Drug Binding to Cardiac Troponin C*

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Quinn Kleerekoper and John A. Putkey‡

From the Department of Biochemistry and Molecular Biology, University of Texas Medical School, Houston, Texas 77030

Compounds that sensitize cardiac muscle to Ca\(^{2+}\) by intervening at the level of regulatory thin filament proteins would have potential therapeutic benefit in the treatment of myocardial infarctions. Two putative Ca\(^{2+}\) sensitisers, EMD 57033 and levosimendan, are reported to bind to cardiac troponin C (cTnC). In this study, we use heteronuclear NMR techniques to study drug binding to \([\text{methyl-\(^{13}\)}C]\)methionine-labeled cTnC when free or when complexed with cardiac troponin I (cTnI). In the absence of Ca\(^{2+}\), neither drug interacted with cTnC. In the presence of Ca\(^{2+}\), one molecule of EMD 57033 bound specifically to the C-terminal domain of free cTnC. NMR and equilibrium dialysis failed to demonstrate binding of levosimendan to free cTnC, and the presence of levosimendan had no apparent effect on the Ca\(^{2+}\) binding affinity of cTnC. Changes in the N-terminal methionine methyl chemical shifts in cTnC upon association with cTnI suggest that cTnI associates with the A-B helical interface and the N terminus of the central helix in cTnC. NMR experiments failed to show evidence of binding of levosimendan to the cTnC-cTnI complex. However, levosimendan covalently bound to a small percentage of free cTnC after prolonged incubation with the protein. These findings suggest that levosimendan exerts its positive inotropic effect by mechanisms that do not involve binding to cTnC.

Cardiac troponin C is the EF-hand Ca\(^{2+}\) receptor in the thin filament of slow skeletal and cardiac striated muscle. The contraction relaxation cycle is regulated by the binding and release of Ca\(^{2+}\) ions from the N-terminal regulatory site II of cTnC. Compounds that bind with high affinity and selectivity to cTnC and increase the affinity of Ca\(^{2+}\) binding to site II could potentially increase the Ca\(^{2+}\) sensitivity of myocardial contraction without altering Ca\(^{2+}\) transients.

Knowledge of the structure and surface topology of cTnC is critical for an understanding of how existing Ca\(^{2+}\)-sensitizing compounds interact with cTnC and to identify potential binding sites for new and more effective compounds. Previous structural models for cTnC based on known structures for Ca\(^{2+}\)-bound fast skeletal troponin C (3, 4) and calmodulin (5) predicted that the N-terminal regulatory domain would have an open conformation with a large contiguous hydrophobic surface. Ca\(^{2+}\)-sensitizing compounds such as bepridil, levosimendan, pimobendan, and trifluoperazine were thought to bind to this predicted hydrophobic surface (6–8). However, the recent NMR solution structures of intact Ca\(^{2+}\)-bound cTnC and the apo and Ca\(^{2+}\)-bound N-terminal domain of cTnC show the N-terminal regulatory domain to be partially closed in the presence of Ca\(^{2+}\) (9, 10). Hydrophobic surfaces in the N-terminal domain exist as discrete patches rather than a contiguous surface. Our recent study localized the binding site for bepridil and TFP in these N-terminal hydrophobic sites as well as in the C-terminal structural domain of cTnC (11).

Here we report studies on the binding of two newer generation Ca\(^{2+}\) sensitisers, EMD 57033 and levosimendan, to cTnC. EMD 57033 is reported to interact with the C-terminal domain of cTnC (12), but its Ca\(^{2+}\) sensitisating effects are thought to result primarily by affecting the actin myosin interface (13). EMD 57033 has an isoform-specific effect on phosphodiesterase activity (14). The Ca\(^{2+}\)-sensitizing effects of levosimendan are thought to result from direct binding to cTnC (12, 15, 16). Our data confirm that EMD 57033 binds to the C-terminal domain of cTnC but that levosimendan does not appear to bind to either free cTnC or the cTnC-cTnI binary complex. However, levosimendan can form covalent adducts with cTnC after prolonged exposure.

**MATERIALS AND METHODS**

**Chemicals**—Levosimendan was kindly provided by Orion-Farmos (Espoo, Finland). Merck kindly provided EMD 57033. Bepridil was purchased from Sigma. L-[methyl-\(^{13}\)C]Methionine, Triac, deuterium oxide, dimethyl sulfoxide, and methanol were obtained from Cambridge Isotope Laboratories. (2,5)-Dihydroxybenzoic acid and trifluoroacetic acid were obtained from Aldrich Laboratories.

**Cloning, Expression, and Purification of cTnI:z-z(A-Cys)—** A mouse cTnI cDNA was isolated from a mouse heart cDNA library by PCR using the following oligonucleotides: N-cTnI: 5'-GGGCCATGGCTGATGAAA-GCACGGATGC-C3'; and C-cTnI: 5'-CACAGTGTCAGGAGAAT-3'. Amplified DNA was purified and digested with Ncol and HindIII and subcloned into pET-23d (Novagen). The cTnI plasmid was transformed into E. coli strain Novabla (Novagen). Positive clones were confirmed by dyeoxy DNA sequence analysis.

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‡ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, University of Texas Medical School, 6431 Fannin St., P. O. Box 77030, Houston, TX 77030. Tel.: 713-500-0661; Fax: 713-500-0652; E-mail: putkey@bmb.med.uth.tmc.edu.

1 The abbreviations used are: cTnC, cardiac troponin C; cTnI, cardiac troponin I; TFP, trifluoperazine; HPLC, high pressure liquid chromatography; NOE, nuclear Overhauser effect; HSQC, heteronuclear single-quantum coherence; 5,5'-dibromoBAPTA, 5,5'-dibromo-1,2-bis(2-aminoxyphenoxylethene-\(\text{N,N',N''}-\text{tetraacetic acid.}

13C]methionine-labeled cTnC when free or the cTnC complex. However, levosimendan covalently bound to a small percentage of free cTnC after prolonged incubation with the protein. These findings suggest that levosimendan exerts its positive inotropic effect by mechanisms that do not involve binding to cTnC.

Congestive heart failure results in desensitization of the myocardium to Ca\(^{2+}\) as well as depressed cardiac contractility. A promising approach to the treatment of congestive heart failure is the development of positive inotropic Ca\(^{2+}\) -sensitizing agents that are capable of increasing the sensitivity of myocardium to Ca\(^{2+}\), thereby compensating for low cardiac output. Ideal calcium-sensitizing compounds would enhance the maximal force of muscle contraction without increasing Ca\(^{2+}\) ion flux into the cell or impairing relaxation in the failing myocardium. In addition, the compound should not inhibit phosphodiesterase activity (1) to minimize the possibility of heart arrhythmias and increased energy consumption in the heart (2).

Based on these criteria, cardiac troponin C (cTnC) is an attractive target for potential Ca\(^{2+}\)-sensitizing compounds.

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Both Cys<sup>81</sup> and Cys<sup>98</sup> were changed to Ser using the Chameleon<sup>TM</sup> double-stranded, site-directed mutagenesis kit from Stratagene and the following primers: C81S, 5'-CCGGTCTTCAGGCAGTTTCAACTGGTTGAGTATG-3'; C98S, 5'-GGAGCTCTGAAGGCTGTTTACGGT-3'. Buffers and chemical shifts were 6012 and 600 Hz, respectively. 1H and 13C chemical shifts were reported relative to the H2O signal at 4.563 ppm and the [13C]Met signal at 14.86 ppm, respectively. 1H and 13C chemical shifts were reported relative to the H2O signal at 4.563 ppm and the [methyl-<sup>13</sup>C]Met signal at 14.86 ppm, respectively. All spectra were processed using the FELIX software package (Bioisom Technologies, Inc.).

**Drug Solutions—Levosimendan is a light-sensitive drug; therefore, all stock solutions of the drug were prepared and stored in the dark. Stock solutions of levosimendan were prepared immediately before use in deuterated Me<sub>2</sub>SO or as described previously (8). After adding the drug to the protein, the sample pH was adjusted when necessary. The stock solution of EMD 50733 was prepared in deuterated DMSO immediately before use. Bepiridil was prepared as reported previously (11).**

**Mass Spectroscopy—Matrix-assisted UV laser desorption mass spectroscopy spectra were acquired using a PerSeptive Voyager Elite Time of Flight Mass Spectrometer equipped with a delayed extraction nitrogen laser (337 nm). A saturated solution of (2,5-dihydroxybenzoic acid in 0.1% trifluoroacetic acid in H<sub>2</sub>O was used as a matrix. 0.5 µl of matrix was mixed with 0.5 µl of sample, placed on the target, and dried at room temperature while protected from light. The sample was a 1:100 dilution in H<sub>2</sub>O of the appropriate drug stock. The spectra in this study represent the sum am of 40 laser shots. In addition to the matrix-assisted UV laser desorption mass spectroscopy analysis, a Kratos MS50 fast atom bombardment was used to analyze the levosimendan provided by Orion-Farmos. 3-Nitrobenzyl alcohol was used as a matrix.**

**Drug Binding and Ca<sup>2+</sup> Titration—Dialysis was carried out at 25 °C using two-chamber equilibrium dialysis units from Sialmed, Inc. Each chamber had a 0.1 ml capacity. Prepared dialysis membranes (Life Technologies, Inc.) had a molecular mass cutoff of 12,000–14,000 daltons. Protein was added to one chamber at a concentration of about 0.2 mM to approximate the concentration used in NMR experiments. Protein was prepared in buffers of 20 mM Tris, pH 7.0, and 200 mM KCl or 20 mM HEPES, pH 7.0, and 100 mM KCl. Drug was added to the opposing chamber at concentrations between 0.2 and 0.4 mM in the corresponding buffer. Sufficient Ca<sup>2+</sup> was added to saturate the Ca<sup>2+</sup> binding sites of the protein. After equilibration (typically 16 h), aliquots of samples from both chambers were diluted at least 50-fold and assayed spectrophotometrically at 406 nm for levosimendan (ε = 93,285 cm<sup>-1</sup>·mol<sup>-1</sup>·cm<sup>-1</sup>) and 296 nm for bepridil (ε = 3,544 cm<sup>-1</sup>·mol<sup>-1</sup>·cm<sup>-1</sup>). After dilution, and at these wavelengths, the protein contributed insignificantly to total absorbance. The concentration of cTnC(A-Cys) in the samples was determined by the method of Bradford (23) and bicinchoninic acid assay (24).**
shows that a 4-fold molar excess of levosimendan has generalized and minor effects on the methionyl methyl chemical shifts of cTnC(A-Cys) in the absence of Ca$^{2+}$. In the presence of Ca$^{2+}$ (Fig. 2B), only Met$^{120}$ and Met$^{157}$ exhibit significant chemical shift changes in the proton dimension upon the addition of 2-fold and 4-fold molar excesses of levosimendan. Observed changes for Met$^{81}$ at high drug:protein ratios are restricted to the carbon dimension. However, the interpretation of these changes with respect to specific drug binding to cTnC must be made cautiously because they are observed only at drug:protein ratios of greater than 1:1. Binding of bepridil and TFP to cTnC(A-Cys) induced significant spectral changes at a drug:protein ratio of 0.5:1 (11). Even the fluorescent dye 4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid, which binds to hydrophobic surfaces, induces large changes in the HSQC spectra of [methyl-$^{13}$C]Met-labeled cTnC(A-Cys) (data not shown).

We next used isotope-filtered NMR techniques in an attempt to identify NOEs between aromatic protons in levosimendan and Met methyl protons in cTnC(A-Cys) that was metabolically labeled with [methyl-$^{13}$C]Met and Phe-(D)$_8$. No NOEs were detected (data not shown). In contrast, similar experiments identified strong NOEs between bepridil and cTnC(A-Cys) (11). cTnC(A-Cys) encodes Ser rather than Cys at positions 35 and 84. It is possible that these mutations affect the ability of levosimendan to bind to cTnC. To test this, we labeled cTnC3, which contains Cys$^{35}$ and Cys$^{84}$, with [methyl-$^{13}$C]Met and monitored Met methyl chemical shifts upon the addition of levosimendan in the presence of Ca$^{2+}$ as shown in Fig. 3. The

![Fig. 1. Titration of apo (A) and 3Ca$^{2+}$-cTnC(A-Cys) (B) with EMD 57033. HSQC spectra were collected in the presence or absence of 1 equivalent of EMD 57033. Solid contours represent the initial cross-peaks in the absence of drug. The protein concentration was 1 mM. The subsequent addition of EMD 57033 to achieve a 2:1 drug:protein ratio (2 mM drug) resulted in no additional changes in the HSQC spectra.](image1)

![Fig. 2. Titration of apo (A) and 3Ca$^{2+}$-cTnC(A-Cys) (B) with levosimendan. A, the HSQC spectra of apo (methyl-$^{13}$C)Met cTnC(A-Cys) in the presence or absence of 4 equivalents of levosimendan. B, the methyl 1H-13C correlations observed for Ca$^{2+}$-bound (methyl-$^{13}$C)Met cTnC(A-Cys) in the presence of 0, 2, and 4 equivalents of levosimendan. In both panels, solid contours represent the initial peaks without drug. Significant chemical shift changes correspond to 0.01 and 0.2 ppm in the proton and carbon dimensions, respectively.](image2)

![Fig. 3. Gradient-enhanced HSQC spectra of 3Ca$^{2+}$ cTnC3 in the presence and absence of levosimendan. The 1H-13C Met methyl correlations without drug are shown as solid contours. Even in the presence of 1.6 equivalents of levosimendan, no significant change is seen for any of the Met groups (dashed line). The spectra were collected on a Varian Unity-Plus 750 using a 5 mm HCN triple resonance probe. The gradient enhanced HSQC pulse sequence was provided by Varian.](image3)
Buffer conditions are given under "Materials and Methods." indicates the amount of free drug measured in the opposing chamber. The dashed line represents the absorbance spectrum of free and bound drug in the chamber containing cTnC(A-Cys). The black line indicates the amount of free drug measured in the opposing chamber. Buffer conditions are given under "Materials and Methods."

no effect on the N-terminal Met residues. Similar to what was observed for cTnC(A-Cys), levosimendan had minor effects on the proton chemical shifts of Met120 (Δ0.01 ppm) and Met157 (Δ0.02 ppm) in C-terminal domain.

Direct Binding Studies—The results in Figs. 2 and 3 and the absence of NOEs between cTnC and levosimendan suggest either that the drug does not bind to cTnC or that it binds with very low affinity and preferentially to the C-terminal domain. Both of these interpretations are not consistent with the Ca2+-sensitizing effects of levosimendan, which is proposed to act via binding to cTnC (8, 15) and to be effective at concentrations as low as 0.03–1 μM levosimendan (25). The line represents a fit of data collected in the absence of levosimendan.

Potential Effect of Levosimendan on Ca2+ Binding to cTnC—The potential effect of levosimendan on the Ca2+ binding affinity of cTnC was determined using Ca2+-sensitive dyes. Fig. 5 shows the absorbance of 5,5′-dibromoBAPTA when titrated with Ca2+ in the presence of cTnC(A-Cys) and in the presence or absence of levosimendan. Binding of Ca2+ to 5,5′-dibromoBAPTA causes a decrease in absorbance at 262 nm. Competition between 5,5′-dibromoBAPTA and cTnC for binding Ca2+ allows determination of the capacity and macromolecular binding constants for Ca2+ binding sites in cTnC. The line represents a fit of experimental data in the absence of levosimendan using the method of Linse et al. (25). It is clear from Fig. 5 that the presence of levosimendan had no effect on the Ca2+ binding properties of cTnC. The fractional change in absorbance was essentially identical for all level of Ca2+ in the presence and absence of levosimendan.

Effect of Levosimendan on Met Methyl Chemical Shifts in cTnC(A-Cys) When Bound to cTnI(A-Cys)33–211—Together, the data in Figs. 2–5 demonstrate that levosimendan at concentrations of less than 1 mm does not bind to free Ca2+-bound cTnC. The modest chemical shift changes seen in Fig. 2 at high concentrations of levosimendan (4 mm) may be due to nonspecific or very weak binding. However, it is possible that levosimendan binds to cTnI only when it is bound to cTnI. To address this possibility, we monitored drug-induced chemical shift changes when cTnC(A-Cys) was bound to cTnI. For these experiments, we generated mouse cTnI(A-Cys)33–211 in which amino acids 1–33 were deleted and in which Cys89 and Cys98...
were converted to Ser. A derivative of mouse cTnI in which the first 33 amino acids had been deleted was shown previously by Guo et al. (17) to be functional and has been used for structural studies (29, 30). We elected to convert both Cys residues to Ser to prevent formation of intramolecular disulfide bonds in native cTnI. A derivative of cTnI in which both Cys residues were converted to Ser was shown to be functional (31).

Table I lists the chemical shifts for the 10 methionyl methyl $^1H$-$^{13}C$ correlations cTnI(A-Cys) when free and when associated with cTnI(A-Cys)$_{33-211}$. All Met methyl chemical shifts in the C-terminal domain of cTnI(A-Cys), except Met$^{137}$, were significantly affected by cTnI(A-Cys)$_{33-211}$. The absolute chemical shift values for Met residues in the C-terminal domain of cTnI(A-Cys) in the presence or absence of cTnI(A-Cys)$_{33-211}$ are in excellent agreement with those of Krudy et al. (29), who used cTnI(C35S) and cTnI$_{33-211}$. The chemical shifts of Met$^{85}$, Met$^{80}$, and Met$^{60}$ in the N-terminal domain are not affected by cTnI(A-Cys)$_{33-211}$. Met$^{47}$ and Met$^{81}$ show a small effect, whereas Met$^{45}$ shows a significant change in the $^1H$ dimension of 0.09 ppm. These data differ from those of Krudy et al. (29), who reported that only methionyl methyl $^1H$ chemical shifts of Met$^{81}$ and Met$^{85}$ in the N-terminal domain of cTnI(C35S) were altered upon association with cTnI$_{33-211}$.

Fig. 6 shows the effect of levosimendan on the cTnI(A-Cys)/cTnI(A-Cys)$_{33-211}$ complex in the presence of Ca$^{2+}$. The $^1H$-$^{13}C$ correlations attributed to Met groups located in the N-terminal domain (Fig. 6A) are shown separately from those in the C-terminal domain (Fig. 6B). Solid contour lines indicate cross-peaks in the absence of drug, whereas dashed contour lines indicate cross-peaks upon the addition of 1 equivalent of drug. The lack of change in the chemical shifts indicates that levosimendan does not bind to the cTnI(cTnC) complex.

**Table I**

| Met residue | cTnI(A-Cys) | A-Cys | cTnI(C35S) | Chemical shift differences $^{a}$ | $^1H$ | $^{13}C$ |
|-------------|-------------|-------|-----------|-------------------------------|---|---|
| 45 | 1.83/16.1 | 1.80/15.9 | 0.03/nc | 0.02 ppm |
| 47 | 2.21/15.4 | 2.12/15.4 | 0.09/nc | 0.02 ppm |
| 60 | 1.95/15.4 | 1.89/15.4 | nc/nc | 0.02 ppm |
| 80 | 1.84/15.8 | 1.83/15.7 | nc/nc | 0.02 ppm |
| 81 | 1.38/15.3 | 1.41/15.6 | 0.03/0.3 | 0.02 ppm |
| 85 | 2.10/15.1 | 2.08/15.1 | nc/nc | 0.02 ppm |
| 103 | 1.91/14.9 | 1.86/15.1 | 0.05/nc | 0.02 ppm |
| 120 | 1.88/15.7 | 1.99/16.7 | 0.10/1.0 | 0.02 ppm |
| 137 | 1.92/15.6 | 1.91/15.9 | nc/0.3 | 0.02 ppm |
| 157 | 1.82/14.3 | 1.07/13.5 | 0.75/0.8 | 0.02 ppm |

$a$ Absolute measurements have an accuracy of ±0.01 ppm for $^1H$ and ±1.0 for $^{13}C$. The accuracy for difference values is ±0.02 ppm for $^1H$ and ±0.2 for $^{13}C$.

Chemical shift differences are derived by subtracting the observed value for the binary cTnI·cTnC complex from that observed for free cTnI(A-Cys).

$^{a}$ nc, no significant change.

**Fig. 6. Effect of levosimendan on Ca$^{2+}$-saturated cTnI(A-Cys)/cTnI$_{33-211}$(A-Cys) complex.** Chemical shift changes for Met residues located in the N-terminal domain (A) are plotted separately from those in the C-terminal domain (B). Solid and dashed contour lines represent data collected in the absence and presence of levosimendan, respectively.

We used analytical HPLC gel filtration to investigate what appeared to be levosimendan covalently bound to cTnI(A-Cys). Elution profiles of various samples were monitored at the absorbance maxima for levosimendan (406 nm). Fig. 8A shows the chromatographic profile of cTnI(A-Cys) that had not been exposed to levosimendan. No peak is observed because the protein does not absorb light at 406 nm. The arrow in Fig. 8A shows the elution position of cTnI(A-Cys) detected at 280 nm. Fig. 8B shows the elution profile of levosimendan. Fig. 8C shows the elution profile of an NMR sample of cTnI(A-Cys) that had been exposed to levosimendan for an extended period of time. Fig. 8D shows the elution profile of the NMR sample after removal of free drug by desalting in the presence of 6 M urea and EGTA. Comparison of these elution profiles demonstrates that a chromophore, which absorbs at 406 nm, remains associated with cTnI(A-Cys) after extended exposure to levosimendan and subsequent removal of free drug under denaturing conditions. This data, as well as the data in Fig. 7, and
SDS-polyacrylamide gel electrophoresis analysis of levosimendan-treated protein strongly suggest the covalent binding of the drug to cTnC(A-Cys).

Mass Spectra of Levosimendan—Both fast atom bombardment and matrix-assisted UV laser desorption mass spectrometry were used to determine whether breakdown products or derivatives of levosimendan, which could covalently bind to protein, accumulate in organic or aqueous solutions. Levosimendan was first analyzed immediately after preparation in Me₂SO as shown in Fig. 9A. The peak at \( m/z \) 281.0 agrees well with the expected mass of levosimendan. The peak at \( m/z \) 550.5 is not identified but was seen in all spectra to varying extents. Levosimendan was stable in Me₂SO because no change in the mass species was apparent after several days of storage (data not shown).

Levosimendan was next prepared in 0.5 M NaOH as described by Pollesello et al. (8) and analyzed immediately (Fig. 9B) or 72 h (Fig. 9C) after dilution into water. The major peak at \( m/z \) 302.9 corresponds well to the mass of the monosodium form of levosimendan. After 72 h in aqueous solution, a number of higher mass peaks appear (Fig. 9C). Peaks at \( m/z \) 561.0, \( m/z \) 583.2, and \( m/z \) 601.1 correspond well to the mass of \( (2\,M^-1\,Na^+2^-) \), \( (2\,M^-1\,Na^+2^-) \), and \( (2\,M^-1\,Na^+2^-) \) forms of levosimendan. These data suggest that levosimendan can dimerize in solution. A time-dependent accumulation of higher molecular mass species was also seen when levosimendan was prepared in Tris or HEPES buffers (data not shown).

DISCUSSION

The primary purpose of the present study was to identify binding sites for levosimendan on cTnC. As part of this overall study, we also report the identification of binding sites for EMD 57033 on cTnC.

EMD 57033 is structurally distinct from bepridil and TFP. It has positive inotropic effects on cardiac myocytes (13) but is believed to act at the actin myosin interface (13). It is reported to bind selectively to the C-terminal domain of cTnC with a \( K_d \) of approximately 40 \( \mu \)M (12) but does not increase the \( Ca^{2+} \) affinity of the regulatory site II (13). Our interest in EMD 57033 was to determine whether it can induce domain-specific changes in the Met methyl chemical shifts that would be consistent with selective binding to the C-terminal domain of cTnC. The data show a single binding site for EMD 57033 in the C-terminal domain of cTnC. Chemical shifts of Met methyl groups in the N-terminal domain of cTnC are unaffected, even at high concentrations of EMD 57033. It is likely that binding

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**Fig. 7.** HSQC spectrum of apo cTnC(A-Cys) after incubation with levosimendan at 40 °C for 24 h. Boxes indicate \(^1\)H,\(^{13}\)C split Met methyl correlations. The methyl \(^1\)H-\(^{13}\)C correlation for Met\(^{81}\) is at a 2-fold lower contour level.

**Fig. 8.** Detection of a covalently bound levosimendan-cTnC(A-Cys) complex. Chromatograms of cTnC(A-Cys) (A), levosimendan (B), the NMR sample containing apo cTnC(A-Cys) and 1 equivalent of levosimendan (see Fig. 5) (C), and the same NMR sample after removal of unbound drug (D). Conditions used are given under "Materials and Methods."
increased in the presence of Ca\(^{2+}\) retention time of levosimendan on a cTnC affinity column was prepared in 0.5 M NaOH and neutralized. The binding of bepridil and TFP to cTnC did not affect the Ca\(^{2+}\) binding properties of cTnC-A. This differs from previous reports (8, 15, 39). All data lead to the conclusion that levosimendan does not bind to cTnC or the cTnC-cTnI complex. This conclusion is in contrast to previous reports. Haikala et al. (15) showed that the retention time of levosimendan on a cTnC affinity column was increased in the presence of Ca\(^{2+}\) or Mg\(^{2+}\). From this it was concluded that levosimendan binds to both the N- and C-terminal domains of cTnC. However, significant effects on retention time were observed only at concentrations of Ca\(^{2+}\) greater than 1–3 mM, and no maximal retention time appeared to be reached even at 30 mM Ca\(^{2+}\). Solution phase equilibrium dialysis shown in Fig. 4 failed to show direct binding of levosimendan to cTnC at a variety of pH values and ionic strengths, whereas bepridil binds to cTnC under similar conditions.

Pollesello et al. (8) studied an N-terminal fragment of cTnC using homonuclear \(^1\)H NMR, and reported NOEs between levosimendan and Met\(^{81}\), Met\(^{85}\), and Phe\(^{77}\) in cTnC. However, these NOEs were weak, and the sequence-specific assignment of Met methyl groups was tentative. Our experiments used full-length cTnC(A-Cys) and cTnC, coupled with high-resolution heteronuclear NMR and unambiguous assignments of the Met methyl groups derived from mutagenesis (19) and the solution structure (9) of full-length cTnC(A-Cys). Our data show that the \(^1\)H chemical shifts of N-terminal Met residues in free cTnC(A-Cys), free cTnC, and the cTnC(A-Cys)/cTnI(A-Cys) binary complex are unaffected by all concentrations of levosimendan tested. Moreover, no NOEs were observed between free cTnC(A-Cys) and levosimendan. The chemical shifts for Met\(^{157}\) and Met\(^{120}\) in free cTnC are affected by levosimendan, but the observed changes are significant only at drug:protein ratios of greater than 1:1, which equates to millimolar concentrations of the drug, and this effect is not seen for the binary complex. These data, coupled with equilibrium dialysis experiments shown in Fig. 4, support the conclusion that levosimendan does not bind to the N-terminal regulatory domain of cTnC. Potential interactions with the C-terminal domain that affect the chemical shifts of Met\(^{157}\) and Met\(^{120}\) may be due to a nonspecific or very weak association that is prevented when cTnI binds to cTnC.

Our demonstration that levosimendan does not bind to cTnC does not question the positive inotropic effects of this compound that have been reported by different groups (26, 32). It simply questions the mechanism by which this effect is achieved. Several groups have reported that the positive inotropic effects of levosimendan result, at least in part, from inhibition of phosphodiesterase activity and accumulation of cAMP (26, 33–35). Our data show that levosimendan, as a dansylated, can covalently link to cTnC with apparent specificity for the N-terminal domain, as evidenced by the splitting of resonances from dansylated cTnC in the presence and absence of drug. We have not directly addressed this issue; however, we see no evidence that levosimendan alters the Ca\(^{2+}\) binding properties of cTnC(A-Cys) using a competitive dye assay in which the protein is not covalently modified.

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Fig. 9. Matrix-assisted UV laser desorption mass spectra of levosimendan. A–C, spectra of levosimendan stock prepared under previously published conditions (8, 15). A is the drug prepared in Me\(_2\)SO. A single major peak with corresponding to levosimendan (expected (M + H\(^+\)) = 281.0) is observed visible. B is levosimendan freshly prepared in 0.5 M NaOH and neutralized. C shows the spectra of the same diluted stock as in B after 72 h.

Pollesello et al. (8) reported that levosimendan increased the Ca\(^{2+}\) binding affinity of site II in cTnC as evidenced by Ca\(^{2+}\)-dependent changes in fluorescence from dansylated cTnC in the presence and absence of drug. We have not directly addressed this issue; however, we see no evidence that levosimendan alters the Ca\(^{2+}\) binding properties of cTnC(A-Cys) using a competitive dye assay in which the protein is not covalently modified.
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