Defining the Binding Site of Levosimendan and Its Analogues in a Regulatory Cardiac Troponin C–Troponin I Complex†

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Received March 14, 2008; Revised Manuscript Received May 26, 2008

ABSTRACT: The interaction of Cardiac Troponin C (cTnC) and Cardiac Troponin I (cTnI) plays a critical role in transmitting the Ca2+ signal to the other myofilament proteins in the activation of cardiac muscle contraction. As such, the cTnC–cTnI interface is a logical target for cardiotoxic agents such as levosimendan that can modulate the Ca2+ sensitivity of the myofilaments. Evidence indicates that drug candidates may exert their effects by targeting a site formed by binding of the switch region of cTnI to the regulatory N domain of cTnC (cNTnC). In this study, we utilized two-dimensional 1H–15N HSQC NMR spectroscopy to monitor the binding of levosimendan and its analogues, CMDP, AMDP, CI-930, imazodon, and MPDP, to cNTnC·Ca2+ in complex with two versions of the switch region of cTnI (cTnI147–163 and cTnI144–163). Levosimendan, CMDP, AMDP, and CI-930 were found to bind to both cNTnC·Ca2+·cTnI147–163 and cNTnC·Ca2+·cTnI144–163 complexes. These compounds contain a methyl group that is absent in MPDP or imazodon. Thus, the methyl group is one of the pharmacophores responsible for the action of these pyridazinone drugs on cTnC. Furthermore, the results showed that the cNTnC·Ca2+·cTnI144–163 complex presents a higher-affinity binding site for these compounds than the cNTnC·Ca2+·cTnI147–163 complex. This is consistent with our observation that the affinity of cTnI144–163 for cTnC·Ca2+ is ~10-fold stronger than that of cTnI147–163, likely a result of electrostatic forces between the N-terminal RRV extension in cTnI144 and the acidic residues in the C and D helices of cNTnC. These results will help in the delineation of the mode of action of levosimendan on the important functional unit of cardiac troponin that constitutes the regulatory domain of cTnC and the switch region of cTnI.

Regulation of cardiac muscle contraction by intracellular Ca2+ is critical for normal heart function. This important task is accomplished by the heterotrimeric troponin complex anchored on the thin filament by its tropomyosin-binding subunit cTnT.1 The inhibitory subunit cTnI acts as a molecular switch moving from actin to the Ca2+-binding subunit cTnC as the intracellular Ca2+ concentration increases. This switch is essential for transmitting the Ca2+ signal to the other myofilament proteins, leading to tension-producing cross bridges between actin and myosin and ultimately activating cardiac muscle contraction (for recent reviews, see refs 1 and 2).

Structural studies of troponin have helped in the elucidation of the molecular mechanism that governs the switching of cTnI to cTnC. cTnC is an “elongated” molecule with two globular N and C domains connected by a flexible linker in solution (3). Apo cTnC was shown to adopt a “closed” conformation with most of its hydrophobic residues buried (4). The energy barrier for “opening” is overcome by the binding of Ca2+ and the switch region of cTnI (residues ~147–163) (5, 6). In the NMR structure of the cNTnC·Ca2+·cTnI147–163 complex (6) and the X-ray structure of the cNTnC·3Ca2+·cTnI31–210·cTnI183–288 complex (7), cTnI147–163 adopts an α-helical conformation and lies across the hydrophobic groove with contacts with key hydrophobic residues in cNTnC. This interaction initiates the movement of the adjoining inhibitory (cTnI28–147) and C-terminal (cTnI163–310) regions of cTnI away from actin and releases the inhibition of the actomyosin ATPase, leading to muscle contraction (I). The cTnI14–71 region binds tightly to cTnC in the absence or presence of cytosolic Ca2+. In the structure of cNTnC·3Ca2+·cTnI31–210·cTnI183–288, the region of residues 34–71 of cTnI forms a long α-helix and makes extensive contacts with the hydrophobic surface of cTnC. Most of the inhibitory region of cTnI is unobserved in cNTnC·3Ca2+·cTnI31–210·cTnI183–288, but a similar region of sTnI is shown to have an extended conformation and interact...
with the central helix area of sTnC in the X-ray structure of the sTnC·4Ca\(^{2+}\)·sTnI\(_{1-182}\)·sTnI\(_{156-262}\) complex (8). Specifically, the inhibitory region of sTnI was found to make electrostatic contacts with the acidic residues on the central DE-helix of sTnC, similar to those observed in the NMR solution structure of cTnC·2Ca\(^{2+}\)·cTnI\(_{128-147}\) (9, 10).

The Ca\(^{2+}\)-sensitive interaction between cNTnC and the switch region of cTnI has been proposed to be an important target for cardiac drugs (for reviews, see refs 1 and 11–15). These drugs are useful in the treatment of heart disease associated with depressed cardiac contractility. They act via a mechanism that modulates the Ca\(^{2+}\) sensitivity of troponin without an overload of Ca\(^{2+}\) that would perturb the regulation of other Ca\(^{2+}\)-based signaling pathways in cardiomyocytes, leading to a series of undesirable side effects such as arrhythmia and death. A good example is levosimendan, which has proven to be a well-tolerated and effective treatment for patients with severe decompensated heart failure (for reviews, see refs 13 and 14). Several cardiotonic agents have been shown to target the binding site formed by cNTnC and the switch region of cTnI. In the NMR structure of the cNTnC·Ca\(^{2+}\)·cTnI\(_{147-163}\)·bepridil complex, bepridil and cTnI\(_{147-163}\) bind concurrently to the hydrophobic pocket of the protein (16). In the structure of the sTnC·4Ca\(^{2+}\)·sTnI\(_{1-182}\)·sTnI\(_{156-262}\) complex, a polycyloxyethylene detergent molecule, anapole, binds to sTnTC together with the switch region of sTnI (sTnI\(_{15-113}\)). This binding mode is likely responsible for the increase in the contractile force of muscle fibers in the presence of anapole (8). Further, we have shown recently that a calmodulin antagonist, W7, binds to cNTnC and the binding can occur in the presence of the switch region of cTnI (17). Thus, these compounds, together with cTnI\(_{147-163}\), may exert their effects by altering the dynamic equilibrium between closed and open cNTnC conformations, enhancing the affinity for cTnI and thereby modulating the calcium sensitivity of troponin. Structures of cNTnC·Ca\(^{2+}\)·levosimendan or cNTnC·Ca\(^{2+}\)·cTnI\(_{147-163}\)·levosimendan are not yet available. NMR chemical shift mapping has shown that levosimendan’s primary binding site is also located in the N-domain of cNTnC in the presence of cTnI (13, 18); however, the exact binding geometry has not been established. To better delineate the binding site of levosimendan in cNTnC, we investigated the binding of levosimendan and its analogues (AMDP, CMDP, CI-930, levosimendan, and imazodan) (Figure 1) to cNTnC. The findings also showed that the pyridazinone compounds bound to cNTnC·Ca\(^{2+}\)·cTnI\(_{144-163}\) with a higher affinity than to cNTnC·Ca\(^{2+}\)·cTnI\(_{147-163}\). This correlates with our observation that the affinity of cTnI\(_{144-163}\) for cNTnC·Ca\(^{2+}\) is ∼10-fold stronger than that of cTnI\(_{147-163}\), likely a result of electrostatic forces between the N-terminal RRV extension in cTnI\(_{144-163}\) and the acidic residues in the C and D helices of cNTnC.

**EXPERIMENTAL PROCEDURES**

**Sample Preparation.** Recombinant human cNTnC (residues 1–89) with C35S and C84S mutations [denoted cNTnC(C35S/C84S)] was used in this study. The engineering of the expression vector and the expression of \(^{15}\)N-labeled proteins in *Escherichia coli* were as described previously (19). Two synthetic cTnI peptides, cTnI\(_{144-163}\) (acetyl-RISADAMMQALLGARAK-amide) and cTnI\(_{144-163}\) (acetyl-RRVRISADAMMQALLGARAK-amide), were obtained from GL Biochem Ltd. (Shanghai, China). The purity was verified by HPLC and the mass verified by electrospray mass spectrometry. CMDP, AMDP, MPDP, and imazodan were purchased from Sigma-Aldrich. AMDP and levosimendan were purchased from Kinbester Co. Ltd. (Hong Kong). CI-930 was obtained from Pfizer. Stock solutions of the compounds in DMSO-\(d_6\) (Cambridge Isotopes Inc.) were prepared, and the vials containing the solutions were wrapped in aluminum foil due to sensitivity to light. All drug stock solutions were made fresh for every titration to minimize possible time-induced degradation. The concentrations were determined gravimetrically. All NMR samples were 500 \(\mu\)L in volume. The buffer conditions were 100 mM KCl, 10 mM imidazole, and 0.17 mM DSS in a 90% H\(_2\)O/10% D\(_2\)O mixture, and the pH was 6.7. DTT, Na\(_3\)IO, and protease...
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The chiral lanthanide shift reagent Eu-(hfc)₃ (Sigma-Aldrich) was titrated into NMR samples to yield 0.86, 1.4, 1.94, 3.02, 4.1, 5.18, 6.26, 7.34, 8.42, 9.23, 12.47, 11.6, and 17.6 mM. A 13.6 mM AMDP sample was prepared, and aliquots of Eu-(hfc)₃ were added to the sample to yield 1.82, 3.6, 5.4, 7.18, and 8.65 mM Eu-(hfc)₃. The one-dimensional (1D) ¹H NMR spectrum of each compound was assigned, and the methyl was monitored throughout the titration with chiral shift reagent Eu-(hfc)₃ (Figure 2). The NMR signal from the methyl of levosimendan did not undergo splitting during the titration with Eu-(hfc)₃, indicating that the drug was of pure R stereospecificity (Figure 2A). Meanwhile, the titration of Eu-(hfc)₃ into the NMR samples of CMDP, AMDP, and CI-930 revealed a splitting of the methyl resonance, indicating that these compounds are racemic mixtures of roughly equal R and S components (Figure 2B).

Titrations of cNTnC(C35S/C84S)·Ca²⁺ with cTnI₁₄₇₋₁₆₃ and cNTnC(C35S/C84S)·Ca²⁺ with cTnI₁₄₄₋₁₆₃. The titration of [¹⁵N]cNTnC(WT)·Ca²⁺ with cTnI₁₄₇₋₁₆₃ was conducted previously (6), and the titration of [¹⁵N]cNTnC(C35S/C84S)·Ca²⁺ (0.6 and 0.7 mM, respectively) with cTnI₁₄₇₋₁₆₃ and cTnI₁₄₄₋₁₆₃ was done in this work. Both cTnI₁₄₇₋₁₆₃ and cTnI₁₄₄₋₁₆₃ are highly soluble in aqueous solution but tend to form gels at high concentrations, likely due to aggregation. Thus, no stock solution was prepared; instead, solid peptide was added at every titration point. The concentrations of [¹⁵N]cNTnC(C35S/C84S)·Ca²⁺ and the peptides were determined by amino acid analysis at every titration point, giving the peptide/protein ratios. The decrease in pH associated with peptide additions was offset by adjusting the pH to 6.7 at every titration point. Both 1D ¹H and 2D ¹H–¹⁵N HSQC NMR spectra were acquired and analyzed at every titration point. Like in the titration of [¹⁵N]cNTnC(WT)·Ca²⁺ with cTnI₁₄₇₋₁₆₃ (6), 3–3.5 equiv of cTnI₁₄₇₋₁₆₃ is required to saturate [¹⁵N]cNTnC(C35S/C84S)·Ca²⁺, whereas 1–1.1 equiv of cTnI₁₄₄₋₁₆₃ is required to saturate [¹⁵N]cNTnC(C35S/C84S)·Ca²⁺.

Titrations of cNTnC(C35S/C84S)·Ca²⁺·cTnI₁₄₇₋₁₆₃ and CMDP, AMDP, CI-930, Imazodan, and MPDP. The [¹⁵N]cNTnC(C35S/C84S)·Ca²⁺·cTnI₁₄₇₋₁₆₃ complex was formed by dissolving 1 equiv of solid protein (0.8 mM) and 3.5 equiv of solid peptide (2.8 mM) in NMR buffer, and the [¹⁵N]cNTnC(C35S/C84S)·Ca²⁺·cTnI₁₄₄₋₁₆₃ complex was formed by dissolving 1 equiv of solid protein (1.0 mM) and 1.1 equiv of solid peptide (1.1 mM) in NMR buffer. For both complexes, 4 equiv of CaCl₂ was added to saturate cNTnC, the pH was adjusted to 6.7, and a slight amount of precipitate was eliminated by filtration. The final NMR sample volume was 500 µL. The protein and peptide concentrations were determined gravimetrically and calibrated on the basis of amino acid analysis of each individual component. Both 1D ¹H and 2D ¹H–¹⁵N HSQC NMR spectra were acquired and analyzed at every titration point.

A stock solution of 97 mM levosimendan was used for the titration of [¹⁵N]cNTnC(C35S/C84S)·Ca²⁺·cTnI₁₄₇₋₁₆₃ in aliquots of 1.0, 1.0, 1.0, 2.0, 4.0, 4.0, and 4.0 µM. A stock solution of 95 mM levosimendan was used for the titration of [¹⁵N]cNTnC(C35S/C84S)·Ca²⁺·cTnI₁₄₄₋₁₆₃ in volumes of 1.0, 2.0, 5.0, 5.0, and 10.0 µM. The sample was mixed thoroughly with each addition. In both cases, levosimendan started to precipitate when the drug to protein ratio reached ~4/1. This precipitate is likely unbound levosimendan, which is insoluble in aqueous solution. The titrations were stopped shortly after the appearance of precipitation. Addition of levosimendan slightly decreased the pH, which was adjusted by adding 1 M NaOH to NMR samples.

Aliquots of 1 µL of a 100 mM CMDP stock solution were added to [¹⁵N]cNTnC(C35S/C84S)·Ca²⁺·cTnI₁₄₇₋₁₆₃ and [¹⁵N]cNTnC(C35S/C84S)·Ca²⁺·cTnI₁₄₄₋₁₆₃. The titration was stopped when white precipitate started to appear (after the 8 µL addition). The change in pH from CMDP addition was negligible.
A stock of 100 mM AMDP was added in aliquots of 0.5, 1.5, 5.0, 5.0, 8.0, and 8.0 µL of [15N]cNTnC(C35S/C84S)·Ca2+·cTnI147−163. AMDP is highly soluble in aqueous solution, and no precipitate was observed even after the final aliquot. A stock of 100 mM AMDP was added in aliquots of 0.5, 1.5, 5.0, 5.0, 8.0, and 10.0 µL to [15N]cNTnC(C35S/C84S)·Ca2+·cTnI144−163. Again, no precipitate was observed during or after the titration. The change in pH from AMDP addition was negligible.

Into a NMR tube containing [15N]cNTnC(C35S/C84S)·Ca2+·cTnI147−163 were titrated aliquots of 1.0, 5.0, 5.0, 5.0, 5.0, and 5.0 µL of a 100 mM CI-930 stock solution. To the [15N]cNTnC(C35S/C84S)·Ca2+·cTnI144−163 complex were added aliquots of 0.25, 0.5, 2.5, 5.0, 5.0, 5.0, and 5.0 µL of a 97 mM CI-930 stock solution. The change in pH from CI-930 addition was negligible.

Imazodan precipitated after an addition of 1 µL of a 100 mM stock solution to the NMR sample of either [15N]cNTnC(C35S/C84S)·Ca2+·cTnI147−163 or [15N]cNTnC(C35S/C84S)·Ca2+·cTnI144−163. This was also the case with MPDP. Further additions resulted in more precipitation. No chemical shift changes were observed for the imazodan and MPDP titrations.

Pure DMSO-d6 was added to a NMR sample containing [15N]cNTnC(C35S/C84S) to test if it would induce chemical shift changes in [15N]cNTnC(C35S/C84S). No chemical shift perturbations were observed in the 2D 1H−15N HSQC spectrum upon addition of DMSO-d6 (data not shown). To confirm that leovosimendan was not interacting with free cTnI147−163 or cTnI144−163, leovosimendan was titrated into solutions containing either peptide. 1D 1H NMR revealed no chemical shift change for cTnI147−163 or cTnI144−163 in the presence of leovosimendan, when compared to the spectra acquired in the absence of the drug (data not shown).

**NMR Spectroscopy.** All of the NMR spectra were recorded using a Varian INOVA 500 MHz spectrometer at 30 °C. All 1D 1H and 2D 1H−15N HSQC spectra were acquired using the water and gHsqc.c pulse sequences, respectively (BioPack, Varian Inc.). The CBCA(CO)NNH and HNCACB pulse sequences, respectively, were used to monitor the binding of unlabeled ligands (such as the cTnI peptides, leovosimendan, or its analogues) to [15N]labeled cNTnC·Ca2+. In these titrations, the chemical shifts of several assigned amide NH cross-peaks in the 2D 1H−15N HSQC NMR spectrum of [15N]cNTnC·Ca2+ were perturbed as a function of added ligand. In addition to the identification of ligands that associate with the 15N-labeled protein, the changes in residue specific backbone shifts, which can be quantified to derive ligand stoichiometry and affinity, can also be used to locate the ligand binding site on the protein. This procedure is commonly known as chemical shift mapping.

**Interaction of cNTnC(C35S/C84S)·Ca2+ with cTnI147−163 and cTnI144−163.** Previously, we titrated cTnI147−163 into [15N]cNTnC(WT)·Ca2+ and demonstrated that the binding of cTnI147−163 to cNTnC(WT)·Ca2+ caused large chemical shift changes in the 2D 1H−15N HSQC NMR spectrum of the protein, consistent with the structural transition in cNTnC from a closed to an open state (6). The dissociation constant (Kd) of cTnI147−163 for cNTnC(WT)·Ca2+ was determined to be 154 ± 10 µM. In this study, we found that the binding of cTnI147−163 to cNTnC(C35S/C84S)·Ca2+ induced similar backbone amide 1H or 15N chemical shift changes, indicating a similar closed to open transition. The stoichiometry of binding of cTnI147−163 to cNTnC·Ca2+ was 1/1 in both the WT and C35S/C84S isoforms. An expanded region of the 2D 1H−15N HSQC NMR spectra of cNTnC(C35S/C84S) free and in the cTnI147−163 complex is depicted in Figure 3A, and peaks are labeled with residue assignments. Many peaks within this region of the spectrum, including V28, G34, V64, G68, and T71, moved in the cTnI147−163 complex to characteristic chemical shifts corresponding to an open conformation of cNTnC(C35S/C84S) (shown by arrows connecting multiple and first single contours). When the longer peptide cTnI144−163 was titrated into cNTnC(C35S/C84S)·Ca2+, the majority of the cross-peaks in the 2D 1H−15N HSQC spectrum of cNTnC(C35S/C84S) shifted along nearly identical pathways as in the titration of cTnI147−163 into cNTnC(C35S/C84S)·Ca2+ but further (shown by arrows connecting multiple and second single contours). The 2D 1H−15N HSQC NMR spectrum of cNTnC(C35S/C84S) in the cNTnC(C35S/C84S)·Ca2+·cTnI144−163 complex has been assigned, and the cross-peaks are labeled with residue assignments (Figure 3A). The pattern of spectral changes is very similar for the two complexes (Figure 3B) except for larger backbone amide 1H or 15N chemical shift perturbations of cNTnC(C35S/C84S) induced by cTnI144−163 in comparison with cTnI147−163. The average chemical shift changes of several residues that were perturbed the most during titration are plotted as a function of cTnI144−163:[cNTnC(C35S/C84S)·Ca2+][cTnI144−163] ratio (Figure 4), and the data were fit to the following equation:

\[
\text{cNTnC(C35S/C84S)·Ca}^{2+} + \text{cTnI}_{144-163} \rightleftharpoons \text{cNTnC(C35S/C84S)·Ca}^{2+} + \text{cTnI}_{144-163}
\]

This yielded a macroscopic dissociation constant of 15 ± 5 µM, which is 10 times smaller than the dissociation constant for cTnI147−163 binding to cNTnC(WT)·Ca2+ (6).
of levosimendan, CMDP, AMDP, and CI-930 (Figure 1). The initial (●) and end points (○) from the titrations of cNTnC(C35S/C84S)·Ca2+·cTnI147–163 (first single contour), and cNTnC(C35S/C84S)·Ca2+·cTnI144–163 (second single contour) complexes. The arrows indicate the direction of chemical shift change during titration of cTnI into cNTnC(C35S/C84S)·Ca2+. Single contours represent the two cNTnC–cTnI complexes at the end points of titrations. The end points were determined by the cessation of chemical shift changes, which indicated a saturated complex. (B) cTnI147–163-induced (white bars) and cTnI144–163-induced (black bars) chemical shift changes of the backbone amides of cNTnC(C35S/C84S). Chemical shifts are presented in hertz and calculated as follows: \( \Delta \delta = [(\Delta \delta_{\text{H}})^2 + (\Delta \delta_{\text{N}})^2]^{1/2} \). Since hertz is used instead of parts per million to calculate the total chemical shift changes (\( \Delta \delta \)), a correction factor of \( 1/5 \) in the 15N dimension is not needed.

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may be due to the different moieties present on the various pyridazinone derivatives. To correlate the drug-induced chemical shift changes of cNTnC(C35S/C84S) to conformational changes in the protein, we plotted the changes for backbone atoms against the protein sequence (Figure 6). The magnitude of the changes was generally small but similar to those reported in levosimendan binding to cTnC·3Ca2+ (25) and to cTnC·3Ca2+·cTnI32–79·cTnI128–180 (18). The changes induced by levosimendan (Figure 6A) or CMDP (Figure 6B) were relatively larger (≤60 Hz) than those (≤20 Hz) induced by AMDP (Figure 6C) and CI-930 (Figure 6D). This indicates that the group attached to the aromatic ring [mesoxalonitrile hydrazone in levosimendan, chloro in CMDP, amino in AMDP, and imidazole in CI-930 (see Figure 1)] influences the drug–protein–peptide interaction.
These four molecules contain a chiral methyl group on the pyridazinone ring which may be in direct contact with the protein and involved in anchoring the drug molecules to cNTnC(C35S/C84S)·Ca\textsuperscript{2+}·cTnI\textsubscript{144–163}. This is supported by the observation that cNTnC(C35S/C84S)·Ca\textsuperscript{2+}·cTnI\textsubscript{147–163} and cNTnC(C35S/C84S)·Ca\textsuperscript{2+}·cTnI\textsubscript{144–163} do not interact with imazodan or MPDP, as represented by the lack of chemical shift perturbations.

To elucidate the binding site of levosimendan on cNTnC·Ca\textsuperscript{2+}·cTnI\textsubscript{144–163}, chemical shift mapping is shown in Figure 7. The solution structure of cNTnC·Ca\textsuperscript{2+}·cTnI\textsubscript{147–163} [Protein Data Bank (PDB) entry 1MXL] was used to map the final chemical shift changes induced by the titration of levosimendan into cNTnC(C35S/C84S)·Ca\textsuperscript{2+}·cTnI\textsubscript{144–163}. The chemical shift perturbations are mapped on the structure by magnitude such that the chemical shift changes for residues colored pink were greater than the mean (\(\mu\)), the chemical shift changes for residues colored dark salmon were one standard deviation above the mean chemical shift change (\(\mu + \sigma\)), and the chemical shifts for residues colored red were two standard deviations above the mean (\(\mu + 2\sigma\)) (see Figure 6A). Chemical shift mapping of CMDP on the structure of cNTnC·Ca\textsuperscript{2+}·cTnI\textsubscript{147–163} (PDB entry 1MXL) resulted in a surface nearly identical to that of levosimendan.

**Binding Affinity Determination.** The calculation of the affinities of the different drugs is illustrated in Figure 8. Data analysis of the titration of CI-930 into cNTnC(C35S/C84S)·Ca\textsuperscript{2+}·cTnI\textsubscript{147–163} and cNTnC(C35S/C84S)·Ca\textsuperscript{2+}·cTnI\textsubscript{144–163} is shown as an example. An expanded region of 2D \(^1\text{H}-^{15}\text{N}\) HSQC NMR spectral changes induced by CI-930 is shown in panels A and C of Figure 8. Plots of chemical shift changes of L48 as a function of [CI-930]\textsubscript{total}/[cNTnC(C35S/C84S)·Ca\textsuperscript{2+}·cTnI\textsubscript{147–163}]\textsubscript{total} and [CI-930]\textsubscript{total}/[cNTnC(C35S/C84S)·Ca\textsuperscript{2+}·cTnI\textsubscript{144–163}]\textsubscript{total} are shown in panels B and D of Figure 8, respectively. In both cases, no plateau could be reached in the titration even at high protein/drug ratios, indicating weak binding. A global fitting approach (www.bionmr.ualberta.ca/bds/software/xcrvfit) was used, in which the data for residues perturbed during titration were fit to the following equation:

\[
\text{protein} + \text{drug} \rightleftharpoons \text{protein}\text{drug}
\]

yielding a \(K_D\) of 11 mM for CI-930 binding to cNTnC(C35S/C84S)·Ca\textsuperscript{2+}·cTnI\textsubscript{147–163} and 2.65 mM for CI-930 binding cNTnC(C35S/C84S)·Ca\textsuperscript{2+}·cTnI\textsubscript{144–163}. For levosimendan, the \(K_D\) values are 8 and 0.7 mM, respectively, and for AMDP, those are 6.5 and 1.5 mM, respectively. We were not able to obtain the dissociation constants for CMDP due to the precipitation of the ligand prior to reaching a 1/1 stoichiometry. The results for levosimendan, AMDP, and CI-930 demonstrate that cNTnC(C35S/C84S)·Ca\textsuperscript{2+}·cTnI\textsubscript{144–163} presents a more complete binding site for the drug molecules than cNTnC(C35S/C84S)·Ca\textsuperscript{2+}·cTnI\textsubscript{147–163}.
to individual atoms throughout the protein sequence, we were able to detect the subtle chemical shift changes of drug binding on the backbone amides of cNTnC(C35S/C84S) in the presence of cTnI_{147-163} or cTnI_{144-163} peptides. These measurements allowed the identification of a combination of specific amino acids in cNTnC and cTnI that form a binding pocket to accommodate the chiral methyl group on the pyridazine ring of levosimendan and its analogues.

The structure of the cNTnC--cTnI complex has been characterized in several studies. We have shown that the binding of cTnI_{147-163} induces a structural opening in cNTnC--Ca^{2+} (5). In the structure of cNTnC--Ca^{2+}--cTnI_{147-163}, the bound cTnI_{147-163} peptide adopts an α-helical conformation spanning residues 4–12 in the 17-residue peptide. With the N-terminus of the peptide interacting with the center of the hydrophobic pocket, the α-helical region interacts with the AB helical interface and stabilizes the opening conformation of cNTnC--Ca^{2+}. The corresponding sTnI_{115-131} peptide adopts a similar structure and a similar mode of interaction with sNTnC as observed in the structure of sNTnC(tryptophan)-2Ca^{2+}-sTnI_{115-131} (26). The backbone atoms of the cNTnC--Ca^{2+}--cTnI_{147-163} structure superimpose to 1.5 Å with the corresponding regions in the X-ray structure of the cardiac troponin complex cTnC--3Ca^{2+}--cTnI_{31-210}·cTnI_{183-288}. Addition of bepridil to cNTnC--Ca^{2+}, cTnI_{147-163} resulted in the cNTnC--Ca^{2+}--cTnI_{147-163}·bepridil ternary complex, with the binding site for bepridil primarily located on the AB interhelical interface and the binding site for bepridil in the center of the hydrophobic pocket (16). A similar binding scenario was observed in the X-ray structure of the sTnC-4Ca^{2+}·sTnI_{115-131}·sTnI_{156-262} complex (8), in which a detergent molecule, anapoe, was found to bind together with the switch region of sTnI to sNTnC-2Ca^{2+} (8). In the structure of sNTnC-4Ca^{2+}·sTnI_{115-131}·sTnI_{156-262}, the three-residue extension (R112, R113, and V114) in the N-terminus of sTnI_{115-131} is involved in electrostatic contacts with a number of acidic residues in both the C and D helices of sNTnC (Figure 9), which may enhance the stability of the open conformation of sNTnC-2Ca^{2+}. Although R144, R145, and V146 are not visualized in the cNTnC--Ca^{2+}·cTnI_{147-163} and cNTnC--3Ca^{2+}·cTnI_{31-210}·cTnI_{183-288} structures, these residues were expected to be located in the proximity of the corresponding acidic residues, D87 and D88, in the D helix of cNTnC. This is based on the observation that the binding of the switch region of TnI to the N domain of TnC is the same in all the structures mentioned above. Thus, it is reasonable to suggest that cNTnC--Ca^{2+}·cTnI_{144-163} would present a more stable complex with a higher-affinity binding site for drug compounds than cNTnC--Ca^{2+}·cTnI_{147-163}. This is in line with the observation in this work.

An earlier study has shown that the binding site of levosimendan is in the N domain of Ca^{2+}-saturated cTnC (27), although this was challenged by a different analysis showing that levosimendan did not bind to cTnC or a cTnC--cTnI complex (28). The later report showed that the inability to observe the levosimendan binding to cTnC was in part due to sample degradation and the use of a recombinant cTnC with C35 and C84 mutated to serines (25). It suggested that C84 is necessary for the interaction of levosimendan and cTnC. Furthermore, this report showed that levosimendan is capable of binding to both domains of

**FIGURE 7: Surface representations of cNTnC--Ca^{2+}·cTnI_{147-163} (PDB entry 1MXL). The cNTnC--Ca^{2+}·cTnI_{147-163} complex is colored yellow with the helices of troponin C labeled. cNTnC--Ca^{2+} is depicted as a surface representation, whereas cTnI_{147-163} is shown in ribbon format. Chemical shift perturbations induced by levosimendan (A) and CMDP (B) binding to cNTnC--Ca^{2+}·cTnI_{144-163} are colored on the basis of the magnitude of the chemical shift. Chemical shifts greater than the mean plus two standard deviations (μ + 2σ) are colored red, greater than the mean plus one standard deviation (μ + σ) dark salmon, and greater than the mean (μ) salmon.**

**DISCUSSION**

The goal of this study was to define the binding site of levosimendan and several structural analogues (Figure 1) in the regulatory domain of cTnC in complex with the switch region of cTnI, as well as to gain insight into the particular pharmacophores responsible for the binding of the ligands to the cNTnC--cTnI complex. An attractive aspect of this complex is that the interaction between cNTnC and the switch region of cTnI is Ca^{2+}-sensitive and the binding of Ca^{2+} and the cTnI switch are coupled to the opening of the cNTnC hydrophobic pocket, revealing an important target for cardiotoxic drugs. We utilized 2D 1H--15N HSQC NMR spectroscopy to monitor the formation of two cTnC--cTnI complexes, cNTnC(C35S/C84S)--Ca^{2+}·cTnI_{147-163} and cNTnC(C35S/C84S)--Ca^{2+}·cTnI_{144-163}, and the titration of six drug compounds to those complexes. Since 2D 1H--15N HSQC NMR spectroscopy can report information pertaining
cTnC. A recent investigation by Sorsa et al. (18) used NMR to demonstrate that levosimendan bound to only the N domain of cTnC in the presence of cTnI32–79 and cTnI128–180. Levosimendan-induced amide chemical shift changes were detected throughout the N domain of cTnC in the presence of the cTnI peptides. A number of N domain resonances either were broadened beyond detection or exhibited multiple chemical shifts. A large number of both polar and nonpolar residues that exhibited chemical shift changes upon levosimendan binding precluded identification of the levosimendan-binding site on cNTnC. Sorsa et al. (18) attributed the numerous N domain chemical shift changes to levosimendan altering either the dynamic equilibrium or the rate of exchange between open and closed N domain conformations by partially stabilizing defunct Ca$^{2+}$ site I in cNTnC$^{\cdot}$Ca$^{2+}$·cTnI. The exact binding location of levosimendan on cTnC remained unclear, although all the studies cited above suggested that its binding site is in the proximity of M81, M85, and F77 (residues on the D helix) within the hydrophobic pocket of cNTnC. Our data are in agreement with these studies and show that levosimendan targets the hydrophobic pocket of cNTnC. The chemical shift mapping depicted in Figure 7 indicates that all backbone amide resonances perturbed modestly (colored salmon) belong to the residues in the hydrophobic pocket, whereas the residues that are more significantly perturbed (colored dark salmon and red) belong to the N and A helices, away from the proposed binding surface. We suggest that the larger chemical shift perturbations of residues, such as Y5 and I4 on the N helix and A23 and F24 on the A helix, may be due to a slight conformational change of cNTnC$^{\cdot}$Ca$^{2+}$·cTnI147–163 upon binding levosimendan, whereas the residues with smaller chemical shift changes identify the binding site of levosimendan in cNTnC$^{\cdot}$Ca$^{2+}$·cTnI144–163 more accurately. The conformational change may be similar to that observed in the binding of bepridil to cNTnC$^{\cdot}$Ca$^{2+}$·cTnI147–163, where in addition to the opening of cNTnC, there is a slight shift in the position of cTnI147–163 (15). Our results demonstrated that the conserved positively charged N-terminal extension (RRV) in cNTnC$^{\cdot}$Ca$^{2+}$-cTnI created the levosimendan binding site with enhanced affinity, with a net result of the stabilization of the hydrophobic pocket and the open conformation of cNTnC$^{\cdot}$Ca$^{2+}$. We propose that the position...
of levosimendan in the cardiac troponin complex is analogous to that of anapoe in the skeletal troponin complex (Figure 9) on the basis of the results of chemical shift mapping (Figures 7). The A-Cys version of cNTnC was used in this study, and our results show that a cTnC complex provides a favorable binding site for levosimendan in the absence of C84. The study (25) reporting that C84 is critical for the interaction of levosimendan and cNTnC suggested that the C84S mutation may slightly alter the conformation of the D helix of cNTnC to render the levosimendan binding site unavailable. Our results show that electrostatic interactions between R144 and R145 of cTnI and D87 and D88 of cNTnC help to stabilize the D helix and thereby the binding site for levosimendan. Covalent modification of C84 by levosimendan provides a plausible mechanism to account for the levosimendan-dependent effects (e.g., splitting of NMR peaks) observed by Sorsa et al. (25). This potential covalent interaction between C84 and levosimendan may not be required for its binding to the troponin complex but may subsequently occur in vivo (29).

To understand the effect of the pharmacophores of levosimendan on binding to cNTnC, we investigated the binding of several levosimendan analogues (Figure 1) to cNTnC·Ca\(^{2+}\)·cTnI\(_{147-163}\) and cNTnC·Ca\(^{2+}\)·cTnI\(_{144-163}\). Among the derivatives, only those containing the chiral methyl group induced chemical shift changes, suggesting that this group constitutes a key pharmacophore responsible for the binding of pyridazinone derivatives to cNTnC(C35S/C84S)·Ca\(^{2+}\). This is probably because this methyl group contributes to the hydrophobic packing in a stereospecific manner. This type of interaction is demonstrated in the binding of EMD 57033 to the C domain of cTnC (30, 31). In the structure of cTnC·2Ca\(^{2+}\)·EMD 57033, the drug molecule is oriented such that the chiral methyl 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 (EMD 57439) is inactive. Studies on the action of stereoisomers of simendan (levosimendan and its + enantiomer, dextrosimendan) on cTnC have presented evidence that the Ca\(^{2+}\) sensitizing effect of levosimendan is in part due to a stereoselective binding to the N domain of cTnC (32). Another cardiotonic drug with structural characteristics similar to those of levosimendan, pimobendan, binds troponin and increases calcium sensitivity in a stereospecific manner as well (33, 34). The three levosimendan analogues that associated with cNTnC·Ca\(^{2+}\)·cTnI\(_{147-163}\) and cNTnC·Ca\(^{2+}\)·cTnI\(_{144-163}\), CMDP, AMDP, and CI-930 were shown in this work to be R/S mixtures (Figure 2); therefore, we cannot unambiguously conclude that binding to the complex is selective for one enantiomer over the other. However, it is possible that the affinity of one of the enantiomers of the pyridazinone compounds with cNTnC·Ca\(^{2+}\)·cTnI\(_{144-163}\) is higher than that of the other.

In addition to the chiral methyl group on the pyridazinone ring, a group attached to the benzene ring may also be important in the binding. In levosimendan, this is a bulky group comprised of the mesoxalonitrile hydrazone moiety. Structure–activity analysis of levosimendan analogues has shown that this group is involved in hydrogen bonding with cTnC, indicating that both hydrogen bonds and van der Waals contacts are formed upon the binding of active levosimendan and CMDP induced larger chemical shift changes than AMDP and CI-930 (Figure 6). This demonstrates that the mesoxalonitrile hydrazone group in levosimendan and the chloro group in CMDP help to form stronger interactions with the binding site compared to the amino group in AMDP or the imidazole group in CI-930. Chemical shift mapping (Figure 7) identifies a similar binding site between levosimendan and CMDP located in the hydrophobic pocket of cNTnC·Ca\(^{2+}\)·cTnI\(_{144-163}\). The large chemical
shift perturbations of residues residing on the N helix of cNTnC induced by CMDP suggest a structural opening of cNTnC·Ca²⁺·cTnI₁₄₄₋₁₆₃. The larger chemical shift changes of residues along the D helix of cNTnC from CMDP binding when compared with those of levosimendan may be due to the proximity of these residues to the electronegative chloro group of CMDP, or a slightly different binding mode between the ligands. The perturbed chemical shifts along the B helix induced by levosimendan may result from a larger displacement of cTnI₁₄₄₋₁₆₃ by the voluminous mesoxaloni-trile hydrazone moiety when compared with CMDP. Assuming the binding mode of levosimendan and its analogues is similar to that of anapoe (Figure 9), the orientation of the pyridazine molecules would be antiparallel to cTnI₁₄₄₋₁₆₃, i.e., the chiral methyl group on the pyridazine ring fits deep in the hydrophobic cavity, whereas the group attached to the aromatic ring makes contacts with the residues in the N-terminus of cTnI₁₄₄₋₁₆₃ and the D helix of cNTnC. Interestingly, EMD 57033 also binds to the hydrophobic pocket of cTnC in a similar manner, with the chiral methyl group deep inside the protein core.

The affinities determined for the binding of levosimendan (K_D ~ 0.7 mM), AMDP (K_D ~ 1.5 mM), and CI-930 (K_D ~ 2.6 mM) to cNTnC·Ca²⁺·cTnI₁₄₄₋₁₆₃ are comparable to those from the previous studies on levosimendan binding to cTnC·3Ca²⁺·cTnI₁₃₂₋₇₉·cTnI₁₂₈₋₁₈₀ (K_D ≥ 0.2 mM) (18), W7 binding to cTnC·3Ca²⁺·cTnI₁₃₄₋₇₁·cTnI₁₂₈₋₁₆₃ (K_D ~ 0.5 mM) (17), and anapoe binding to sTnC·4Ca²⁺·sTnI₁₁₈₋₁₈₂·sTnI₁₅₆₋₂₀₂ (K_D ~ 0.64 mM) (8). This might support the notion that the binding of cardiotonic drugs to the regulatory unit of cardiac troponin occurs in a similar mode. These K_D values are in the high micromolar range, which is generally considered weak for protein—drug interactions. Levosimendan is known to exert multiple effects on perfused hearts at concentrations as low as 0.3 μM; the effect of levosimendan on skinned muscle fibers was half-maximal by 0.3 μM and maximal by 10 μM (36), and similar levels of levosimendan modulated contractility in cardiomyocytes (37). Several factors could contribute to this phenomenon. Micellization of drug molecules in aqueous solutions has been shown to cause low apparent affinities (38, 39). It is possible that cTnC in the highly organized and cooperative myofilaments may have a higher affinity for levosimendan than it does when cTnC is isolated in solution. On the other hand, the weak levosimendan—cTnC interaction may allow the drug to gently modulate cardiac contractility in a rhythmic fashion so that cardiac relaxation is not compromised. This is in accordance with a study reporting a K_D of >2 mM for the interaction of pineal hormone melatonin and calmodulin. The low affinity has been used to explain many in vitro effects and pharmacological dosages of melatonin (40). The small chemical shift changes observed in this study suggest that these drug molecules do not induce large conformational changes in cNTnC·Ca²⁺·cTnI₁₄₄₋₁₆₃. This feature is potentially useful for the rapid mapping of the drug molecules to the already determined structure of cNTnC·Ca²⁺·cTnI₁₄₇₋₁₆₃ via measurement of the intermolecular NOE restraints between cNTnC·Ca²⁺·cTnI₁₄₇₋₁₆₃ and the drug molecules, a project currently in progress in our laboratory.

ACKNOWLEDGMENT

We thank David Corson and Melissa Rakovszky for protein purification, Olivier Julien, Robert Boyko, and Ryan Hoffman for help using software such as nmrView and nmrPipe, Jeff Devries for NMR spectrometer maintenance, Jeffrey Way and Sean Walker from Reify Corp. for early discussions, and Pfizer for the gift of CI-930.

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