Binding of Levosimendan, a Calcium Sensitizer, to Cardiac Troponin C*

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Levosimendan is an inodilatory drug that mediates its cardiac effect by the calcium sensitization of contractile proteins. The target protein of levosimendan is cardiac troponin C (cTnC). In the current work, we have studied the interaction of levosimendan with Ca2+-saturated cTnC by heteronuclear NMR and small angle x-ray scattering. A specific interaction between levosimendan and the Ca2+-loaded regulatory domain of recombinant cTnC was observed. The changes in the NMR spectra of the N-domain of full-length cTnC35S due to the binding of levosimendan to the primary site, were indicative of a slow conformational exchange. In contrast, no binding of levosimendan to the regulatory domain of cTnCCTn2 (where all the cysteine residues are mutated to serine), was detected. Moreover, it was shown that levosimendan was in fast exchange on the NMR time scale with a secondary binding site in the C-domain of both cTnC35S and cTnCCTn2. The small angle x-ray scattering experiments confirm the binding of levosimendan to Ca2+-saturated cTnC but show no domain-domain closure. The experiments were run in the absence of the reducing agent dithiothreitol and the preservative sodium azide (NaN3), since we found that levosimendan reacts with these chemicals, commonly used for preparation of NMR protein samples.

The number of patients suffering from heart failure is increasing along with the aging of population. Calcium sensitizers have been proposed as a treatment for congestive heart failure since they exert a positive inotropic effect without increasing the intracellular calcium concentration (1). Levosimendan, a potent calcium sensitizers that improves the force development of the muscle contraction without increasing the cytosolic Ca2+ ion concentration (2), was discovered using troponin C as target protein.

Troponin C (TnC) is responsible for the contraction trigger in the muscle. It belongs to the family of calcium binding EF-hand proteins and consists of two domains. The N-terminal half (NTnC) is responsible for the calcium-dependent regulation of the contraction, and the C-terminal half is a structural domain always loaded with divalent cations under physiological conditions. Troponin C interacts with troponin I (TnI), and this interaction is modulated by the binding of calcium. Studies of skeletal troponin C, a homologous protein, show that a hydrophobic patch is exposed in the open conformation of the calcium-loaded regulatory domain, which is a binding site for TnI (3). This has also been proposed to be a potential binding site for calcium sensitizers (4, 5). Contrary to skeletal troponin C, the binding of Ca2+ to cTnC does not induce an opening of the conformation. Consequently there is, in vitro, no exposure of a hydrophobic region (6–9). The simultaneous binding of cardiac troponin I and Ca2+ to cTnC, however, opens the structure of the N-terminal domain (10, 11). This structural and functional difference between TnC in skeletal and cardiac muscle is still to be clarified.

Levosimendan has been reported to bind to the regulatory domain of cardiac troponin C in a calcium-dependent manner (5, 12). However, the interaction of levosimendan with cTnC has been under debate for some time. Pollesello et al. (5) report the binding of levosimendan to the Ca2+-saturated form of cTnC. A possible binding site for calcium sensitizers in the vicinity of Asp-88 was located by using point-mutated and danylabeled human recombinant cTnC, NMR, and molecular modeling (4, 5). However, very recently Kleerekoper and Putkey (13) reported that levosimendan did not bind to cTnC. To clarify this controversial situation we studied the stability of levosimendan and levosimendan-cTnC under various solution conditions and the interaction of levosimendan with cTnC by heteronuclear NMR spectroscopy and small angle x-ray scattering. The results are also of general importance to studies of the structure-activity relationship by NMR.

MATERIALS AND METHODS

Levosimendan Samples—For every experiment with levosimendan, a fresh 30 mM stock solution was prepared by dissolving dry levosimendan powder into 30 mM potassium carbonate. The solution was gently shaken for ~30 s at room temperature until a clear solution was obtained. The stock solution was analyzed by high performance liquid chromatography (HPLC) for purity.

Levosimendan was purchased from Orion Pharma and dissolved in 30 mM potassium carbonate.

The abbreviations used are: TnC, troponin C; cTnC, cardiac TnC; NTnC, N-terminal half of TnC; TnI, troponin I; cTnC35S, cardiac troponin C with Cys-35 mutated to Ser; cTnCCys, cardiac troponin C with both cysteine residues 35 and 84 mutated to Ser; cTnCCTn2, N-terminal domain of cardiac troponin C; DTT, dithiothreitol; HSQC, heteronuclear single-quantum correlation; NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy; ni, number of time increments; nt, number of transients; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; Bis-Tris, bis(2-hydroxyethyl)iminotri(hydroxymethyl) methane.

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9337
chromatography and by mass spectrometry to ensure that no degradation had occurred during the course of the sample preparation. Levosimendan solutions were thereafter diluted in the same buffer solution used for protein samples (20 mM Bis-Tris, 10 mM CaCl₂, pH 6.8).

**Protein Sample Preparation**—In this study, we used three different cTnC molecules. Recombinant 13N-labeled N-terminal fragment of human cardiac troponin C (residues 1–91) was cloned, expressed, purified as previously described (8). The cDNA for cTnC C35S, was generated by site-directed mutagenesis of the cTnC C35S cDNA previously subcloned into the pET23d + expression vector (Novagen). The polymerase chain reaction-based gene splicing by overlap extension strategy was used to incorporate base changes encoding for Ser at codon 84 (14). The cTnC C35S cDNA was subsequently subcloned into NcoI and BamHI sites in the pET23d + expression vector. Isotopically enriched cTnCC35S and cTnC C35S, were expressed and purified as previously described (15 and 16).

Protein samples were initially prepared in the presence of DTT to avoid disulfide formation (17). Before the binding experiments, protein solutions containing DTT were washed with a large volume of DTT-free and NaN₃-free buffer and concentrated by centrifuge ultrafiltration (3,000 Centricon, 5 °C, Sorvall SS-34 rotor, 7,500 rpm). The washing buffer contained 20 mM Bis-Tris, 10 mM CaCl₂ at pH 6.8. Protein concentrations, generally between 0.2 and 0.5 mM, were determined by the method of Bradford (18) using bovine serum albumin as a standard. The NMR samples were prepared to the volume of 300 μL, containing 5% D₂O, and the pH was adjusted to 6.8 at room temperature with a few microliters of dilute NaOH or HCl when necessary (pH was not corrected for deuterium effects). An aliquot from the levosimendan stock solution was instantly added after the preparation to the protein solution up to a 3-fold excess compared with the protein concentration, and pH was readjusted to 6.8 with dilute HCl. A small aliquot of the final Ca²⁺-saturated cTnC sample with levosimendan was kept at 40 °C and analyzed with matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) at time points 0, 1, 4, 24, and 72 h.

**NMR Spectroscopy**—All spectra were acquired by a Varian Unity Inova 600- or 800-MHz spectrometers at 40 °C. One-dimensional proton spectra were collected to monitor the state of levosimendan under various experimental conditions. Two-dimensional 13N heteronuclear single-quantum correlation spectra (13N-HSQC) of cTnC and cTnC C35S were recorded at 800 MHz using 256 time increments (ni and 16 transients (nt) and spectral widths of 11,000 Hz in proton dimension and 2,200 Hz in nitrogen dimension in the presence of 0.05% NaN₃ (1 mM levosimendan) (C) and 8 mM DTT (1 mM levosimendan) (D). The spectrum of levosimendan in the presence of NaN₃ is referenced according to the left doublet. Other spectra were referenced to the water signal. Levosimendan molecule (E) and its adduct with sodium azide (F) as well as a reaction product of levosimendan in the presence of DTT (G) are shown on the right.

**RESULTS**

**Stability of Levosimendan in the Samples**—The stability of levosimendan was monitored by recording one-dimensional proton NMR spectra in the presence and absence of a reducing agent DTT and a bacterial inhibitor sodium azide (NaN₃). The effects of these substances on the stability of levosimendan were tested because they had been commonly used in the preparation of protein samples for NMR analysis. A freshly made levosimendan sample (Fig. 1 A) showed the characteristic AA′BB′₁H signal pattern of para-disubstituted phenyl ring in the aromatic region of the proton NMR spectrum. After an incubation of 20 h at 40 °C, a broadening of the signal at 8.0 ppm and a decrease in intensity with a doublet component at 7.5 ppm was observed (Fig. 1 B). Surprisingly, just a few minutes after the addition of NaN₃ to a levosimendan solution, an incubation of 20 h at 40 °C, the signal intensity showed a reduction in intensity combined with the appearance of a new resonance (Fig. 1 C) indicative of a formation of a new compound. In an NMR tube, this reaction proceeded to completeness, and a single product was obtained. We suspect that the cyano groups reacted with the azide group to form a cyclic adduct (Fig. 1 F) by a 1,3-dipolar addition mechanism (21).

In the presence of a large excess of DTT, sometimes a visible precipitate appeared in the levosimendan sample, and the ¹H signals of the aromatic protons of levosimendan almost completely disappeared in a few minutes, as shown in Fig. 1 D. The cyano groups of levosimendan might react by a reductive addi-
tion mechanism with the reducing agent DTT. A possible reaction product with DTT is presented in Fig. 1G. Notably, both levosimendan and DTT have two reactive groups, which can lead to formation of a polymer, causing the precipitation of the sample and the broad lines in the $^1H$ spectrum (Fig. 1D).

**Binding of Levosimendan to Ca$^{2+}$-cTnC—Two-dimensional $^{15}$N-HSQC spectra were acquired to determine the interaction of levosimendan with the Ca$^{2+}$-saturated form of cTnC. The $^{15}$N-HSQC spectrum (Fig. 2) revealed changes in the chemical shifts of several cross-correlation peaks. Some cross-peaks were split into two signals upon the addition of levosimendan, whereas other cross-peaks experienced frequency shifts. All resonance doublings took place in the N-terminal half of the (Ca$^{2+}$)$_2$-cTnC$_{35S}$, whereas shift changes appeared also in the C-terminal half.

The observed resonance doublings and chemical shift changes might have been due to at least two different processes. One process has slow kinetics with a residence time of Levosimendan $>$ 0.1 s, since it does not result in any observable broadening of the two resonances. The other employs fast kinetics with a residence time of < 0.001 s, resulting in shift changes of up to 20 Hz without any major line broadening. In the following will we assume that the binding site with the slow kinetics is the primary binding site and that the fast kinetics are caused by binding to one or more secondary binding sites. As can be seen from Fig. 3A, no resonance doublings were observed beyond residue 92, and therefore, we concluded that the primary binding site is in the N-terminal domain. A comparison of the data in Figs. 2, B and C, shows that the primary binding site did not exist in cTnC$_{A-Cys}$. Evidently Cys-84, but not Cys-35, is important for levosimendan binding to the primary site. It is also important to note that even though there are no resonance doublings in the C-terminal half of cTnC$_{35S}$, this half of the molecule is essential for the binding to the primary site, since no resonance doubling is observed in the isolated N-terminal half (1–91) of cTnC (Fig. 2D). These observations may explain the discrepancies in the results obtained earlier when studying the binding of levosimendan to cardiac troponin C. In the C-domain of cTnC$_{35S}$ and cTnC$_{A-Cys}$, there seems to be a secondary binding sites, judged by the relatively small chemical shift changes (Fig. 3B and C). By mapping these chemical shift changes to the cTnC structure (1AJ4 from the Protein Data Bank), it appears that the two distinct interaction sites in the C-domain of cTnC are not spatially related to each other.

The $^{13}$C signals of the methionine methyl groups are sensitive markers for binding of ligands to cTnC, and they have been used to study the interaction between cTnC and cardiac troponin I (15, 22, and 23) and between cTnC and various drugs (13, 24). Constant time $^{13}$C-HSQC spectra were used to follow changes in these groups, as the methionine methyls can be easily distinguished from other methyls by their negative correlation peaks. Splitting of the methyl signals of N-terminal methionine residues Met-47, Met-81, and Met-85 (Fig. 4) were observed. This is in agreement with the result of the $^{15}$N-HSQC experiment, as the amide proton signals of both Met-81 and Met-85 split into doublets due to drug binding (Met-47 could not be assigned in the $^{15}$N-HSQC). In the C-terminal domain, the methyl groups of Met-120 and Met-157 experience only small chemical shift changes (Met-120, $\delta^1H$ 0.03 ppm; $\delta^{13}$C, 0.04 ppm; and Met-157, $\delta^1H$ 0.02 ppm).

To gain further insight into the binding of levosimendan with Ca$^{2+}$-saturated cardiac TnC, two-dimensional NOESY spectra of free levosimendan, (Ca$^{2+}$)$_2$-cTnC$_{35S}$, and the drug-protein complex were acquired (Fig. 5). For a small ligand like levosimendan with a short rotational correlation time ($\tau_c$), NOEs are weak and negative but become stronger and positive when the ligand binds to the target protein. In water, the intramolecular NOE signals of levosimendan change their signs when the drug is titrated to the protein solution. This shows that there is a pronounced change in the correlation time of levosimendan in...
All the NMR data were collected immediately after the addition of levosimendan to troponin C. Interestingly, we noticed that the new signals and the shift changes induced by levosimendan binding disappeared when the sample was stored for several days at 40 °C (data not shown). Notably, the spectral changes reappeared upon the addition of fresh levosimendan to the sample.

Small angle x-ray scattering of the Ca\(^{2+}\)-saturated form of cTnCC\(_{\text{GSS}}\) and its complex with levosimendan are rather similar. The scattered intensity obeys Guinier law at the smallest \(k\) values, and there is no sign of protein aggregation (Fig. 6A) (26). There is only a small change in the form of the distance distribution function, \(P(r)\), when levosimendan binds to cTnCC\(_{\text{GSS}}\) (Fig. 6B). The maximum distance increases from \(63 \pm 5\) to \(70 \pm 5\) Å, and the radius of gyration increases from \(20.2 \pm 0.5\) to \(21.7 \pm 0.6\) Å. The determined radius of gyration, \(20.2 \pm 0.5\) Å, for troponin C without levosimendan, is in good agreement with a previous study on troponin C (27).

**DISCUSSION**

Our present finding that levosimendan reacts with common additives in protein solutions used in NMR studies clearly shows the need for careful studies of the stability of molecules used in binding experiments. In the drug discovery strategy structure-activity relationship by NMR (28–30), for example, it appears now of utmost importance to know the stability of the compounds to be tested at the experimental conditions which are used.

The binding of levosimendan to cardiac troponin C has been under debate for some time. Previous studies (5, 12, and 31) gave evidence for levosimendan binding. However, contradictory results that show no binding have also been reported (13). In our hands, in the course of the titration of (Ca\(^{2+}\))\(_3\)-cTnCC\(_{\text{GSS}}\) with levosimendan, some odd behavior was observed. The pH of the protein-drug solution changed during the experiments, and sometimes levosimendan precipitated out of the protein solution as a bright yellow precipitate, making it difficult to reproduce the measurements (data not shown). We have now found that commonly used additives in protein samples, DTT and Na\(\text{N}_3\), react with levosimendan (Fig. 1). Sodium azide forms an adduct with levosimendan, and consequently it was no longer added to our protein samples. To prepare cTnC samples without DTT was of concern because of the possible formation of intra- and intermolecular disulfide bonds. However, we observed no intermolecular disulfide bond formation in our DTT-free cTnCC\(_{\text{GSS}}\) samples after a couple of days of incubation, as analyzed by MALDI-TOF. Moreover, intramolecular disulfide bonds are not possible in cTnCC\(_{\text{GSS}}\), with only one cysteine residue.

The controversial results of levosimendan binding to cTnC are difficult to explain only by drug reactivity under different experimental conditions. We believe that the differences to some extent also originate from the fact that various protein sequences have been used. In fact, the recombinant N-terminal fragment of human cTnC contains two cysteine residues, Cys-35 and Cys-84. In full-length chicken cTnC\(_{\text{GSS}}\), Cys-35 is mutated to serine, and in full-length chicken cTnC\(_{\text{GSS}}\), both cysteine residues are changed to serines. The residues Cys-35 and Cys-84 of cTnC are conserved among various species, suggesting their importance for the function of the protein. However, it has been previously reported that the conversion of cysteines to serines does not alter calcium binding to cTnC but might modify the structure of cTnC, as indicated by changes in its dye binding properties (17). The \(^{15}\text{N}\)-HSQC spectra show that binding of levosimendan to Ca\(^{2+}\)-saturated forms of cTnCC\(_{\text{GSS}}\) and cTnC\(_{\text{A-Cys}}\) are different. The small chemical shift changes attributed to the secondary binding sites are similar,
but the observed resonance doublings in the N-domain of cTnCC35S are completely missing from the 1H-13C HSQC spectrum of the A-Cys form of cTnC. This observation proves that the C84S mutant makes a difference in levosimendan binding to the primary binding site. We therefore conclude that the primary binding site critically depends on Cys-84. The isolated N-terminal fragment of cTnC also shows interaction with levosimendan (Fig. 2D). However, the binding seems to be different compared with the full-length cTnC C35S. The N-terminal fragment does not contain an intact primary binding site for levosimendan. This is reasonable because Cys-84 is only a few residues away from the chain end at Gly-91.

It would naturally be very interesting to localize the primary binding site of levosimendan in the cTnC. This is, however, presently not possible since there are effects all over the N-terminal half of cTnC. The fact that most of the residues in the N-domain of cTnC C35S show resonance-doubling indicates that the binding of levosimendan to the primary site causes a conformational change involving most of cTnC. The exchange rate for this conformational change is slow, $k < 10 \text{ s}^{-1}$, since we do not observe any line-broadening effects. (Ca$^{2+}$)$_3$-cTnC exists in two conformations, i.e. the open and closed states. The exchange between open and closed conformations is intermediate on the NMR time scale, and the equilibrium favors the closed form (8, 9). An obvious explanation for peak doublings would be that levosimendan binds only to the open conformation, but there is a large difference between the $k_{on}$ and $k_{off}$ values, the $k_{off}$ being significantly slower as compared with $k_{on}$. Another possible explanation is that levosimendan binds to both forms, but preferentially to the open one, since in the presence of levosimendan the equilibrium reaches about a 50:50 ratio for the two states. The exchange between the two states of cTnC is significantly slower in the presence of levosimendan. At the present stage of the work, we were not able to completely rule out either possibility.

It is interesting to compare the levosimendan binding to troponin C with the binding of other molecules (e.g. bepridil, EMD57033, and trifluoperazine (24, 32)). Recently, it has been shown by x-ray crystallography that the structure of cTnC opens in response to bepridil binding (33). Three bepridil mol-

![Fig. 4. Binding of levosimendan to Ca$^{2+}$-saturated cTnC followed by constant time 1H-13C HSQC spectra at 800 MHz. 1H-13C HSQC spectra of Ca$^{2+}$-saturated cTnC in the absence (A) and presence (B) of three equivalents of levosimendan. Assignments of the Met methyl correlations are according to Lin et al. (25). Met-47, Met-81, and Met-85 experience the largest changes in shifts. For each of these residues the simultaneous presence of two correlations reveals the presence of two conformations. Assignments of the correlations in the complex are based on similarity to free cTnC C35S.](image)

![Fig. 5. 13C-edited NOESY of Ca$^{2+}$-saturated cTnC with 13C-labeled levosimendan at 800 MHz. The 13C-edited NOESY of the 13C-labeled aromatic ring of levosimendan with unlabeled cTnC was acquired at 40 °C. Trace A through the low-field peak of the aromatic region is indicated by the arrow. The levosimendan binding to cTnC C35S leads to an increase of effective rotational correlation time that gives rise to observable intramolecular NOEs within the drug molecule (dashed lines). Weak correlations seen at the high-field are thought to arise from the interaction between levosimendan and cTnC C35S. Trace B is from the same level of the reference NOESY spectrum of 13C-labeled levosimendan without cTnC in the same conditions.](image)
molecules bind to one cTnC A-Cys molecule. One bepridil molecule binds to the N-terminal half and opens its structure, and the other two bepridil molecules mimic the TnI binding to the C-terminal half. The chemical shift changes induced by levosimendan binding are smaller than those caused by bepridil binding (data not shown). In contrast to the case of bepridil binding to cTnC, our small angle x-ray scattering data show no sign of a domain-domain closure in the presence of levosimendan (Fig. 6). If anything, it seems that levosimendan binding to the primary binding site, located close to the end of N-domain, prevents the domains from moving closer to each other and might actually increase the maximal distance ($r_{\text{max}}$). Alternatively, this might be caused by a levosimendan induced structural change in the regulatory domain of cTnC. In vivo cTnC is not expected to experience a large spatial reorganization of domains within the troponin complex. This is in accordance with Ca$^{2+}$ sensitizers, affecting only the regulatory domain. However, the final evidence will be obtained once the structure of the levosimendan-(Ca$^{2+}$)$_3$-cTnCC$^{35S}$ complex in solution is determined.

**CONCLUSIONS**

Our data unambiguously show several interaction sites for levosimendan on the Ca$^{2+}$-loaded form of cardiac troponin C (cTnC$^{35S}$). Levosimendan does bind to cTnC$^{35S}$, but only in the absence of NaN$_3$ and DTT, which cause degradation of levosimendan. Thus, the current observations explain the discrepancies between earlier studies of levosimendan binding to cTnC. Our results suggest that the primary binding site is located in the regulatory domain (cNTnC) of cTnC and that there are two secondary binding sites at the C-terminal half of cTnC possibly analogous to the case of three bepridil molecules binding to cTnC$_{A-Cys}$ (33). Likewise, levosimendan may contribute to the opening of the regulatory domain. However, levosimendan does not cause a domain-domain closure. At present, we are not able to determine the precise locus of the primary binding site on the N-terminal domain due to the numerous changes in the spectra upon levosimendan binding. However, results from experiments with cTnCA-Cys show that the presence of Cys-84 is of critical importance for levosimendan binding. The results presented give us a reason to believe that the binding of levosimendan to the calcium-saturated regulatory domain of cTnC is the mechanism behind its known Ca$^{2+}$-sensitizing effect.

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**FIG. 6.** Small angle x-ray scattering data. A, scattered intensities ($I$) of troponin C (solid line) and troponin C in the complex with levosimendan (filled circles) as a function of the magnitude of the scattering vector $k$. For clarity, only every third measuring point is plotted in the figure. The Guinier region is shown in the inset. Error bars are based on statistical precision of the experimental intensity curves. B, experimental distance distribution functions, $P(r)$, of troponin C (solid line) and troponin C with levosimendan (filled circles) and $P(r)$ calculated from the coordinates of bepridil-cTnCA-Cys (1DTL from the Protein Data Bank) (dotted line). The difference in the shapes of the $P(r)$ functions indicates that levosimendan only slightly affects the relative orientation of the two domains of troponin C.
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