 3A; Table II, C-D interhelical angle). These observations demonstrate that the inability of Glu$^{40}$ to coordinate Ca$^{2+}$ results in a more compact conformation for the B-helix, and possibly the C-helix, than is observed in skeletal TnC (Fig. 3A); in effect, Ca$^{2+}$ binding to sites I and II of skeletal TnC locks open the whole regulatory domain, whereas Ca$^{2+}$ binding to site II of cardiac TnC only partially opens up the regulatory domain. (This discussion assumes that the structure of the apo form of the regulatory domain of cardiac TnC is similar to that of skeletal TnC, as has been recently demonstrated.$^3$) The model of Glu$^{40}$ acting as a pivot for the N-domain is further supported by a recent structural study of a skeletal TnC mutant in which Glu$^{40}$ is replaced by Ala$^{41}$, such that residue 41 can no longer coordinate the Ca$^{2+}$ ion present at site I (29). In the Ca$^{2+}$-saturated state of this protein, the single substitution results in a kink at Ala$^{41}$ and a closed conformation for the B-helix, similar to what is seen in Ca$^{2+}$-saturated cardiac TnC.

The structure of defunct site I shows that Leu$^{29}$, which comes just after the insertion at Val$^{28}$, forms an extra half-turn at the end of the A-helix, as evidenced by $d(H^i, i + 3)$ and $d(H^j, i + 3)$ NOE connectives from Ile$^{26}$ to Leu$^{29}$. Site I, being Ca$^{2+}$-free, is not as well defined as the rest of the molecule (root mean square deviation of 0.78 Å for backbone atoms of residues 30–33), and is shown to be more flexible than the rest of the regulatory domain by relaxation measurements.

The structural C-domain of cardiac TnC (Figs. 1B and 3B) is predictably similar to those in skeletal TnC and calmodulin, although the interhelical angles of the two EF-hands in the C-domain indicate that this domain is in fact slightly more compact in cardiac TnC than in its counterparts (10–20° more closed in the E-F and G-H interhelical angles; see Table II). Overall, the backbone atoms of residues 95–157 of cardiac TnC superimpose within 1.9 Å with their equivalent residues (96–158) in the NMR structure of skeletal TnC with Ca$^{2+}$-saturated N-domain, and 1.3 Å with the same region in the crystal structure of skeletal TnC with apo N-domain.

DISCUSSION

We have shown for the first time the three-dimensional structure of Ca$^{2+}$-saturated cardiac TnC, which reveals an unexpected compact regulatory domain as a direct consequence of an inactive Ca$^{2+}$-binding site I. These results provide a structural precedent for a Ca$^{2+}$-binding regulatory protein in which one of the two sites in the paired set of EF-hands is inactive (for example, some invertebrate TnCs also have this feature; Ref. 31). This unique structural feature sets cardiac TnC apart from other “calcium sensor” EF-hand Ca$^{2+}$-binding proteins such as skeletal TnC and calmodulin, as well as “calcium buffer” EF-hand proteins such as parvalbumin and calbindin. The compact regulatory domain is a surprising result because it violates the general rule with Ca$^{2+}$-binding proteins that a small conformational change accompanies Ca$^{2+}$ binding in buffering proteins, and that a large conformational change accompanies Ca$^{2+}$ binding in regulatory proteins such as cardiac TnC (32).

Thus, it has long been believed that the mechanism for the activation of cardiac TnC involves the exposure of a large hydrophobic patch upon Ca$^{2+}$ binding as observed for other calcium sensors. In fact, cardiac TnC models based on the conformational changes observed in skeletal TnC have been widely used to interpret the functional, Ca$^{2+}$-binding and drug-binding properties of cardiac TnC (7–9), despite the unique inactive Ca$^{2+}$-binding site I in cardiac TnC. The present results show that the hydrophobic exposure in the Ca$^{2+}$-saturated regulatory domain of cardiac TnC (Fig. 4B) is dramatically

$^3$ L. Spyropoulos, M. X. Li, S. K. Sia, S. M. Gagné, M. Chandra, R. J. Solaro, and B. D. Syles, unpublished data.
The program RASTER3D (37). occupied by Ca$^{2+}$. On the other hand, as an alternative to the chemical environment of Met$^{81}$, which is mostly buried in this structure (accessible surface area of $14 \text{ Å}^2$), changes upon the binding of cardiac TnI (33). This may also imply that cardiac TnC opens up to different degrees in response to events of muscle contraction such as TnI phosphorylation. At present, there is no compelling evidence to either favor or discount either model for cardiac TnI-TnC binding.

Cardiac TnC is a potential target in therapy for patients with acute myocardial infarctions and subsequently congestive heart failure, where the diseased myocardium is “desensitized” to increases in cytosolic Ca$^{2+}$ levels. A novel group of positive inotropic agents known as “calcium sensitizers” (10) is known to increase the affinity of cardiac TnC for Ca$^{2+}$, possibly by binding to a hydrophobic patch in the N-domain of cardiac TnC (8). The exposed hydrophobic patches in Ca$^{2+}$-saturated cardiac TnC can now be identified (Fig. 4B). Although several residues (e.g., Phe$^{77}$, Met$^{81}$, and Met$^{85}$) have been implicated in earlier studies as possible binding sites for these drugs (8), the proposed modes of binding must now be re-evaluated since most of these residues lie on the side of the D-helix facing the B-helix, and therefore are more buried by the B-helix than previously suspected (B-D interhelical distance of 12 Å for cardiac TnC versus 18 Å for skeletal TnC). In addition, significant surface topology differences between the cardiac TnC model and the solution structure (Fig. 4) warrants for a reinterpretation of most of the previous drug binding studies performed based on the now-disproved model (8). Thus, the

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4 A. L. Hazard, N. L. Stricker, J. A. Putkey, and J. J. Falke, unpublished data.
solution structure of cardiac TnC presented here will allow for the accurate modeling of the binding of calcium-sensitizing drugs to cardiac TnC, in addition to revealing the structural basis for the regulation of cardiac versus skeletal muscle contraction.

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