Structure of the C-domain of Human Cardiac Troponin C in Complex with the Ca$^{2+}$ Sensitizing Drug EMD 57033*

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Ca$^{2+}$ binding to cardiac troponin C (cTnC) triggers contraction in heart muscle. In heart failure, myofilaments response to Ca$^{2+}$ are often altered and compounds that sensitize the myofilaments to Ca$^{2+}$ possess therapeutic value in this syndrome. One of the most potent and selective Ca$^{2+}$ sensitizers is the thiodiazinone derivative EMD 57033, which increases myocardial contractile function both in vitro and in vivo and interacts with cTnC in vitro. We have determined the NMR structure of the 1:1 complex between Ca$^{2+}$-saturated C-domain of human cTnC (cCTnC) and EMD 57033. Favorable hydrophobic interactions between the drug and the protein position EMD 57033 in the hydrophobic cleft of the protein. The drug molecule is orientated such that the chiral group of EMD 57033 fits deep in the hydrophobic pocket and makes several key contacts with the protein. This stereospecific interaction explains why the (−)-enantiomer of EMD 57033 is inactive. Titrations of the cCTnC-EMD 57033 complex with two regions of cardiac troponin I (cTnI$^{115–131}$ and cTnI$^{128–147}$) reveal that the drug does not share a common binding epitope with cTnI$^{128–147}$ but is completely displaced by cTnI$^{115–131}$. These results have important implications for elucidating the mechanism of the Ca$^{2+}$ sensitizing effect of EMD 57033 in cardiac muscle contraction.

In order to function properly, heart muscle must respond efficiently to the transient increases in cytosolic Ca$^{2+}$ levels in the myocardial cell. In the syndrome of heart failure, there is strong evidence that the amount of Ca$^{2+}$ available for contraction is depressed as is the maximum force generating capability of the myofilaments (for reviews, see Refs. 1–3). Treating this condition by elevating intracellular Ca$^{2+}$ has the potential threat of inducing arrhythmias. In contrast, the ability to sensitize cardiac muscle to Ca$^{2+}$ has the considerable advantage of increasing tension with little or no change in intracellular Ca$^{2+}$ and thus no increase in the energy required to release and transport Ca$^{2+}$ (for a review, see Ref. 1). A logical target for such Ca$^{2+}$ sensitizing drugs is cardiac troponin C (cTnC)$^1$ due to its role as the Ca$^{2+}$ binding receptor on the thin filament of cardiac muscle.

cTnC is a member of the EF-hand family of Ca$^{2+}$-binding proteins. Its structure represents a dumbbell with the N- and C-domains connected by a flexible linker in solution (4). Both domains contain a core of hydrophobic residues. Once exposed, these hydrophobic residues are essential for the binding of cTnI to cTnC and transmitting the Ca$^{2+}$ signal to other proteins in the thin filaments, and ultimately signal the activation of the myosin-actin ATPase reaction (for reviews, see Refs. 5 and 6). Structural studies have shown that the apo N-domain of cTnC (cNTnC) adopts a “closed” conformation with most of its hydrophobic residues buried (7), like the apo N-domain of sTnC (sNTnC) (8, 9). However, the binding of Ca$^{2+}$ has strikingly different structural consequences in cNTnC and sNTnC. In sNTnC, the N-domain switches from a closed to an “open” conformation upon binding Ca$^{2+}$ (9), while the N-domain of cTnC remains in a closed state in the Ca$^{2+}$ bound state (7). Consequently, a large hydrophobic surface is exposed in the Ca$^{2+}$-saturated sNTnC, but not in the Ca$^{2+}$-saturated cNTnC. This is mainly due to the fact that sNTnC contains two functional Ca$^{2+}$-binding sites, while cNTnC contains only one (10).

The exposed hydrophobic surface on the Ca$^{2+}$-saturated sNTnC has been shown as the sNTnC-binding site (11–14). Although Ca$^{2+}$ binding to cNTnC induces little structural changes, it sets the stage for cTnI binding. In the end, both cNTnC and sNTnC adopt similar conformations in binding their respective TnI regions. Specifically, sTnI$^{115–131}$ was found to bind to the hydrophobic cleft of Ca$^{2+}$-saturated sNTnC (13) and the corresponding cTnI$^{147–161}$ also interacts with the hydrophobic cleft of Ca$^{2+}$-saturated cNTnC and stabilizes the opening conformation of cNTnC (15). This region of TnI has been identified by many biological and biophysical studies to be the region responsible for binding to the regulatory domain of TnC and this interaction modulates the binding of the N-terminal and inhibitory regions of TnI to the C-domain of TnC (for reviews, see Refs. 16 and 17).

Unlike the apo N-terminal domain, the apo C-terminal do-

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1 The abbreviations used are: TnC, troponin C; cTnC, cardiac troponin C; cCTnC, C-domain of cTnC; cNTnC, N-domain of cTnC; sTnC, skeletal troponin C; sNTnC, N-domain of sTnC; sTnC, C-domain of sTnC; cTnI, cardiac troponin I; cTnI$^{128–147}$, synthetic peptide (residues 128–147) of cTnI; cTnI$^{147–161}$, synthetic peptide (residues 147–161) of cTnI; sTnI, skeletal troponin I; sTnI$^{115–131}$, synthetic peptide (residues 96–115) of sTnI; sTnI$^{140}$, synthetic peptide (residues 1–40) of sTnI; CaM, calmodulin; HSQC, heteronuclear single-quantum coherence; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy; DIPSI, decoupling in the presence of scalar interactions; r.m.s.d., root mean square deviation.
main in both sTnC and cTnC possesses a largely unstructured state. Upon binding two Ca\(^{2+}\) ions, this domain folds into a compact globular structure (18, 19) and exhibits a similar but slightly less open conformation as that of the Ca\(^{2+}\)-saturated sTnC. Two regions of TnI have been shown to interact with the Ca\(^{2+}\)-bound C-domain (12). These include the inhibitory region corresponding to sTnI\(_{25-115}\) or cTnI\(_{129-147}\), and the N-terminal region corresponding to sTnI\(_{1-40}\) or cTnI\(_{34-71}\). The inhibitory region is the critical functional region in the interaction of TnC with TnI and its movement from TnC to actin-tropomyosin is believed to be the major switch between muscle contraction and relaxation, while the N-terminal region plays primarily a structural role (12). NMR studies of the inhibitory peptides have yielded some structural information on the interaction of inhibitory region with TnC (20–22), however, the exact binding sites for the inhibitory region on TnC has been under debate. The crystal structure of TnC in complex with sTnI\(_{1-47}\) has shown that sTnI\(_{1-32}\) forms a long α-helix and binds to the hydrophobic groove of sTnC (23). The corresponding region of cTnI\(_{132-80}\) has also been shown to bind within the hydrophobic patch of cTnC (18).

In view of the importance of the exposed hydrophobic surfaces on both domains of cTnC for the binding of cTnI, it is clear that one way to enhance the Ca\(^{2+}\) sensitivity of cardiac muscle would be to stabilize the interaction of cTnC and cTnI by amplifying the hydrophobic cTnI-binding interface on cTnC. This can be accomplished by employing certain pharmacological agents that bind to the hydrophobic cleft but do not interfere with cTnC-cTnI interaction. Indeed, a variety of small hydrophobic compounds including the calmodulin antagonists bepridil, trifluoperazine, and calmidazolium have been shown to interfere with cTnC-cTnI interaction. However, most of these compounds primarily a structural role (12). NMR studies of the inhibitory region explains why the (+)-enantiomer of EMD 57033 makes several key contacts with the protein. This stereospecific group of EMD 57033 fits deep in the hydrophobic pocket and makes several key contacts with the protein. This stereospecific interaction explains why the (+)-enantiomer of EMD 57033 is inactive. This structure provides a structural basis for the Ca\(^{2+}\)-sensitizing effect of EMD 57033. We also examined the possible competition of the drug with cTnI peptides and found that the drug does not share the common binding epitope with the inhibitory region of cTnI but is displaced completely by the N-terminal region of cTnI. These results have important implications in understanding the mechanism underlining Ca\(^{2+}\) sensitizing effects of EMD 57033 in cardiac muscle contraction.

**EXPERIMENTAL PROCEDURES**

**Sample Preparation**—The engineering of the expression vector for the cCTnC-(191–161) protein was as described in Chandra et al. (36). The expression and purification of [\(^{15}N\)]- and [\(^{15}N\)\(^{13}C\)]-labeled protein in E. coli follows the procedures as described previously for sTnC (37), except a further purification step was done using a Superdex-75 column (Amersham Pharmacia Biotech) with a buffer containing 0.15 M NaCl and 50 mM Tris, pH 8.0. The handling and characterization of the stock solutions of EMD 57033 are as described in Li et al. (35). Two stock solutions (94 and 53 mM, respectively) of EMD 57033 in MeSO-\(d_6\) were prepared. The synthetic peptides cTnI\(_{129-147}\), taty-QKIFDLRGGKPRTLRRV-amide, and cTnI\(_{34-71}\), acetyl-ADKKSISASKRIQLKTLIIAQKQLEEREGK-amide were prepared as described for sTnI\(_{125-115}\) and sTnI\(_{1-40}\) by Triplet et al. (12). Solid peptides were dissolved in double distilled water to make stock solutions. The concentrations were 60 mM for cTnI\(_{129-147}\), and 32 mM for cTnI\(_{34-71}\), respectively, as determined by amino acid analysis. All NMR samples were 500 μl in volume. The buffer conditions were 100 mM KCl, 10 mM imidazole, 0.2 mM 2,2-dimethyl-2-silapentanesulfonic acid, and 0.01% NaN\(_3\) in 90% H\(_2\)O, 10% D\(_2\)O, and the pH was 6.7. For structure determination, NMR samples contained 1–2 mM [\(^{15}N\)]-cTnC or [\(^{15}N\)\(^{13}C\)]-cTnC saturated with 10 mM Ca\(_{2+}\). The EMD 57033 was titrated to the Ca\(_{2+}\)-saturated cTnC until ratios reached 1:1. The NMR samples used for titrations with cTnI\(_{129-147}\) or cTnI\(_{34-71}\) were prepared in a similar manner as described above. The titrations of cCTnC by cTnI\(_{129-147}\) or cTnI\(_{34-71}\) follow the procedures as described previously (35).

**Stability of EMD 57033 in Aqueous Solution**—EMD 57033 is only marginally soluble in aqueous solutions. Once it is bound to cTnC, the complex is stable and is stable for 4–5 days, which is long enough for the typical three-dimensional NMR experiment. Two-dimensional (\([\(^{1}H\),\(^{15}N\)]-HSQ NMR spectra acquired before and after every three-dimensional NMR experiment were compared and their identity was taken as an indication of sample stability throughout the three-dimensional NMR experiment. However, EMD 57033 tends to dissociate from the complex and precipitates out of the aqueous environment after 4–5 days. This is reflected in the disappearance of the EMD 57033-induced chemical shift changes (Fig. 1), which, however, can be reproduced upon the addition of more EMD 57033. This indicates that the protein is still intact and it is the drug that is disappearing from the solution. \(^{15}N\)-Filtered DIPSI spectra of EMD 57033 in the cCTnC-EMD 57033 NMR sample detected no new resonances except those of intact EMD 57033, implying that the drug is not breaking down, but precipitating out of the solution. This conclusion was further supported by evidence from mass spectrometry measurements. \(^{1}H\) NMR spectra were used to check any possible modification of EMD 57033 by chemicals present in the NMR buffer, such as imidazole and NaN\(_3\), which have been shown to interact with leusovemandin (38), and the results show no sign of reaction between EMD 57033 and the chemical reagents.

**NMR Spectroscopy**—Most NMR data used in this study were collected at 30 °C using Unity 600 MHz, Unity Inova 500 MHz, and Inova 800 MHz spectrometers. All three spectrometers are equipped with triple resonance probes and Z-pulsed field gradients (XYZ gradients for the 800 MHz). Unity 300 spectrometer was also used to collect the spectra of EMD 57033 in MeSO-\(d_6\). Two-dimensional (\([\(^{1}H\),\(^{15}N\)]-HSQC NMR spectra were acquired using the sensitivity enhanced gradient pulse sequence developed by Lewis E, Ray and co-workers (39, 40). For cCTnC in the cCTnC-EMD 57033 complex, the chemical shift assignments of the backbone and side chain atoms and NOE interpretations...
distance restraints were determined using the two-dimensional and three-dimensional NMR experiments described in Table I. For the bound EMD 57033, the proton chemical shifts of the drug were assigned by using two-dimensional $^{15}$N/13C-filtered NOESY (80 ms mixing time) and two-dimensional $^{15}$N/13C-filtered DIPSI experiments (43.2-ms spin-lock time). The pulse sequences for these two experiments were based on Ogura et al. (41) with extensive modifications done in this lab (Leo Spyracopoulos, University of Alberta). One-dimensional $^1$H and two-dimensional DIPSI spectra of EMD 57033 in Me$_2$SO-$d_6$ were obtained using the Unity 300 MHz spectrometer. The intermolecular proton distances were obtained using three-dimensional $^{15}$N/13C-filtered F$_3$-filtered, F$_2$-edited NOESY HSQC experiments employing linear frequency ramped broadband inversion pulses for $^1$H (80-ms mixing time) (42).

Data Processing and Peak Calibration—All two-dimensional and three-dimensional NMR data was processed using NMRPipe (43), and all one-dimensional NMR data were processed using VNMR (Varian Associates). The spectra were analyzed using NMRView (44). For cCTnC in the complex, intramolecular distance restraints obtained from the NOESY experiments were calibrated according to Gagné et al. (45). Intramolecular proton distances for EMD 57033 in the complex were calibrated based on NOEs corresponding to known distances (neighboring protons on aromatic ring are separated by 2.48 Å). Intermolecular NOEs obtained from the $^{15}$N/13C-filtered/edit ed experiment were categorized as either strong (1.8–3.0 Å), medium (1.8 to 4.0 Å), or weak (1.8 to 5.5 Å). Dihedral angle restraints were derived from data obtained from HNHA, HNHB, and NOE-HSQC experiments according to Sia et al. (4).

Structural Calculations—Using an initial set of intramolecular NOE restraints for cCTnC, 100 structures of cCTnC without EMD 57033 were calculated starting from an extended conformation. The calculations were done using simulated annealing protocol implemented in X-PLOR (46) with 10,000 high-temperature steps (time step of 30 ps) and 6000 cooling steps (time step of 30 ps). Approximately 50% of the initial structures converged. These structures were used as templates for further rounds of refinements. Dihedral angle restraints and 12 artificial distance restraints from chelating oxygens to the two Ca$^{2+}$ ions were added at later stages of the refinement process. The structure of the cCTnC-EMD 57033 complex were calculated starting from the extended conformations of cCTnC and EMD 57033 using simulated annealing protocol with the same conditions as above. The calculations were carried out using the distance and dihedral restraints for cCTnC, 12 distance restraints to Ca$^{2+}$ ions, 14 intramolecular distance restraints between cCTnC and EMD 57033, as well as 8 intramolecular distance restraints for the bound EMD 57033 molecule (see Table II). The final family of solution structures presented in this article consists of 30 of the lowest energy structures.

Coordinates—The coordinates for the structure have been deposited in the RCSB Protein Data Bank (1IH0). Chemical shifts assignments for cCTnC and EMD 57033 have been deposited in the BioMagResBank (4994).

RESULTS AND DISCUSSION

Structure of the cCTnC-EMD 57033 Complex—We have shown clearly that EMD 57033 forms a 1:1 complex ($K_D = 8 \mu M$) with intact cTnC and the binding site resides in the C-domain (35). For the purpose of this study, we have made a complex between an isolated Ca$^{2+}$-saturated C-domain (residues 91–161) of human cTnC and EMD 57033. This reduces the NMR spectral overlap and facilitates the structure determination process. The two-dimensional $[^1$H,${^15}$N]-HSQC NMR spectrum of the Ca$^{2+}$-saturated cCTnC, shown in Fig. 1, indicates that it is a well structured domain characterized by the dispersion of amide proton signals. Titration of EMD 57033 induces progressive shifts of the cross-peaks. All the chemical shift changes fall into the fast exchange limit on the NMR time scale. The linear movement of the cross-peaks indicates that EMD 57033 binding to cTnC occurs with a 1:1 stoichiometry. At the end of titration, a stable 1:1 cCTnC-EMD 57033 complex ($K_D = 10 \mu M$) was formed.

The two-dimensional $[^1$H,$^15$N]-HSQC NMR spectrum (Fig. 1) of cCTnC in the cCTnC-EMD 57033 complex was highly resolved, which allowed the chemical shifts of the backbone and the side chain atoms to be readily assigned using $^{15}$N- or $^{13}$C-labeled protein. Distance restraints for cCTnC in the cCTnC-EMD 57033 complex were obtained by analyzing three-dimensional $^{15}$N- or $^{13}$C-NMR experiments. Dihedral angle restraints for cCTnC in the cCTnC-EMD 57033 complex were obtained from three-dimensional HNHA and HNHB experiments. The NMR experiments performed are summarized in Table I.

The proton NMR chemical shifts assignments and intramolecular distance restraints for the bound EMD 57033 required the collection of $^{15}$N/13C filtered two-dimensional DIPSI and two-dimensional NOE-HSQC experiments using unlabeled drug bound to $^{15}$N/13C-labeled cCTnC. These experiments removed all of the resonances arising from $^{15}$N/13C-labeled cCTnC. One-
Structure of the C-domain of Cardiac Troponin C Bound to EMD 57033

The two EF-hands are joined by a short twisted (residues 111–113, 147–149) is well defined with a backbone distance restraints for cCTnC, and 12 restraints to Ca²⁺ including 974 for cCTnC, 8 for EMD 57033, 14 intermolecular distance restraints (approximately 14 restraints per residue) all the intramolecular NOEs. A total of 1000 experimental restraints for cCTnC, and 12 restraints to Ca²⁺ because of a lack of calibration for those NOEs (see "Experimental Procedures").

The four helices, E, F, G, and H, are well defined, superimposing with individual backbone r.m.s.d.s of 0.23 Å (E, residues 95–103), 0.27 ± 0.09 Å (F, residues 114–123), 0.36 ± 0.11 Å (G, residues 130–140), and 0.26 ± 0.07 Å (H, residues 150–156). The two EF-hands are joined by a short twisted antiparallel β-sheet. The two Ca²⁺-binding sites are relatively well defined with backbone r.m.s.d. of ∼0.61 Å. The β-sheet (residues 111–113, 147–149) is well defined with a backbone r.m.s.d. of 0.29 ± 0.08 Å. The N- and C-terminal residues (residues 91–94, and 158–161) are less well defined (r.m.s.d., 1.4 ± 0.4) than the helices and the β-sheet. EMD 57033 consists of three main organic groups, which are the thiadiazinone (A ring in Fig. 3A), the tetrahydroquinolinyl (B and C rings in Fig. 3A), and the dimethoxybenzoyl (D ring in Fig. 3A) moieties, respectively. The three functional groups have rigid conformations, however, the bonds that connect the thiadiazinone-tetrahydroquinolinyl units and the tetrahydroquinolinyl-dimethoxybenzoyl units can rotate freely (see Fig. 3A). The drug molecule is completely assigned and the chemical shifts for all the protons are labeled in Fig. 3A. Eight intramolecular NOEs of the bound drug were observed and shown in Fig. 3, B and C.

The relative orientations of the three moieties in the cCTnC-EMD 57033 complex are determined by both the intramolecular NOEs within the drug molecule and the intermolecular NOEs between cCTnC and the drug. In the ensemble of structures (Fig. 2A), the interplanetary angles for the thiadiazinone-tetrahydroquinolinyl and the tetrahydroquinolinyl-dimethoxybenzoyl units are 44° ± 21° and 69° ± 14°, respectively. When the heavy atoms of EMD 57033 superimpose onto the average structure in the cCTnC-EMD 57033 complex, the average r.m.s.d. is 0.29 ± 0.07 Å. This r.m.s.d. increases to 1.15 Å when the backbone atoms of residues 95–158 of cCTnC in the ensemble of solution structures for the cCTnC-EMD 57033 complex are superimposed onto the average cCTnC structure. As a result, the stereochemical quality of the drug molecule assembly is slightly lower than the structures of cCTnC in the cCTnC-EMD 57033 complex. This is due in part to the loosely imposed intermolecular restraints between cCTnC and EMD 57033 because of a lack of calibration for those NOEs (see "Experimental Procedures").

Binding Interface between EMD 57033 and cCTnC in the cCTnC-EMD 57033 Complex—Strip plots taken from the three-dimensional ¹⁵N/¹³C F₂-filtered, F₂-edited spectrum of the cCTnC-EMD 57033 complex are shown in Fig. 4A. In this spectrum, only NOEs arising from the drug protons (attached to ¹²C) and terminating on protein protons (attached to ¹³C) are observed. For example, the H78 methyl protons from the thiadiazinone unit show strong NOE contacts with the protons attached to the methyl groups of Leu¹¹⁷ located in the H-helix (Fig. 4B, c) and of Ile¹¹² and Ile¹⁴⁸ located in the β-sheet of cCTnC (Fig. 4B, a); the H9, H12, and H13 protons from the B ring (Fig. 3A) show NOE contacts with the protons attached to the methyl groups of Leu¹³⁶ located in the G-helix (Fig. 4B, b); and the two H17 protons from the C ring (Fig. 3A) show NOE contacts with the protons attached to the methyl groups of Leu¹³³ located in the H-helix (Fig. 4B, a).

### Table I

| Experiment name | Nuclei | ¹H freq. | nt | x-pts | y-pts | z-pts | z-sw | y-sw | z-sw | Mix | Ref. |
|-----------------|-------|----------|----|-------|-------|-------|------|------|------|-----|------|
| Two-dimensional [¹H,¹³C]-HSQC | ¹H,¹³C | 500 | 32 | 1024 | 128 | 7000 | 3771 | 60 |
| Two-dimensional [¹H,¹⁵N]-HSQC | ¹H,¹⁵N | 600 | 8 | 960 | 96 | 7500 | 1800 | 39, 40 |
| Three-dimensional ¹⁵N-edited DIPSI-HSQC | ¹H,¹⁵N | 600 | 8 | 960 | 128 | 32 | 7500 | 6400 | 1642 | 80.2 | 40 |
| Three-dimensional ¹⁵N-edited NOESY-HSQC | ¹H,¹⁵N | 500 | 16 | 1024 | 128 | 32 | 7500 | 5500 | 1500 | 80 | 40 |
| HNHA | ¹H,¹⁵N | 500 | 8 | 896 | 48 | 48 | 7000 | 4000 | 1408 | 61 |
| HNHB | ¹H,¹⁵N | 500 | 8 | 1024 | 96 | 32 | 7000 | 5400 | 1408 | 62 |
| CBCA(CO)NNH | ¹H,¹⁵N | 500 | 16 | 768 | 50 | 32 | 6000 | 7794 | 1500 | 63 |
| HNCA(CO)NN | ¹H,¹⁵N | 500 | 16 | 888 | 50 | 32 | 6000 | 7794 | 1500 | 63 |
| HCC(TOC)SY | ¹H,¹⁵N | 500 | 16 | 768 | 128 | 32 | 6000 | 3000 | 1996 | 64 |
| Three-dimensional ¹⁵N/¹³C-filtered NOESY | ¹H,¹⁵N/¹³C | 600 | 16 | 810 | 128 | 32 | 7500 | 6500 | 3165 | 80 | 65 |
| Two-dimensional ¹⁵N/¹³C-filtered NOESY | ¹H(¹⁵N/¹³C),¹H,¹⁵N/¹³C | 500 | 32 | 4096 | 512 | 6200 | 6200 | 80 | 41 |
| Two-dimensional ¹⁵N/¹³C-filtered DIPSI | ¹H(¹⁵N/¹³C),¹H,¹⁵N/¹³C | 500 | 32 | 4096 | 512 | 6200 | 6200 | 43.2 | 41 |
| Three-dimensional ¹⁵N/¹³C-filtered/edited NOESY | ¹H(¹⁵N/¹³C),¹H,¹⁵N/¹³C | 500 | 16 | 640 | 128 | 32 | 6000 | 5000 | 3000 | 80 | 42 |

a The number of transients acquired for each FID.
b The frequency of ¹H in MHz.
c The number of transients acquired for each FID.
d x,y,z-pts and sw is the number of complex points and sweep width in each respective dimension (x is the directly detected dimension).
e Mixing in milliseconds. In the case of DIPSI or TOCSY experiments, this is the spin-locking time.

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3 L. Spyracopoulos, unpublished data.
Met157 located in the H-helix (Fig. 4B, d). These experimental intermolecular restraints serve to orient the drug molecule in the cTnC-EMD 57033 complex. The protein is shown in dark red and Ca$^{2+}$ ions are shown as cyan spheres. The thiadiazinone functional group of the drug is colored in blue; the tetrahydroquinolyl group of the drug is colored in gold; and the dimethoxybenzoyl group is shown in gray. C, molecular surface of cTnC in the cTnC-EMD 57033 complex. The side chain atoms of hydrophobic residues (Ala, Ile, Leu, Met, Phe, and Val) are shown in yellow. Negatively charged residues (Asp and Glu) in red, positively charged residues (Arg and Lys) in blue. Polar residues (Ser, Thr, and Tyr) are shown in cyan. EMD 57033 is embedded in the hydrophobic cleft of the protein. The orientation of the complex is the same as in B. C was created with the program GRASP (47).
Restraint violations

| Restraint type | Total | intra-residue | sequential (|j - j'| = 1) | Medium-range (2 ≤ |j - j'| ≤ 4) | Long-range (|j - j'| ≥ 5) |
|----------------|-------|---------------|---------------- |---------------- |---------------- |
| NOE restraints | 1000 (approximately 14/residue) | 432 | 251 | 197 | 120 |

Artificial restraints to Ca$^{2+}$

- 94–123, 128, 130–160.

The r.m.s.d. for helices are calculated from EMD 57033-induced structural opening of cCTnC is also measured by an increase of ~20 Å$^2$ of exposed apolar surface area for cCTnC in the cCTnC-EMD 57033 complex, compared with the free C-domain of cTnC. Although EMD 57033 induced little G/H interhelical angle changes, the multiple contacts between Leu$^{136}$ of the G helix with several ring protons of EMD 57033 resulted in a more flexible G helix in the present structure than the C-domain in the cTnC structure (r.m.s.d. of 0.36 Å for cCTnC in the cCTnC-EMD 57033 versus 0.24 Å for the C-domain in cTnC). Another significant EMD-induced change on cCTnC is the unwinding of residues 156–161 at the end of helix H. This last helix has always been well defined to the C-terminal end in many EF-hand domains such as sNTnC (9, 48), cTnC (7), and CaM domains (49). However, in this complex, helix H ends at residue 156, the last portion of the C-terminal residues (157–161) forms an extended structure and appears to be more flexible than usual. This can be attributed to the interactions between EMD 57033 and residue Met$^{157}$ of cCTnC.

Interestingly, the same extended conformation at the end of the H helix was observed in the C-domain of sTnC bound to the N-terminal region of sTnI (sTnI$_{128–147}$) (23). This suggests that the nature of the interactions between EMD 57033 and cCTnC may be similar to that between sTnI$_{128–147}$ and sTnC. An overlay (Fig. 5B) of the cTnC in the cTnC-EMD complex and sCTnC in the cTnC-sTnI$_{128–147}$ complex shows that the backbone of cCTnC and sCTnC adopt very similar conformations (r.m.s.d. = 1.3 Å). It also shows that the binding of EMD 57033 to cCTnC possesses similar features as the binding of sTnI$_{128–147}$ to sTnC. In fact, residues Ile$^{110}$, Leu$^{115}$, Phe$^{119}$, Leu$^{134}$, Ile$^{146}$, and Met$^{155}$ of sCTnC, corresponding to the six EMD 57033-contacting residues in cCTnC, are all involved in the interaction with sTnI$_{128–147}$ (23). It is interesting to note that binding of sTnI$_{128–147}$ to sTnC does not greatly perturb the fold of this domain from a peptide-free conformation. In the free state, sCTnC exhibits E/F and G/H interhelical angles of ~105° and ~115°, respectively (Table III). These angles do not change very much upon sTnI$_{128–147}$ binding to sTnC. On the other hand, both EMD 57033 and cTnI$_{33–80}$ induce interhelical angle changes in cTnC and the bound cCTnC exhibits a more open conformation. This is analogous to the ligand induced structural changes in the N-domain of sTnC and cTnC. Ca$^{2+}$ binding switches sNTnC from a closed to open conformation (9) and the open sTnC is ready to bind sTnI$_{128–147}$ with no need to open further (13). On the other hand, Ca$^{2+}$ binding to cTnC induces little structural changes but sets the stage for cTnI binding and cTnC undergoes a large closed to open transition upon binding cTnI$_{147–163}$ (15) or Bepridil (50). Thus, the interaction of both domains of cTnC with cTnI would require more free energy than those of sTnC with sTnI, because cTnI has to overcome the energy barrier of opening the domains of cTnC (see discussions in McKay et al. (51)). This energy cost may be compensated by employing pharmacological agents, which bind to both domains of cTnC and thereby help to stabilize the open conformation of cTnC. The ideal situation would be that the interaction between the drug and cTnC occurs without interfering with cTnI binding to cTnC. The analysis presented herein raises issues about the effect of EMD 57033 on the interaction of cTnC and cTnI. Since two regions of cTnI (cTnI$_{125–147}$ and cTnI$_{34–71}$) were identified to interact with the C-terminal domain of cTnC (12), we examined the binding of these two synthetic peptides (cTnI$_{125–147}$ and cTnI$_{34–71}$) to the cCTnC-EMD 57033 complex as discussed below.

**Competition of EMD 57033 with cTnI$_{128–147}$ and cTnI$_{34–71}$—**

Previously, we have shown that both EMD 57033 and cTnI$_{128–147}$ bind with a 1:1 stoichiometry to cTnC but do not compete for the same binding sites on cTnC (35). EMD 57033 binding is not affected by cTnI$_{128–147}$ nor does the drug affect cTnI$_{128–147}$ binding. In the end, a stable ternary cTnC-EMD 57033-cTnI$_{128–147}$ complex is formed. In the present work, sim-
imilar titrations were performed on cCTnC and similar conclusions were obtained (data not shown). Both EMD 57033 and cTnI_{128–147} can bind to cTnC simultaneously and the binding affinities ($K_D = 10 \mu M$ for EMD 57033 and $K_D = 100 \mu M$ for cTnI_{128–147}) are similar to those determined from binding to intact cTnC (35). However, unlike cTnI_{128–147}, the N-terminal region of cTnI, cTnI_{34–71}, can displace EMD 57033 completely from cCTnC. When the 38-residue cTnI_{34–71} peptide is titrated to the Ca$^{2+}$-saturated cCTnC, it binds tightly ($K_D = 1 \mu M$) to form a stable cCTnC-cTnI_{34–71} complex. Fig. 6A shows a superimposition of the two-dimensional $^{1}H,^{15}N$-HSQC NMR spectra of cCTnC-EMD 57033 and cCTnC-cTnI_{34–71}. The only difference between Fig. 6, A and B, is the starting spectra. The former started with the spectrum of cCTnC and the latter started with the spectrum of cCTnC-EMD 57033. The end spectra are identical and represent that of the cCTnC-cTnI_{34–71} complex. Thus, cTnI_{34–71} associates tightly with cCTnC regardless of the presence of EMD 57033.

It is necessary to put these results into perspective with respect to the interaction of cTnC and cTnI. The noncompetitive binding sites of cTnI_{128–147} and EMD 57033 on cTnC (35) or cCTnC (present data) suggest that the inhibitory region of cTnI does not block the binding of EMD 57033 to cTnC, and vice versa. This conclusion is important in terms of addressing the Ca$^{2+}$ sensitizing role of EMD 57033 because the inhibitory region of cTnI constitutes a major switch between muscle contraction/relaxation by moving between cTnC and actin-tropomyosin (52). A good Ca$^{2+}$ sensitizer would not interfere with the inhibitory function of cTnI. A pertinent question is whether

**Fig. 3.** A, the chemical structure of EMD 57033. The four rings that form the three functional groups are labeled as A-D. The two bonds that connect the three units are indicated by **curved arrows** to emphasize their ability to rotate freely. The number designation and the chemical shift assignments of most protons of the drug are indicated. The residues of cCTnC, which are involved in interacting with protons of EMD 57033, are also indicated. B and C, strips from the two-dimensional $^{15}N/^{13}C$-filtered-NOESY NMR experiment showing the intramolecular NOE contacts of EMD 57033.
**FIG. 4.** NOE contacts between the protein and the drug. *A*, strip plots from $^{13}$C portion of the three-dimensional $^{15}$N/$^{13}$C-filtered/edited-NOESY NMR experiment illustrating the NOEs between cCTnC and EMD 57033. The carbon chemical shift is shown on the left. The cCTnC proton to which the strip corresponds is labeled on the right. The proton chemical shifts of the drug are indicated at the top. The two peaks circled in the Leu$^{121}$,C$_6$ strip are artifacts in the spectra. *B*, detailed views of the interactions between EMD 57033 and cCTnC. Dotted lines represent the NOE distances observed in *A*.

**FIG. 5.** *A*, the backbone overlay of cCTnC in the cCTnC-EMD 57033 complex (dark red) and the free C-domain of cTnC (green), PDB accession code 1AJ4. The two Ca$^{2+}$ ions are shown as cyan-colored spheres. The three units of the drug are colored in the same scheme as in *A*. Note the slight opening of helix E and F as well as the unwinding of the C-terminal helix. *B*, the backbone overlay of cCTnC in the cCTnC-EMD 57033 complex (dark red) with the C-domain of skeletal TnC (purple), bound to sTnI$_{1-47}$ (light green), PDB accession code 1A2X. The Ca$^{2+}$ ions and EMD 57033 are colored in the same scheme as in *A*. The overlay shows that the binding of EMD 57033 to cCTnC mimics the binding of sTnI$_{1-47}$ to sCTnC.
this is also the case with intact cTnI, especially considering the present clear-cut results that EMD 57033 cannot compete with cTnI34–71 for cCTnC. The very tight association of sTnI1–40 and the sTnC have been shown by several groups. In addition to the sTnC-sTnI1–47 crystal structure (23), early functional studies of this region by Ngai and Hodges (53) have shown that sTnI1–40 can effectively compete with sTnI or sTnI96–115 inhibitory peptide for sTnC and a recent NMR study has shown that sTnI1–40 binds strongly to sTnC with a $K_D$ of $2 \mu M$ and can displace sTnI96–115 completely (19). This raises questions regarding how the inhibitory region binds to TnC to release inhibition if the N-terminal region of TnI is always present. In order to rationalize these results, two models have been proposed for the interaction of TnC and TnI. One suggests that these two regions of TnI share overlapping binding sites on the C-domain of TnC, which are alternatively occupied by either one or the other depending on the interactions between the N-domain of TnC and the C-domain of TnI or the C-domain of TnT (12). The second model proposes that the N-terminal region of TnI always binds to the C-domain of TnC, regardless of the Ca$^{2+}$-dependent interactions between the N-domain of TnC and the C-domain of TnI (23, 54, 55), while the inhibitory region interacts with the central helix area (including part of the D helix in the N-domain and part of the E-helix in the

**TABLE III**

| Calcium-binding protein | Interhelical angles (°) | Accession code |
|-------------------------|-------------------------|----------------|
| cCTnC (2Ca$^{2+}$)-EMD 57033 | 92 ± 4 113 ± 7 | 1H01 |
| C-domain of Ca$^{2+}$ saturated cTnC (2Ca$^{2+}$) | 115 ± 4 121 ± 4 | 1A34 |
| C-domain of Ca$^{2+}$ saturated sTnC (2Ca$^{2+}$) | 105 ± 6 111 ± 7 | 5TNC |
| cCTnC (2Ca$^{2+}$) bound to sTnI$_{12}$–80 | 94 ± 8 89 ± 9 | 1FI5 |
| C-domain of sTnC (2Ca$^{2+}$) bound to sTnI$_{1–47}$ | 99 109 | 1A2X |

*The parentheses indicate the number of the Ca$^{2+}$ ions contained in the domain.

* A large angle defines a closed conformation, whereas a small angle defines an open conformation. The axis for an $\alpha$-helix is defined by two points, taken as the average coordinates of the first and last 11 backbone atoms of the $\alpha$-helix.

**FIG. 6.** Two-dimensional $[^{1}H,^{15}N]$-HSQC NMR spectra of the titrations of (A) cCTnC with cTnI$_{34}$–71, (B) the cCTnC-EMD 57033 complex with cTnI$_{34}$–71. A, the cross-peaks corresponding to free cCTnC are shown in multiple contours, whereas the peaks corresponding to the cCTnC-cTnI$_{34}$–71 complex are shown as single contours. B, cross-peaks corresponding to the cCTnC-EMD 57033 complex are shown as multiple contours, whereas cross-peaks corresponding to the cCTnCcTnI$_{34}$–71 complex are shown as single contours.
C-domain) of TnC in a Ca\(^{2+}\)-dependent manner. The latter model does not adequately explain the experimental data (20, 35, 56–59), supporting a binding site for the inhibitory region of TnI primarily in the C-domain of TnC, especially when only isolated C-domains of TnC were used in the study (19, 57).

Based on our titration data of cTnI\(_{128-147}\) binding to cTnC (35) and cTnT (present work) and the competitive binding of sTnI\(_{1-40}\) and sTnI\(_{96-115}\) to sTnC (19), we suggest that cTnI\(_{128-147}\) binds primarily to the C-domain of TnC and in order for this binding to occur, the interaction between cTnI\(_{128-147}\) and cTnC has to be weakened. This can be accomplished by the mechanisms proposed for model 1 (12). Since cTnC does not act alone, and the contractile proteins work in a highly organized and cooperative manner in muscle contraction, it is possible that cTnC in myofilaments may have a lower affinity for cTnI\(_{128-147}\) than it does in isolation. Although EMD 57033 is too small to compete with the extensive contacts between the long α-helix of cTnI\(_{128-147}\) and cTnC, the fact that EMD 57033 interacts with many of the same residues on cTnC as sTnI\(_{1-47}\) on sTnC suggests that the drug may play a role in disrupting and therefore weakening the interaction of cTnI\(_{128-147}\) with cTnC in the myofilaments, and consequently, in enhancing the binding of the inhibitory region of TnI to TnC. Since this interaction is Ca\(^{2+}\)-dependent, the apparent Ca\(^{2+}\) sensitivity of the contractile system can be modulated by EMD 57033.

Implications in the Ca\(^{2+}\) Sensitizing Effect of EMD 57033 in Cardiac Muscle Contraction—The number of patients suffering from congestive heart failure is rising accompanying the aging of baby boomers. Ca\(^{2+}\) sensitizers have been proposed as a treatment for this common disease. These agents increase myocardial contractility by generating more force for a given amount of cytosolic free Ca\(^{2+}\). This allows an achievement of positive inotropic effect more economically as compared with other positive inotropic drugs that exert effect by simply enhancing Ca\(^{2+}\) influx into myocytes and therefore add intracellular Ca\(^{2+}\) overload. Numerous studies have documented the Ca\(^{2+}\) sensitizing effects of this class of agents under in vivo and in vitro conditions (for a review, see Ref. 1). Among which, EMD 57033 is one of the most potent and selective Ca\(^{2+}\) sensitizers available. Earlier physiological studies have shown that EMD 57033 exerts detectable positive inotropic effects on isolated cardiac myocytes at concentrations as low as 1 μM (27), and in skinned cardiac muscle fibers, less than 10 μM EMD 57033 induced significant Ca\(^{2+}\) sensitivity of force development (26). In a recent study, 0.3–1 μM EMD 57033 is shown to have positive inotropic effects on both normal and failing cardiac myocytes (32). The cTnC-EMD 57033 complex structure presented in this work provides a structural basis for the understanding of the mechanism underlying the Ca\(^{2+}\) sensitizing effect of EMD 57033 in cardiac muscle contraction. In the present structure of the cTnC-EMD 57033 complex, the drug molecule is orientated such that the chiral group of EMD 57033 fits deep in the hydrophobic pocket and makes several key contacts with the protein. This stereospecific interaction explains why the (−)-enantiotomer of EMD 57033 is inactive (27). Since the methyl group attached to the chiral carbon of EMD 57033 makes extensive contacts with methyl groups of Ile\(_{117}\), and Ile\(_{148}\), these contacts may be weakened or lost if the stereospecificity of the chiral carbon is changed. This is especially true for the interactions between residues on the short β-sheet and the drug. These interactions would be eliminated if the chirality of the drug has reversed, not even the rotation of ring A can completely restore all the contacts between the drug and these residues. Interestingly, the (−)-enantiotomer of EMD 57033, EMD 57439 is also capable of stimulating muscle contraction, but through a different mechanism. EMD 57439 is a potent phosphodiesterase III inhibitor, and as such, is capable of producing an increase in cAMP level inside the cell. This will result in the activation of protein kinase A and ultimately, an increase in Ca\(^{2+}\) concentration in cardiac muscle cells (26, 28). The fact that EMD 57439 has no Ca\(^{2+}\) sensitizing activity and EMD 57033 is only a weak phosphodiesterase III inhibitor points to the sensitivity of protein toward stereospecificity of ligands.

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