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Original Article

Interaction of levosimendan with cardiac troponin C in the presence of cardiac troponin I peptides

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Abstract

The interaction between troponin C (TnC) and troponin I (TnI) is essential for the regulation of muscle contraction. There are several binding sites for TnI on TnC that are differentially occupied depending on the phase of the contraction/relaxation cycle. TnI and TnC interact in an antiparallel fashion with each other. The C-domain of cTnC and the N-domain region of cTnI(residues 33–70) always interact under physiological conditions, whereas the interaction between regulatory regions of TnC and TnI (residues 128–166) is calcium dependent. Previously, it has been shown that levosimendan, a calcium sensitizer used as a treatment for acute heart failure, can interact with both domains of isolated cTnC. To understand which interaction is relevant for the mechanism of calcium sensitization, we used a more complete troponin model obtained by complexing $cTnI_{32–79}$ and $cTnI_{128–180}$ with calcium-saturated $cTnC_{CS}$. The cTnI peptides bound to $cTnC_{CS}$ to form a 1:1:1 complex. The interaction of levosimendan with this complex was followed by $^{1}H–^{15}N$ heteronuclear correlation spectroscopy. It was clear that based on chemical shift changes, $cTnI_{32–79}$ blocked the levosimendan interaction sites on the C-domain, whereas $cTnI_{128–180}$ did not compete with levosimendan for the binding site on the N-domain. Hence, the effective binding site of levosimendan on cTnC resulting in the calcium-sensitizing effect is located in the regulatory domain (N-domain).

Keywords: Cardiac troponin C; Cardiac troponin I; Calcium sensitizer; NMR; Drug interaction

1. Introduction

The contractile system works in a highly organized manner to bring about muscle contraction and relaxation. The troponin complex is essential for the regulation of this function. This heterotrimeric protein complex is anchored on the thin filament by its tropomyosin-binding unit troponin T (TnT). The contraction is regulated by a calcium-dependent interaction between troponin C (TnC, the calcium-binding unit) and troponin I (TnI, the inhibitory unit). Muscle contraction is initiated by calcium binding to the N-domain of TnC altering the interaction between TnC and TnI. This causes the inhibitory region of TnI to detach from actin and bind to TnC. Inhibition of the actomyosin ATPase is thus removed and muscle contracts.

Two decades ago a new class of drugs was proposed for treatment of cardiac failure [1–3]. Such drugs would function by making the regulatory thin filament proteins more sensitive to calcium and/or stabilize calcium-induced changes resulting in stronger contraction of the myocardial muscle without an increase in intracellular calcium concentration. Cardiac troponin C (cTnC) was considered to be an ideal target for these drugs, which were named as calcium sensitizers as the calcium binding to cTnC is responsible for the initiation of the contraction. Since then it has become clear that there are several potential mechanisms that increase the calcium sensitivity of the contractile apparatus. It has been

Abbreviations: TnC, troponin C; cTnC, cardiac troponin C; cCTnC, C-domain of cardiac troponin C; cNTnC, N-domain of cardiac troponin C; $cTnC_{CS}$, cardiac troponin C with Cys35 mutated to Ser; TnI, troponin I; TnT, troponin T; Bis–Tris, *bis*(2-hydroxyethyl)imino-*tris*(hydroxymethyl) methane; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; NMR, nuclear magnetic resonance; HSQC, heteronuclear single-quantum coherence; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

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proposed among others that pharmacological agents, which augment the free energy of interaction between cTnC and cTnI would increase the apparent calcium sensitivity [4]. This would be achieved by a drug that stabilized the open conformation of the N-domain of cardiac troponin C (cNTnC) without interfering with the binding of TnI. This is the proposed mechanism for bepridil [5,6]. Another, less likely, mechanism for a drug to increase the apparent calcium sensitivity is to bind to the C-domain of cardiac troponin C (cCTnC) as EMD57033 [7]. Bepridil is not considered as a lead for drug development of calcium sensitizers any longer, since it was found to be active on other target proteins [8,9]. Also EMD57033 is not considered as a potential drug, since it has been reported to impair the relaxation of the cardiac muscle in some in vitro and ex vivo experiments [10,11], even though in vivo studies with dog hearts indicated no impairment of relaxation [12].

Levosimendan is a calcium sensitizer that has already been accepted for clinical use as an inotropic drug. Previous studies have demonstrated its capability to increase the contraction force in the cardiac myocytes without a concomitant increasing in calcium concentration [13,14]. It has been suggested that the effect of levosimendan on heart muscle results from its binding to the regulatory domain of cTnC. In a model of levosimendan/cNTnC, the binding site for levosimendan appeared to be located in the hydrophobic pocket of the N-domain [15,16]. It was concluded that the most likely calcium-sensitizing mechanism of levosimendan could be due to a stabilization of the calcium-saturated form of cTnC. However, the possibility of TnI binding competing with the drug binding was, at that time, left open.

Levosimendan is capable of binding to both domains of isolated cTnC [17,18]. The primary binding site was suggested to be in the cNTnC. Additionally, there were two secondary binding sites found on the hydrophobic regions of the C-terminal domain. These sites are shown to coincide with the binding sites for cTnI [19]. We have obtained a protein complex of cardiac troponin C with Cys35 mutated to Ser ($cTnC_{CS}$) with the $cTnI_{32–79}$ and $cTnI_{128–180}$ peptides to study the effect of levosimendan on a molecular level more precisely. The changes induced by levosimendan on this complex were followed by $^{1}H–^{15}N$ correlation spectroscopy. In this study, we describe the binding of levosimendan to the 1:1:1 complex of $cTnC_{CS}$/$cTnI_{32–79}$/$cTnI_{128–180}$ and discuss the molecular mechanism of this drug.

2. Materials and methods

2.1. Cardiac troponin C samples

^{13}C and ^{15}N labeled, chicken recombinant cTnC (Swiss-Prot ID P09860) with Cys35Ser mutation, $cTnC_{CS}$ was over expressed and purified as described earlier [20]. Lyophilized protein was dissolved in 20 mM *bis*(2-hydroxyethyl)imino-*tris*(hydroxymethyl)methane (Bis–Tris)/10 mM $CaCl_2$. Five percent D_2O was added to the final nuclear magnetic resonance (NMR) samples and the pH was adjusted to 6.8 (not corrected for deuterium effects). The protein concentrations used in structure–function studies varied from 0.1 to 0.2 mM. The sample made for the backbone assignment had a protein concentration of 0.5 mM. No protease inhibitors, reducing agents or bacterial inhibitors were added to any of the NMR samples to avoid degradation of levosimendan [17]. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and reversed phase chromatography were used to determine the purity of the protein samples.

2.2. Cardiac troponin I peptides

Human $cTnI_{32–79}$ and $cTnI_{128–180}$ peptides (Swiss-Prot ID P19429) were synthesized with an automated solid-phase synthesizer using 9-fluorenylmethoxycarbonyl (Fmoc) amino acids [21]. Peptide purity was confirmed by reversed phase chromatography and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF). $cTnI_{32–79}$ was mutated, Cys79Ser, to prevent unwanted interaction between cystein residues in the protein.

2.3. Protein complex formation

Stock solutions (10 mM) of both peptides of cTnI were prepared by dissolving the lyophilized peptides in pure water (Milli-Q H_2O). The $cTnC_{CS}$/$cTnI_{32–79}$/$cTnI_{128–180}$ complex was formed by adding small aliquots of the peptide stock solutions to the protein sample. Complex formation was followed by NMR.

2.4. Levosimendan samples

Levosimendan, the (–) enantiomer of {[4-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)phenyl]hydrazono}-propanedinitrile (CAS registry number 141505-33-1), was synthesized at Orion Pharma, Espoo, Finland.

Levosimendan titrations were made with 30–60 mM stock solutions. They were prepared by dissolving a weighed amount of levosimendan in dimethyl sulfoxide (DMSO). Stock solutions were light protected and stored at room temperature. The stability of the stock solutions was determined from one-dimensional proton NMR spectra.

2.5. NMR spectroscopy

The NMR spectra were recorded on a Varian INOVA 800-MHz spectrometer at the University of Helsinki and 750-MHz spectra were recorded at the SON NMR Large Scale Facility in Utrecht. The temperature in all experiments was 40 °C. Fast $^{1}H–^{15}N$ heteronuclear correlation spectroscopy (F-$^{1}H–^{15}N$ heteronuclear single-quantum coherence (HSQC)) was used to follow both the protein complex formation and drug-induced changes [22]. The number of transients was 16 or 32 and the number of increments was 128 or 256 in the $^{1}H–^{15}N$-HSQC spectra. The spectral width of the nitrogen dimension was 2200 Hz and of the proton dimen-

sion was 11,000 Hz. The backbone assignment of the cTnC$_{CS}$ in complex with cTnI$_{32–79}$ and cTnI$_{128–180}$ was achieved from three-dimensional HNCACB [23–25] and HN[CO]CA [26] spectra. The HNCACB spectrum was recorded using 20 transients with 64, 54, and 1024 complex points in ^{13}C, ^{15}N, and ^{1}H dimensions, respectively. The spectral width of the carbon dimension was 12,000 Hz, the nitrogen dimension 2200 Hz, and the proton dimension was 10,000 Hz. The HN[CO]CA spectrum was recorded using eight transients with 64, 54, and 1024 complex points in ^{13}C, ^{15}N, and ^{1}H dimensions, respectively. The spectral width of the carbon dimension was 5500 Hz, the nitrogen dimension 2200 Hz, and the proton dimension was 10,000 Hz, The NMR data was processed with NMRPipe and NMRView [27], and analyzed with SPARKY [28].

3. Results

3.1. cTnC$_{CS}$/cTnI$_{32–79}$/cTnI$_{128–180}$ complex formation

Titration of cTnI$_{128–180}$ to calcium-saturated cTnC$_{CS}$ occurred with smooth and linear chemical shift changes in resonances from the N-domain and linker region (data not shown). As no further changes were detected after a 1:1 protein-to-peptide ratio, we concluded that the formation of a 1:1 protein complex with high affinity had taken place. A light white precipitate became visible at a protein to peptide ratio 1:1.3. As the fully occupied complex was already formed at 1:1 ratio of cTnC$_{CS}$:cTnI$_{128–180}$, this was used in all other experiments.

Similarly, cTnI$_{32–79}$ peptide was titrated alone against $(Ca^{2+})_3$-cTnC$_{CS}$. Chemical shift changes were linear up to a protein to peptide ratio of 1:1 and were detected for resonances from the cCTnC$_{CS}$ (data not shown). When the cTnI$_{32–79}$ peptide concentration exceeded the concentration of cTnC$_{CS}$, the behavior of the chemical shift changes changed. Another set of resonances, residing mainly in the N-domain, was affected, and the direction by which some peaks from the C-domain shifted also changed. This indicated that there was at least a second binding site in cTnC$_{CS}$ for cTnI$_{32–79}$. cTnI$_{32–79}$ has been reported to bind to the cCTnC, the first formed 1:1 complex was most likely the biologically important [19,20,29–31]. The other binding site(s) for cTnI$_{32–79}$ could be blocked by other subunits of the complex as discussed below. cTnI$_{32–79}$ did not precipitate even at concentration 2.5 times higher than cTnC.

Adding cTnI$_{128–180}$ to the cTnC$_{CS}$/cTnI$_{32–79}$ complex, almost caused the same effect on resonances from the cNTnC$_{CS}$ as when added to the isolated cTnC$_{CS}$ and similarly for cTnI$_{32–79}$ on the C-domain in cTnC$_{CS}$. Furthermore, the spectrum of the ternary complex was the same independent of the order in which the peptides were added. The assignment of 107 of total 161 residues could be reliably achieved from three-dimensional HN[CO]CA and HNCACB NMR spectra with the help of the assignments of cTnC/cTnI$_{1–167}$ and cTnC/cTnI$_{1–80}$/cTnI$_{129–166}$ complexes by Abbott et al. [32]. The assignments of the complexes made of full-length cTnI or shorter cTnC-binding peptides of cTnI showed a very good agreement. Thus, the 1:1:1 complex of cTnC$_{CS}$/cTnI$_{32–79}$/cTnI$_{128–180}$ most likely has a relevant conformation for drug-binding studies.

3.2. Levosimendan-induced structural changes

Levosimendan-induced chemical shift changes in the cTnC$_{CS}$/cTnI$_{32–79}$/cTnI$_{128–180}$ complex were followed by ^{1}H–^{15}N-HSQC NMR spectroscopy. Titrations were made in both directions, i.e. the drug was titrated into the cTnC$_{CS}$/cTnI$_{32–79}$/cTnI$_{128–180}$ complex sample and also the peptides were added into cTnC$_{CS}$/levosimendan sample. This was done to ensure that the same complex was formed regardless of the order of the compounds added.

Levosimendan binding only caused significant chemical shift changes on resonances from the regulatory domain of cTnC$_{CS}$ in the presence of cTnI peptides (Fig. 1). The magnitude of the chemical shift changes was larger than observed for the isolated cTnC$_{CS}$ [17]. Chemical shift changes were dispersed throughout the whole cNTnC$_{CS}$ upon levosimendan binding. The residues, for which significant chemical shift changes occurred, have been indicated in the three-dimensional structure of the cNTnC with cTnI$_{147–163}$ (1MXL from PDB) (Fig. 2). A few clear resonance doublings (6) could be found in the complex spectrum after the addition of levosimendan. The number of clear resonance doublings was significantly fewer than for the isolated cTnC$_{CS}$ spectrum (40). Resonance doublings, where the intensity of the original peak decreased and a new peak in the vicinity clearly simultaneously appeared are marked as filled diamonds in Fig. 1.

Levosimendan did not inhibit the cTnI peptide binding. In the presence of levosimendan, both peptides of cTnI bound to cTnC$_{CS}$ and the end result was the same regardless of the order of peptide addition (Fig. 3). The binding of cTnI$_{128–180}$ to the cTnC$_{CS}$/levosimendan complex resulted in a disappearance of several N-domain peaks. The disappearing peaks were for the same residues that in isolated cTnC underwent resonance doublings upon levosimendan binding.

4. Discussion

The interaction between cTnC and cTnI is essential in the regulation of muscle contraction. Calcium acts as a trigger in this mechanism by binding to cNTnC and influencing the interaction between cTnC and cTnI. In a relaxed state, the inhibitory region of cTnI is bound to the actin filament thereby inhibiting actomyosin ATPase. Calcium binding to the cNTnC results in the conformational changes necessary to form a new cTnC/cTnI interaction. Thus, TnI switches from actin to troponin and the inhibition is removed. Tropomyosin is now able to role over actin and expose myosin-binding sites and contraction occurs. Modulation of this interaction between cTnC and cTnI has been hypothesized as

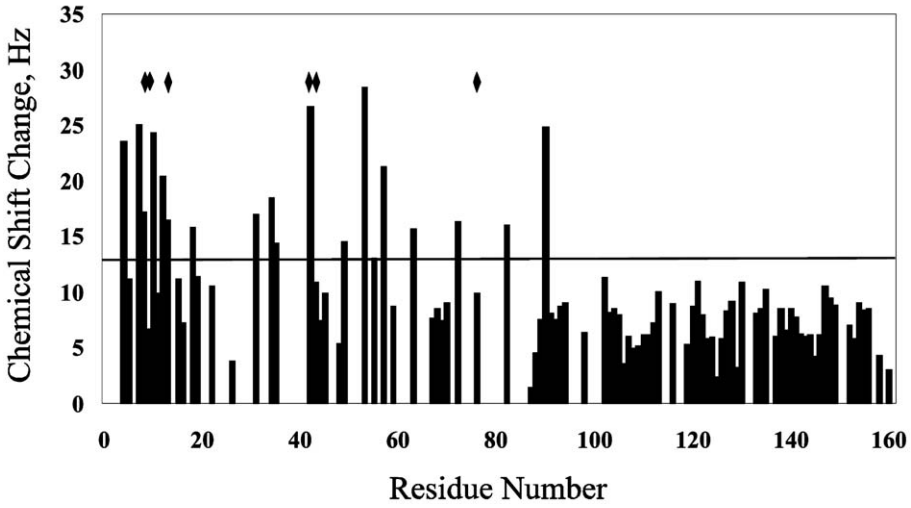

Fig. 1. Levosimendan induced chemical shift changes on the $cTnC_{CS}$/$cTnI_{32-79}$/$cTnI_{128-180}$ complex followed by F-^{1}H–^{15}N-HSQC at 750 MHz. The chemical shift change was calculated as a distance in Hz from the original peak. The solid line represents the average chemical shift changes of the C-domain + 1 S.D. The filled diamonds mark the resonance doublings.

a potential mechanism of calcium sensitizers [6,7,33]. It is necessary that the calcium-binding affinity to the TnC/TnI complex increases in the presence of a drug in order to cause an increased calcium sensitivity.

All previous structure–function studies of levosimendan interaction with cTnC were made using isolated cTnC. It has also been reported that levosimendan has an affinity towards the whole troponin complex but no structural information has been available [18]. Previously, we reported that levosimendan has at least three binding sites on isolated cTnC [17]. The binding to the N-domain resulted in doubling of most of the resonances. Resonance doublings as well as chemical shift changes were detected throughout the whole sequence of the N-domain and no exact binding site could be determined. In the C-domain, chemical shift changes indicate two binding sites that were in the proximity of cTnI-binding site.

To gain more insight regarding the effective mechanism of levosimendan and its interaction with cTnC, we complemented our model of the contraction switch by including cTnC-binding peptides $cTnI_{32-79}$ and $cTnI_{128-180}$. They include the primary cTnC-binding region (32–70) and the regulatory region (147– 166) and also the functionally important inhibitory region (128–147) (Fig. 4). The full-length cTnI could not be used because levosimendan bound to it non-specifically. The cTnI peptides had a high affinity for cTnC and based on smooth chemical shift changes along the peptide titrations, a 1:1:1 complex was formed. Levosimendan was added to this $cTnC_{CS}$/$cTnI_{32-79}$/$cTnI_{128-180}$ complex and conformational changes were followed by ^{1}H–^{15}N-HSQC. The quality of the spectra of the complex was not as good as for isolated cTnC and broadening beyond detection of some resonances resulted in several gaps in the sequential data. However, we were able to assign about two-thirds of the complex peaks and follow over 90% of them upon levosimendan binding.

Levosimendan binds to the N-domain of calcium-saturated $cTnC_{CS}$ in the ternary complex (Fig. 1). The chemical shift changes induced by levosimendan on the $cTnC_{CS}$/$cTnI_{32-79}$/$cTnI_{128-180}$ complex were somewhat larger than those observed for isolated $cTnC_{CS}$. However, since the changes were numerous and located all along the N-domain, no exact binding site could be determined for the levosimendan on the cTnC at this point. An unexpected result, not observed in the absence of cTnI peptides was the numerous chemical shift changes observed in the N-helix of cTnC in the complex with cTnI peptides. This could be an indication of a cardiac-specific triggering mechanism that has previously been reported to involve an inactive calcium-binding site I together with the N-terminal helix [34].

All the chemical shift changes in the $cCTnC_{CS}$ complexed with $cTnI_{32-79}$/$cTnI_{128-180}$ were small upon levosimendan binding. The hydrophobic region, where the largest levosimendan-induced changes were observed in the isolated cTnC, did not undergo any significant chemical shift changes in the presence of $cTnI_{32-79}$. It is, therefore, clear that in this region $cTnI_{32-79}$ is competing with levosimendan and the drug was not capable of interacting with the $cCTnC_{CS}$. This also implies that the protein complex formation had already been completed and no detectable amount of free cTnC was left to interact with levosimendan. TnI is bound to the cCTnC during the entire contraction–relaxation cycle, and thus blocks the levosimendan binding to this domain. Therefore, it is unlikely that the levosimendan interaction interferes, or is relevant to, the interaction of TnT with the cCTnC. However, it is clear that we cannot exclude other, possibly indirect, effects of levosimendan to the contractile apparatus.

All titration data supported our conclusions that the effective binding site for levosimendan is in the cNTnC and that cTnI blocks the C-domain binding of levosimendan. It should be noticed that we did not characterize an exact

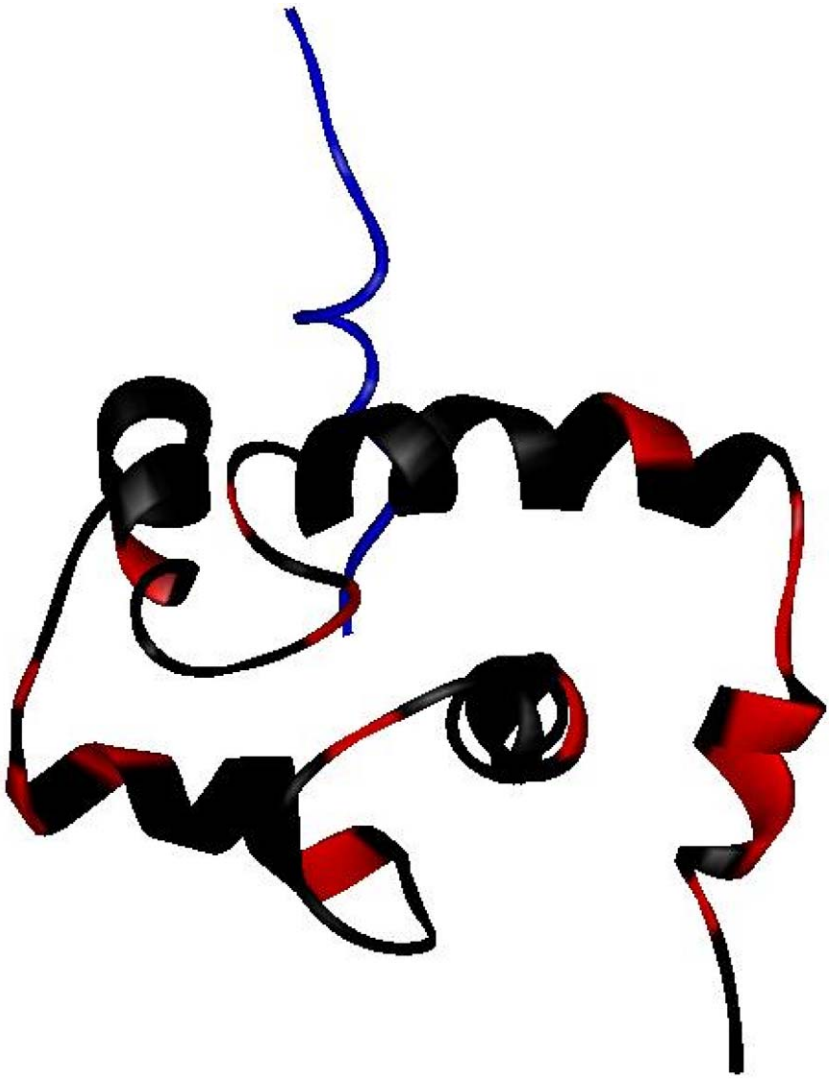

Fig. 2. Chemical shift changes induced by levosimendan binding to the $cNTnC_{CS}$ in the presence of $cTnI_{32–79}$ and $cTnI_{128–180}$ plotted on the three-dimensional structure of cTnC/cTnI (1MXL from PDB). The cTnI peptide is colored blue and the levosimendan-induced changes are marked with red. The N-helix is the short helix to the right.

binding site since we observed chemical shift changes on several amino acids of the N-domain. The affinity constants calculated for residues in different parts of the domain is, therefore, different (from 200 μM upward) and an exact K_d value for the affinity of levosimendan to the cNTnC cannot be given. In addition, many amino acids could be involved in small structural changes due to eventual non-specific low-affinity binding site of levosimendan on cTnC.

It should also be noticed that despite we used a more complete troponin model than cTnC alone, this is still a crude simplification of the cTnC/cTnI/cTnT– tropomyosin complex, which is bound to the actin filaments. In that complex, all elements contribute to the modulation, amplification, and transmission of the calcium-induced switch to the formation of actin–myosin cross-bridges. It would, therefore, be more meaningful to compare the EC_{50} of levosimendan as calcium sensitizer of the contractile apparatus to the K_d of the binding of levosimendan to cTnC when this protein is functionally connected to the whole regulatory complex [35].

It is possible that the levosimendan binding shifts the equilibrium from the closed or inactive conformation towards the open, active conformation of the N-domain, thus making the cTnI binding more favorable. It reduces the ATPase inhibition by making the cTnI binding to cTnC more effective, which results in the calcium-sensitizing effect. This agrees well with recently published results for bepridil binding to the cNTnC [5,6]. In those studies, it was concluded that bepridil stabilizes the open conformation and thus increases the calcium sensitivity. In our study, many of the levosimendan-induced chemical shift changes were located in the same region, where bepridil was found to induce changes [5] (Fig. 2). Thus, we tried to confirm if levosimendan and bepridil compete for the same binding site, however, we found that the two molecules interact with each other.

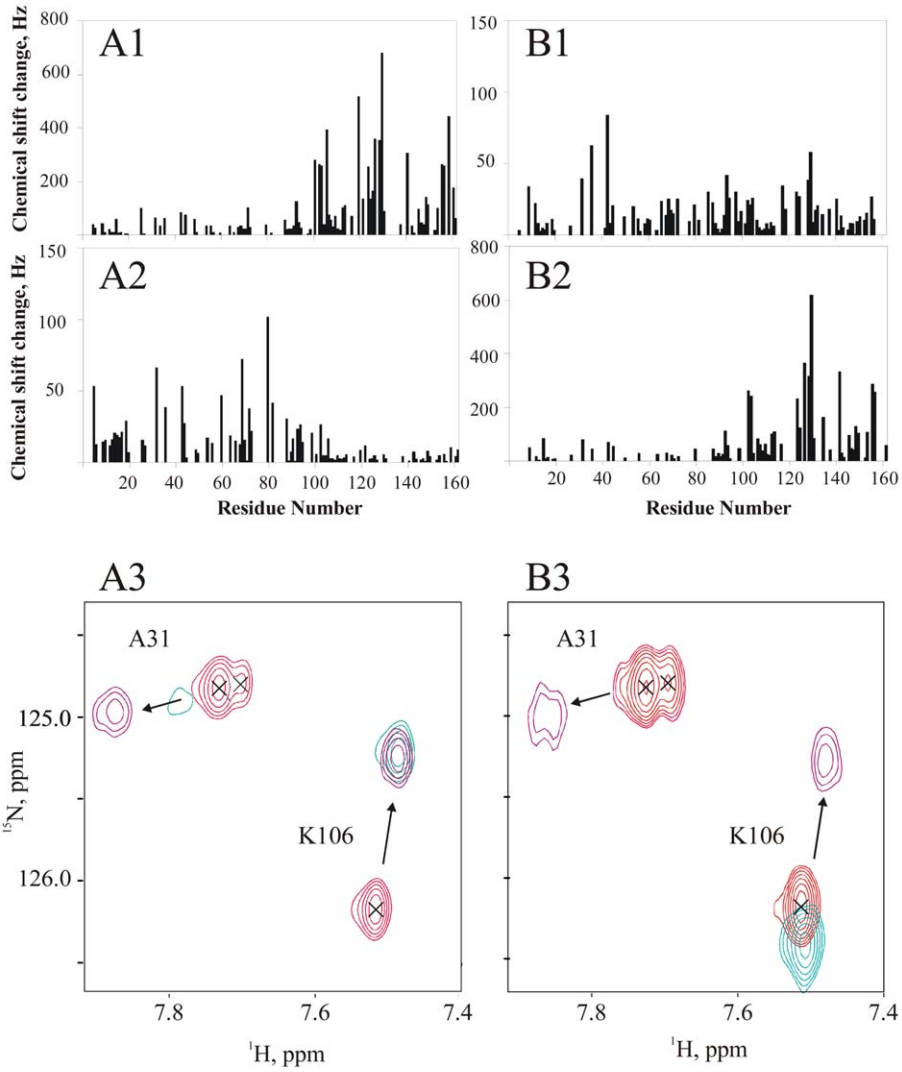

Fig. 3. $cTnI_{32–79}$ and $cTnI_{128–180}$ peptides induced chemical shift changes in the $cTnC_{CS}$/levosimendan complex followed by F-^{1}H–^{15}N-HSQC at 750 MHz. The difference to the original peak was calculated as a distance in Hz. (A1) Addition of 1:1 $cTnI_{32–79}$ to $cTnC_{CS}$/levosimendan. (A2) To that complex $cTnI_{128–180}$, which was added to form 1:1:1 protein complex. (A3) Overlaid spectra of the above titration points. Titration of peaks of Ala31 is represents the N-domain and Lys106 represents the C-domain binding of cTnI. Red marks the $cTnC_{CS}$ with levosimendan. Light blue represents the 1:1 addition of the first peptide and magenta is 1:1:1 addition of the second peptide. (B1) Addition of 1:1 $cTnI_{128–180}$ to $cTnC_{CS}$/levosimendan. (B2) To that complex $cTnI_{32–79}$ was added to form 1:1:1 protein complex. (B3) Overlaid spectra of the above titration points. Red marks the $cTnC_{CS}$ sample with levosimendan. Light blue represents the 1:1 addition of the first peptide and magenta is 1:1:1 addition of the second peptide.

The non-phosphorylated cardiac-specific N-terminal extension of cTnI interacts with the regulatory domain of cTnC [19,32,36]. It is considered to stabilize the interaction between the regulatory region of cTnI and cNTnC by shifting the equilibrium towards the open conformation. It is known that phosphorylation of the S22 and S23 in the cardiac-specific N-terminal extension reduces calcium sensitivity [37]. This suggested stabilization of the open conformation greatly resembles the proposed mechanism for calcium sensitizers and thus makes it extremely interesting in respect of the calcium sensitization in myofibrils.

Fig. 4. cTnC interaction regions of cTnI. Cardio-specific N-terminal extension (1– 32) is able to interact with the cNTnC but is not essential for muscle contraction. Residues 32–70 interact with the cCTnC Ca^{2+}/Mg^{2+} dependently. Inhibitory region of cTnI (128–147) interacts with actin if calcium is not bound to the cNTnC. When calcium binds to the cNTnC, $cTnI_{128–147}$ switches from actin to cTnC. This interaction is modulated by $cTnI_{147–166}$ that binds to the cNTnC calcium dependently. Gray regions mark the peptides used in this study.

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