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Tia Sorsa

The Interaction of the Calcium Sensitiser Levosimendan with Cardiac Troponin C

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ABSTRACT

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Cardiac failure is one of the leading causes of mortality in developed countries. As life expectancies of the populations of these countries grow, the number of patients suffering from cardiac insufficiency also increase. Effective treatments including the use of calcium sensitisers are being sought. They cause a positive inodilatory effect on cardio-myocytes without deleterious effects (arrhythmias) resulting from increases in intracellular calcium concentration.

Levosimendan is a novel calcium sensitiser that has been proved to be a well-tolerated and effective treatment for patients with severe decompensated heart failure. Cardiac troponin C (cTnC) is its target protein. However, there have been controversies about the interactions between levosimendan and cTnC. Some of these controversies have been addressed in this dissertation. Furthermore, studies on the calcium sensitising mechanism based on the interactions between levosimendan and cTnC as followed by nuclear magnetic resonance (NMR) are presented and discussed.

Levosimendan was found to interact with both domains of the calcium-saturated cTnC in the absence of cardiac troponin I (cTnI). In the presence of cTnI, the C-domain binding site was blocked and levosimendan interacted only with the regulatory domain of cTnC. This interaction may have caused the observed calcium sensitising effect by priming the N-domain for cTnI binding thereby extending the lifetime of that complex. It is suggested that this is achieved by shifting the equilibrium between open and closed conformations.

Keywords: cardiac troponin C, calcium sensitisation, drug interaction, regulation of muscle contraction, nuclear magnetic resonance

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PREFACE

The research for this thesis has been carried out at the NMR laboratory of the Institute of Biotechnology at the University of Helsinki and at the Target Protein Laboratory at Orion Pharma during the years 1998 to 2003. I thank Professor Mart Saarma, the Director of the Institute of Biotechnology, University of Helsinki, and Ph.D. Heimo Haikala at Orion Pharma for providing excellent facilities to carry out this work and for believing in this research.

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Professor Arto Annila at the Department of Physics, at the University of Helsinki, I thank for being the driving force for the use of residual dipolar couplings and SAXS along the more classical NMR methods. Arto's analytical approach to every aspects of life made the coffee-table discussions interesting.

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My warmest thanks are due to my family and all my friends for reminding me that there is life outside the lab too. I'm thankful to my parents for their endless concern of my well being, their support and extensive help through out the years. Especially I want to thank my mami for being a good professional example. My brother Timo I thank for his friendship and for giving a good resistance through out the years. I'm grateful to my aunt Tarja for her supportive interest and practical help. All my little friends Siiri, Meri, and my goddaughter Janette deserve extra thanks for showing tremendous capability to learn new things during the times I've tried to learn NMR.

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Helsinki, September 2003

Tia Sorsa

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which will be referred to by their respective Roman numerals in the text.

I Pääkkönen, K., Sorsa, T., Drakenberg, T., Pollesello, P., Tilgmann, C., Permi, P., Heikkinen, S., Kilpeläinen, I., and Annila, A. (2000) Conformations of the regulatory domain of cardiac troponin C examined by residual dipolar couplings. *European Journal of Biochemistry* **267**, 6665-6672.

II Sorsa, T., Heikkinen, S., Abbott, M.B., Abusamhadneh, E., Laakso, T., Tilgmann, C., Serimaa, R., Annila, A., Rosevear, P.R., Drakenberg, T., Pollesello, P., and Kilpeläinen, I. (2001) Binding of levosimendan, a calcium sensitizer, to cardiac troponin C. *Journal of Biological Chemistry* **276**, 9337-9343.

III Sorsa, T., Pollesello, P., Rosevear, P.R., Drakenberg, T., and Kilpeläinen, I. Stereoselective binding of levosimendan to cardiac troponin C causes calcium sensitization. *European Journal of Pharmacology.* Submitted

IV Sorsa, T., Pollesello, P., Permi, P., Drakenberg, T., and Kilpeläinen, I. (2003) Interaction of levosimendan with cardiac troponin C in the presence of cardiac troponin I peptides. *Journal of Molecular and Cellular Cardiology* **35**, 1055-1061

Tia Sorsa's contribution to the work in publications I to IV is as follows:

I Responsible for the sample preparation including both protein and phage production and purification as well as final NMR sample preparation. Participated in the experimental work and in the interpretation of the results.

II Responsible for the protein production and purification as well as the final sample preparation for NMR and SAXS measurements. Participated in planning the experimental work and acquiring the NMR spectra for the drug-protein interaction experiments. Processed and analysed the NMR data. Participated in the interpretation of the data and wrote the publication.

III Responsible for the protein production and purification as well as for sample preparation for NMR experiments. Planned the experimental work and performed the protein-ligand interaction NMR experiments. Processed, analysed and interpreted the NMR data and wrote the publication.

IV Responsible for the protein production and purification. Prepared the NMR samples, acquired the HSQC spectra for complex formation as well as for ligand interaction studies. Processed and analysed the NMR data. Interpreted the results and wrote the publication.

The author has also contributed to the following papers related to but not included in the thesis:

Levijoki, J., Pollesello, P., Kaivola, J., Tilgmann, C., Sorsa, T., Annila, A., Kilpeläinen, I., Haikala, H. (2000) Further evidence for the cardiac troponin C mediated calcium sensitization by levosimendan: structure-response and binding analysis with analogs of levosimendan. *Journal of Molecular and Cellular Cardiology* **32**, 479-491.

Permi, P., Sorsa, T., Kilpeläinen, I., Annila, A. (1999) HN(alpha/beta-COCA-J) experiment for measurement of (1)J(C'C(alpha)) couplings from two-dimensional [^{15}N, ^{1}H] correlation spectrum. *Journal of Magnetic Resonance* **141**, 44-51.

Pääkkönen, K., Annila, A., Sorsa, T., Pollesello, P., Tilgmann, C., Kilpeläinen, I., Karisola, P., Ulmanen, I., Drakenberg, T. (1998) Solution structure and main chain dynamics of the regulatory domain (Residues 1-91) of human cardiac troponin C. *Journal of Biological Chemistry* **273**, 15633-15638.

ABBREVIATIONS

ATPase	adenosine triphosphatase
BSA	bovine serum albumin
Bis-Tris	Bis(2-hydroxyethyl)-imino-tris(hydroxymethyl)methane
cCTnC	C-domain of cardiac troponin C
cNTnC	N-domain of cardiac troponin C
cTn	heterotrimeric cardiac troponin complex
cTnC	cardiac troponin C
CTnC	C-domain of troponin C
$cTnC_{A\text{-}Cys}$	cardiac troponin C with mutations of C35S and C84S
$cTnC_{CS}$	cardiac troponin C with mutation of C35S
cTnI	cardiac troponin I
cTnT	cardiac troponin T
DMSO	dimethyl sulfoxide
DTT	dithiolthreitol
FRET	fluoresence resonance energy transfer
HSQC	heteronuclear single quantum correlation
kDa	kilodalton
MALDI-TOF	matrix-assisted laser desorption ionization time-of-flight
NMR	nuclear magnetic resonance
NOESY	nuclear Overhauser enhancement spectroscopy
NTnC	N-domain of troponin C
PAGE	polyacrylamide gel electrophoresis
PDB	Protein Data Bank
PKA	protein kinase A
PKC	protein kinase C
RDC	residual dipolar coupling
RP	reversed-phase chromatography
SAXS	small-angle X-ray scattering
SAR	structure-to-activity relationship
SDS	sodium dodecyl sulfate
SR	sarcoplasmic reticulum
sTnC	skeletal troponin C
sTnI	skeletal troponin I
TFP	trifluoperazine
Tm	tropomyosin
Tn	heterotrimeric troponin complex
TnC	troponin C
TnI	troponin I
TnT	troponin T

1 INTRODUCTION

This thesis is based on a study of the interaction between the target protein cardiac troponin C (cTnC) and a novel calcium sensitiser levosimendan. In the introduction (chapter 1) the basic principles of muscle contraction and the main components of the contractile apparatus and their roles in muscle contraction are briefly described. The concepts used in the description are as they are understood and presented in contemporary scientific literature. The target protein, cTnC, and that of its rather complex interaction with troponin I (cTnI) are discussed. A few examples of other calcium sensitisers and their putative mechanisms are briefly covered. In the materials and methods part (chapter 2), the samples, methods and conditions of the studies presented in this thesis are described. More detailed information of samples and protocols can be found in the original publications I to IV (Appendices). In the results and discussion (chapter 3), the principal results are presented and discussed with respect to the interaction between levosimendan and cTnC. These results are compared with two other calcium sensitising molecules.

1.1 Muscle structure and function

1.1.1 Assembly of the contractile apparatus

Each striated muscle cell contains myofibrils formed by repeating units of sarcomeres arranged in series. Each sarcomere is composed of parallel and overlapping thin and thick filaments (Fig. 1). In skeletal and cardiac muscle, sarcomere structure and the general mechanism of the muscle contraction are the same. Thin and thick filaments of the sarcomere slide past each other and the sarcomere length shortens resulting in muscle contraction. This contraction movement is controlled by free intracellular calcium that regulates complex, interlinked protein-protein interactions that eventually result in the formation of the energy generating strong cross-bridges. The skeletal and cardiac muscle types differ in thin filament activation properties. These tissue specific differences are explained by varying physiological requirements. Such requirements arise from differences in the balance between the different conformational states of the regulatory proteins expressed in skeletal and cardiac muscle (McKay *et al.*, 2000).

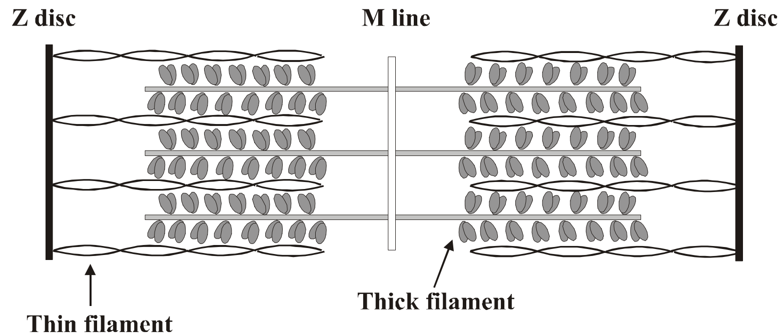

Fig. 1 **A sketch of a sarcomere.** It is formed of parallel thin and thick filaments between Z discs. When muscle contracts the filaments slide past each other and the length of the sarcomere shortens. Strong cross-bridges form between thin and thick filaments.

The thick filament of the sarcomere is a bipolar polymer composed of myosin molecules (Fig. 2). Myosin consists of two heavy and four light chains. A bundle of myosin heavy chain coiled-coil tails form the backbone of the thick filament. The globular heads of the heavy chain N-terminus named subfragment 1 (S1) point out from the thick filament at regular intervals. These myosin heads interact with the thin filaments of actin to form strong cross-bridges and an actomyosin complex, which facilitate and eventually result in the contraction of the sarcomere (Haselgrove and Huxley, 1973). Minor scaffolding components of the thick filaments, C-, X- and H proteins, keep myosin molecules together (Starr *et al.*, 1985; Seiler *et al.*, 1996). M-line proteins connect the thick filaments together at the center of the sarcomere and titin protein contributes to the elasticity and stability of the sarcomere (Wang *et al.*, 1998).

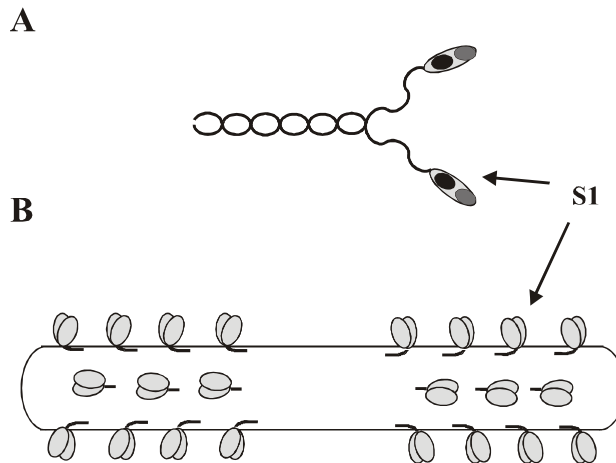

Fig. 2. **A scheme of myosin and a thick filament structure.** A) Myosin is formed of coiled-coil tail and globular heads (S1) that include the binding sites for actin and ATP. Myosin heads interact with thin filaments forming strong cross-bridges leading to muscle contraction. B) A bipolar myosin polymer shows S1 heads sticking outward at regular intervals for actin interaction.

The thin filament has a two-stranded helical structure (Fig. 3). The backbone of the thin filament is composed of polymerized globular actin monomers (G-actin). Actin monomers consist of two equal sized domains that can be further divided into two sub domains. The smaller sub domains 1 and 2 are solvent exposed and available for myosin interaction. Whereas the larger sub domains 3 and 4 interact with the corresponding sub domains of the adjacent strand (Holmes *et al.*, 1990; Lorenz *et al.*, 1995).

Tropomyosin (Tm) is located in a groove of the helical actin filament called F-actin (Fig. 3) (Moore *et al.*, 1970). It is a long (~ 40 nm) and flexible, largely α-helical coiled-coil dimer, which overlaps the neighboring tropomyosins in a head-to-tail configuration (Phillips *et al.*, 1986). Each Tm spans over seven actin monomers of the thin filament (Potter, 1974). The overlapping regions of adjacent tropomyosins are mainly responsible for the affinity of Tm for actin (Phillips *et al.*, 1986). It binds to the actin filament by electrostatic interaction (McLachlan and Stewart, 1976; Lorenz *et al.*, 1995). However, Tm on the thin filament is not fixed in one position. It rolls over the surface of the thin filament depending on the phase

of the contraction cycle. This movement is influenced by Ca^{2+} and it affects myosin S1 binding to actin (recent reviews by Gordon *et al.* (2000; 2001)).

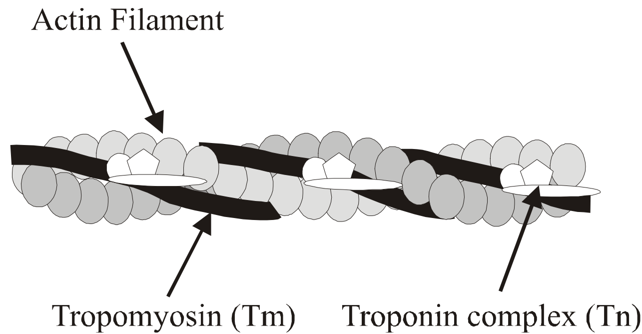

Fig. 3. **A scheme of a thin filament.** Tm is in the groove of the actin formed helical core and in regular intervals troponin complex is attached on the thin filament.

Troponin complex (Tn), together with Tm, forms the calcium dependent trigger of the contractile apparatus, i.e. a molecular switch on the thin filament. Tn is a heterotrimeric protein assembly. It consists of a calcium binding unit troponin C (TnC), an actomyosin ATPase inhibitory unit troponin I (TnI), and a Tm binding unit troponin T (TnT) (Greaser and Gergely, 1971; Ohtsuki *et al.*, 1986; Filatov *et al.*, 1999). One Tn complex interacts with one Tm molecule at regular intervals. Moreover, it is able to regulate the interaction of 12 to 14 actin monomers with myosin (Potter, 1974; Yates and Greaser, 1983; Solaro and Van Eyk, 1996; Ohtsuki and Shiraishi, 2002) (Fig. 4).

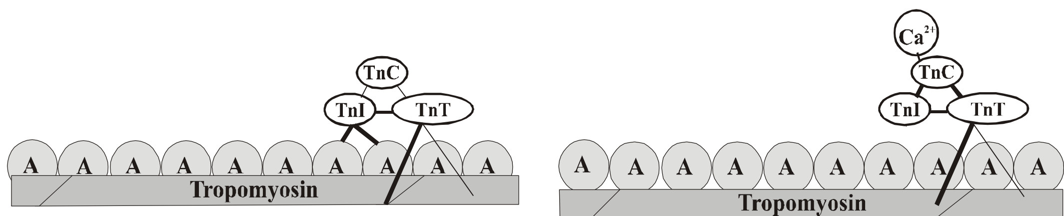

Fig. 4. **A diagram of the regulation of myosin/actin interaction by the Tm/Tn complex.** The lines between the subunits indicate binding and the thickness of the line marks the strength of the binding. According to Gordon *et al.* (2000) and modified from Heeley *et al.* (1987).

TnT is an asymmetric protein that attaches the Tn complex to a defined position on the thin filament. It is needed for full, Ca^{2+} dependent activity of the thin filament. TnT consists of two domains, an extended N-terminal domain T1 (residues 1-158) and a globular T2 domain (residues 159-259). The extended T1 domain interacts strongly with Tm (Mak and Smillie, 1981). It is suggested this interaction mediates the activation of actomyosin ATPase by stabilising the inactive state (Malnic *et al.*, 1998; Maytum *et al.*, 2002). The C-terminus of T2 interacts with the N-terminus of TnI, CTnC, and the thin filament thus maintaining the cohesion of the complex (Pearlstone and Smillie, 1982; Morris and Lehrer, 1984; Schaertl *et al.*, 1995; Tobacman, 1996; Blumenschein *et al.*, 2001). The interaction of TnT with Tm is calcium sensitive. Consequently, in the absence of calcium the T2 region binds to Tm and blocks the ATPase activation whereas in the presence of calcium the interaction with TnI/TnC is modified and it is detached from Tm (White *et al.*, 1987; Farah *et al.*, 1994; Potter *et al.*, 1995; Malnic *et al.*, 1998; Tobacman *et al.*, 2002).

Calcium binding to TnC initiates the cascade of events leading to muscle contraction. The interaction between TnC and TnI is essential for further transmission of the contraction signal to the other components of the thin filament. Therefore, their structures, roles and interactions are discussed in more detail later in the text.

1.1.2 Regulation of muscle contraction

Cardiac muscle contraction is involuntary whereas skeletal muscle contracts voluntarily. In both cases the calcium ion initiates the contraction. The complex mechanism of calcium directed regulation of striated muscle contraction has been studied extensively over the years (for recent reviews see Leavis and Gergely (1984), Zot and Potter (1987), da Silva and Reinach (1991), Grabarek *et al*. (1992), Gergely *et al*. (1993), Farah and Reinach (1995), Tobacman (1996), Lehrer and Geeves (1998), Squire and Morris (1998), Gordon *et al*. (2000; 2001). Skeletal muscle has been studied more than cardiac muscle and results from studies on striated skeletal muscle regulation have been analogously applied to cardiac muscle. The focus is on the cardiac isoform but both isoforms are included in the text for comparison and also because no confirmed data about some of the events in cardiac muscle contraction is currently available.

Several models for the regulation of the muscle contraction have been proposed in the literature. These are based on structural, biochemical and physiological data (for review see Gordon *et al*. (2000)). A three state model has been suggested by McKillop and Geeves (1993). In their model, about three Tm positions on the actin filament and two steps of myosin binding to actin have been proposed. This agrees well with current structural and biochemical data and has been generally accepted as the model to describe the regulation of the thin filament activation (McKillop and Geeves, 1993; Head *et al.*, 1995; Vibert *et al.*, 1997; Maytum *et al.*, 1999; Xu *et al.*, 1999). The three states of the thin filament are named blocked (**B**), closed (**C**) and open (**M**). The two steps of myosin binding to actin initially involve a weak binding followed by a stronger binding. In the blocked state no myosin binding to actin occurs. In the closed state, there is a weak interaction between myosin and actin that becomes stronger binding in the open force-generating state.

Regulation of the contraction cycle of both cardiac and skeletal muscles are calcium dependent (Fig. 5) (Ebashi and Endo, 1968). Calcium release from the sarcoplasmic reticulum (SR) triggers a cascade of events that include changes in protein-protein interactions and structural changes in proteins leading to muscle contraction (for recent reviews see Leavis and Gergely (1984), Ohtsuki *et al*. (1986), Zot and Potter (1987), Farah and Reinach (1995), and Solaro and Rarick (1998)). In resting muscle, the free intracellular calcium concentration is low ($[Ca^{2+}]_i \sim 10^{-7}$ M) and TnI inhibits the ATPase activity of actomyosin by binding to actin/Tm (Schaub and Perry, 1969; Potter and Gergely, 1974; Hitchcock, 1975). Tm occupies a position on the thin filament where it is able to block (**B**) the interaction between actin and myosin hence the cross-bridge formation can be inhibited. When calcium is released from the SR to cytosol ($[Ca^{2+}]_i \sim 10^{-5}$ M), it binds to TnC enabling TnI-binding. The ATPase inhibition ceases when the inhibitory region of TnI switches from actin to calcium-saturated TnC (Weeks and Perry, 1978). Moreover, Tm moves on the thin filament from blocked (**B**) to the closed state (**C**) (Xu *et al.*, 1999; Lehman *et al.*, 2001). This movement allows weak interactions between actin and myosin to form. Myosin and Tm binding to actin induce a conformational change on actin (Rosol *et al.*, 2000; Tobacman and Butters, 2000). After which the connection between actin/Tm and Tn breaks and Tm moves further to the open state (**M**) on the surface of the thin filament (Lehman *et al.*, 2001). Thus, myosin binding sites are exposed to strong binding, and muscle contraction occurs.

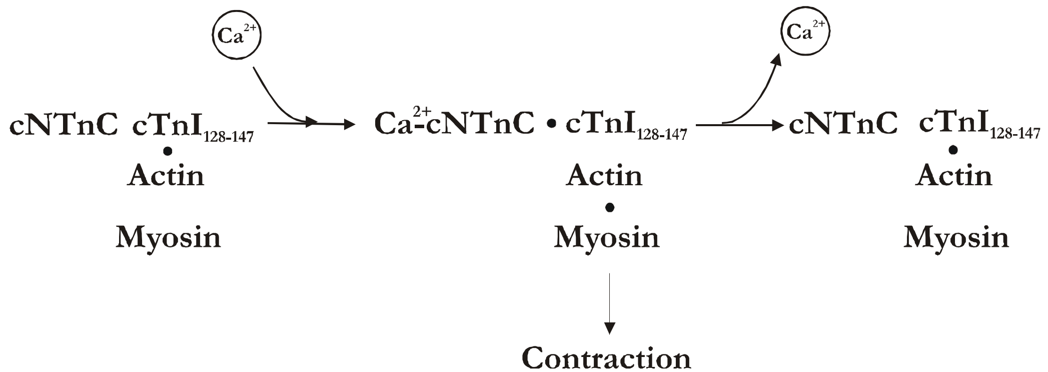

Fig. 5. **Calcium dependent regulation of cardiac muscle contraction.** In the relaxed state (diastole), there is no binding between actin and myosin. This is the blocked state (**B**). Upon calcium binding to cTnC, the inhibitory region of cTnI switches from actin to cTnC. This leads to the closed state (**C**) with weak binding between actin and myosin. Tm is, in turn, moved further to the open state (**M**) allowing stronger interaction between actin and myosin and muscle contracts (systole). Black spheres indicate binding.

1.2 Troponin C

Striated muscle troponin C is expressed in two isoforms in vertebrates, in fast skeletal muscle skeletal troponin C (sTnC) and in slow skeletal and cardiac muscles cardiac troponin C (cTnC). Skeletal and cardiac TnC sequences are about 70 % identical (Fig. 6) (Romero-Herrera *et al.*, 1976; Roher *et al.*, 1986). The most significant sequential difference between skeletal and cardiac TnC isomers is the inactive calcium-binding site of the N-domain of cTnC. Mutations of D29L and D31A and the insertion of V28 in the cardiac sequence make the calcium binding site I incapable of binding calcium (van Eerd and Takahashi, 1975).

```
sp|P02590|TPCC_HUMAN      MDDIYKAAVE QLTEEQKNEF KAAFDIFVLG AEDGCISTKE LGKVMRMLGQ NPTPEELQEM 60
sp|P02585|TPCS_HUMAN      -TDQQAEARS YLSEEMIAEF KAAFDMF-DA DGGGDISVKE LGTVMRMLGQ TPTKEELDAI 58
                            *    * .  *:**   ** *****:*  .   .* **.** **.******* .** ***: :

sp|P02590|TPCC_HUMAN      IDEVDEDGSG TVDFDEFLVM MVRCMKDDSK GKSEEELSDL FRMFDKNADG YIDLEELKIM 120
sp|P02585|TPCS_HUMAN      IEEVDEDGSG TIDFEEFLVM MVRQMKEDAK GKSEEELAEC FRIFDRNADG YIDPEELAEI 118
                          *:******** *:**:***** *** **:*:* *******::  **:**:**** *** ***  :

sp|P02590|TPCC_HUMAN      LQATGETITE DDIEELMKDG DKNNDGRIDY DEFLEFMKGVE 161
sp|P02585|TPCS_HUMAN      FRASGEHVTD EEIESLMKDG DKNNDGRIDF DEFLKMMEGVQ 159
                          ::*:** :*: ::**.***** *********: ****::*:**:
```

Fig. 6. **A comparison of the amino acid sequences of human skeletal and cardiac troponin C.** Swiss-Prot ID P02590 and P02585 for human cardiac and skeletal TnC, respectively. An asterisk indicates homology in the sequence and a dot close similarity. The sequence alignment was made with Clustal W 1.82 multiple sequence alignment software.

1.2.1 Structure of troponin C

TnC is an 18 kDa acidic protein. High resolution crystal and NMR structures of both skeletal (Herzberg and James, 1985; Sundaralingam *et al.*, 1985; Herzberg and James, 1988; Satyshur *et al.*, 1988; Slupsky and Sykes, 1995; Houdusse *et al.*, 1997; Soman *et al.*, 1999) and cardiac TnC (Sia *et al.*, 1997) show an elongated, largely α-helical, dumbbell-shaped protein (Fig. 7). Both isomers consist of globular N- and C-domains that are connected by a linker helix, often referred to as D/E linker. An N-terminal helix (N-helix) together with A, B, C, and D helices compose the regulatory or the N-domain of TnC (NTnC) and helices E, F, G, and H form the structural or the C-domain (CTnC). The domains of the isolated TnC appear to be structurally and functionally independent from each other. The central D/E linker of isolated TnC between the domains is flexible and allows N- and C-domains to move independently of each other (Kleerekoper *et al.*, 1995). There appears to be a pH dependent transition of the isolated TnC (Wang *et al.*, 1987; Wang and Leavis, 1990). The crystal structure determined at pH ~5 reveals an elongated molecule with an extended D/E linker helix. Whereas closer to the physiological pH (pH ~7), isolated cTnC assumes a slightly more compact structure and the N- and C-domains are closer to each other (Wang and Leavis, 1990). Apart from the pH dependent transition, the differences in linker helix crystal and solution structures can also result from crystal packing forces that stabilise the more extended structure as is the case with calmodulin (Heidorn and Trewhella, 1988). The

function of the linker is to keep the domains apart though in optimal proximity to their target sites on troponin I (Sheng *et al.*, 1991; Babu *et al.*, 1993; Ramakrishnan and Hitchcock-DeGregori, 1995). TnI restricts the linker flexibility and the positions of the domains are set so that the hydrophobic regions of each domain faces each other (Dvoretsky *et al.*, 2002).

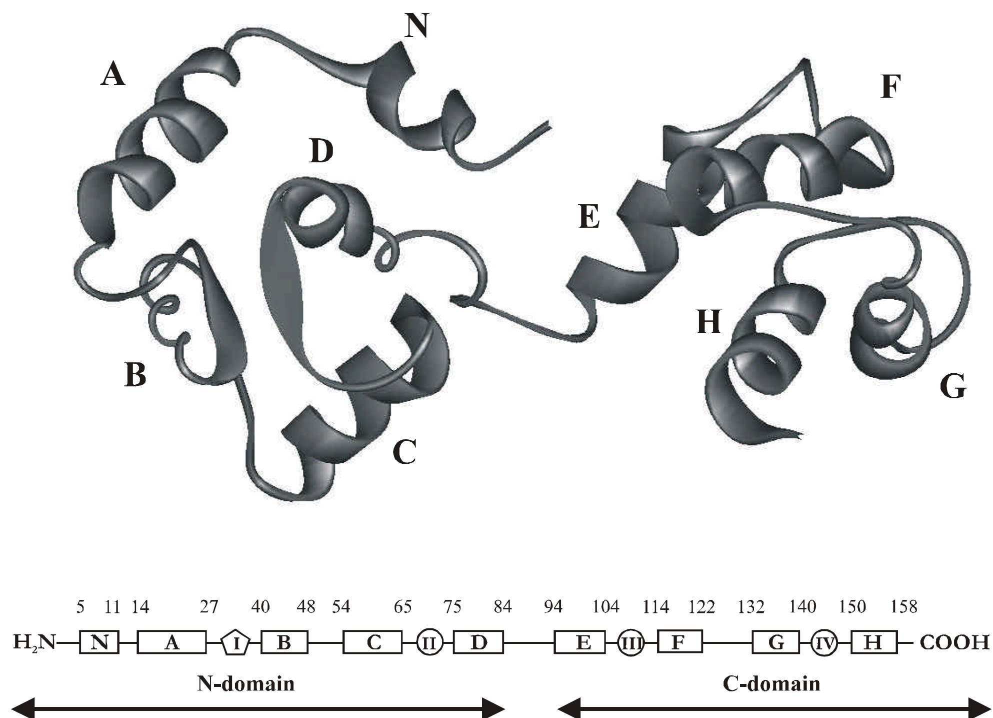

Fig. 7. **A ribbon presentation of calcium saturated cTnC (Protein Data Bank (PDB) entry 1AJ4, (Sia et al., 1997), www.rcsb.org/pdb).** Below the 3D-structure is shown the secondary structure of cTnC.

1.2.2 Calcium binding protein

The calcium ion is a common factor in various cellular mechanisms and therefore there are numerous proteins that have the ability to bind calcium. For example, Ca^{2+}-binding proteins function in cellular signal transmission, cytosolic Ca^{2+} buffering, and also take part

in Ca^{2+}-dependent enzymatic activities. Many of these calcium-binding proteins contain an EF-hand calcium binding motif(s) first described by Kretsinger and Nockolds (1973). In the EF-hand motif, two helices, which are almost perpendicular to each other, are connected by a loop comprising 12 amino acids. Certain side chains of the loop residues provide oxygen ligands for Ca^{2+} chelation. Calcium binding to the EF-hand motif, which is reversible, often results in significant structural changes. EF-hand motifs are usually arranged in pairs interacting via short β-strands in the loops.

TnC belongs to the calmodulin group of EF-hand calcium binding proteins. It functions as a calcium dependent trigger for the control of muscle contraction (Potter and Gergely, 1975; Babu *et al.*, 1987; Babu *et al.*, 1989). Binding of calcium ions to the regulatory domain of TnC alters the interaction between TnC and TnI and other thin filament proteins. Thus, the contraction signal is further transmitted to other subunits of the contractile apparatus.

There are two helix-loop-helix EF-motifs in the regulatory domain of TnC. The two calcium-binding sites of sNTnC and one active site in cNTnC have a specific affinity for calcium (K_{Ca} 2×10^5 M^{-1}) (Potter and Gergely, 1975; Zot and Potter, 1982). These N-domain sites are referred to as regulatory sites because calcium binding to them triggers the muscle contraction (Potter and Gergely, 1975; Sheng *et al.*, 1990; Putkey *et al.*, 1991). The paired calcium binding sites of the N-domain are structurally and energetically coupled in the both TnC isoforms (Li *et al.*, 1995; Li *et al.*, 1997; Spyracopoulos *et al.*, 1998). There are hydrogen bonds and van der Waals forces between the β-strands of the EF-hands (Herzberg and James, 1988; Satyshur *et al.*, 1988; Strynadka *et al.*, 1997). Calcium binding to cNTnC causes perturbations throughout the N-domain sequence, primarily in the defunct calcium binding site I and in the active binding site II (Li *et al.*, 1997). Calcium binding to site II in cTnC decreases the flexibility of the backbone and also the defunct calcium-binding loop becomes more rigid (Spyracopoulos *et al.*, 1998).

In the C-domain of TnC, there are also two EF-hand motif calcium-binding sites. These sites have a higher affinity to calcium than the sites on the N-domain of TnC, K_{Ca} about 2×10^7 M^{-1} and also a measurable affinity for magnesium ions, K_{Mg} ~5×10^3 M^{-1} (Potter and Gergely, 1975; Leavis *et al.*, 1978; Holroyde *et al.*, 1980). The structure of the C-

domain appears to be similar regardless of the bound metal ion (Finley *et al.*, 2000). Ca^{2+}/Mg^{2+}-binding to the C-domain of TnC results in two hydrogen bonds between the calcium binding loops and the conformation becomes more structured compared to the apo-form (Krudy *et al.*, 1992; Lin *et al.*, 1994). It is generally believed that these C-domain sites are always occupied with either Mg^{2+} or Ca^{2+} under normal physiological conditions (Zot and Potter, 1982; Grabarek *et al.*, 1992). Therefore, they are referred to as structural calcium binding sites. In fact, calcium/magnesium binding to either site III or IV of TnC is enough for anchoring TnC to the thin filament (Negele *et al.*, 1992; Brito *et al.*, 1993; Szczesna *et al.*, 1996).

1.2.3 Trigger mechanism of muscle contraction

Binding of the calcium ion to TnC initiates the muscle contraction process. Specifically, calcium ion binding to sites I and II of sTnC and site II of the cTnC triggers the muscle contraction signal (Grabarek *et al.*, 1990; Szczesna *et al.*, 1996). The signal is further transmitted to other subunits of the contractile apparatus by structural changes and alterations in protein-protein interactions (Perry, 1999). The activation step induced by calcium binding differs between skeletal and cardiac isoforms. In the apo-form, both isomers are energetically in the lowest state and the conformation of the N-domain of cTnC and sTnC stays closed. This means that helices B and C are closer to the unit formed of helices N, A, and D (NAD), due to hydrophobic interactions (Sia *et al.*, 1997; Spyracopoulos *et al.*, 1997).

When the regulatory sites of sTnC are occupied by calcium ions, the domain structure is open and exposes a large hydrophobic surface (Herzberg and James, 1985; Herzberg *et al.*, 1986; Herzberg *et al.*, 1987; Gagné *et al.*, 1995; Strynadka *et al.*, 1997). Studies with mutated sTnC, E41A-sTnC, which is incapable of binding calcium to site I, revealed that this site is the link between the calcium binding and the opening of the regulatory domain (Gagné *et al.*, 1997). Furthermore, the Glu41 residue, which is the last residue on the 12 residue calcium binding loop on site I is particularly important in this process. The binding of a second calcium ion releases sufficient energy to overcome the energy barrier to open the structure of the sNTnC (Li *et al.*, 1995). Helices B and C move

relative to the NAD unit exposing a hydrophobic region where TnI binds (Gagné *et al.*, 1995; Strynadka *et al.*, 1997; McKay *et al.*, 1998; McKay *et al.*, 1999).

In the apo-form, cNTnC is more structured than its skeletal counterpart. Short β-strands in calcium binding loops are formed in the absence of calcium in cNTnC (Brito *et al.*, 1991). The active calcium binding site II in cNTnC alone is responsible for triggering cardiac muscle contraction (Holroyde *et al.*, 1980; Johnson *et al.*, 1980; Putkey *et al.*, 1989). Similarly, the binding of the Ca^{2+} to site II of sTnC does not induce large conformational changes and the regulatory domain primarily stays closed (Sia *et al.*, 1997; Spyracopoulos *et al.*, 1997; Pääkkönen *et al.*, 1998). It was proposed that there is an equilibrium between open and closed states of the calcium saturated cNTnC with the closed conformation dominating resulting in an average structure that resembles the relatively closed conformation state (Pääkkönen *et al.*, 1998). A similar state of open and closed forms in equilibrium has been demonstrated more clearly for a mutant (E104Q/E140Q) in the C-domain of calmodulin (Evenäs *et al.*, 1998).

Despite the cardiac calcium-binding site I being inactive, its first 41 residues including the defunct site and the first N-terminal helix, are considered to be important for the normal function of cTnC. Calcium binding primes the N-domain for cTnI binding even though not enough energy is released to open the structure. The defunct site modifies the calcium dependent contraction (Putkey *et al.*, 1989). It forms a conformationally active region which modulates the overall conformational entropy and hence the populations of closed and open states of cNTnC (Abusamhadneh *et al.*, 2001). The N-helix stabilises the N-domain of TnC and is involved in the fine-tuning of the calcium binding affinity and the transmission of the signal in the activation of the thin filament (Gulati *et al.*, 1992; Chandra *et al.*, 1994; Smith *et al.*, 1994; Fredricksen and Swenson, 1996; Smith *et al.*, 1999).

Myocytes are sensitive to changes in pH. For example, a pH decrease in ischemic heart leads to a decrease in the contraction force (Fabiato and Fabiato, 1978). This results from protons competing with calcium ions for binding to cTnC, which in turn reduces the positive inotropic effect caused by calcium (Blanchard *et al.*, 1984; Blanchard and Solaro, 1984; Parsons *et al.*, 1997). Protons are also reported to alter TnC/TnI interactions that transmit the calcium-binding signal (el-Saleh and Solaro, 1988).

1.3 Troponin I

TnI, the inhibitory subunit of the Tn complex, is responsible for calcium dependent inhibition of actomyosin ATPase (Leavis and Gergely, 1984; Perry, 1999). TnI is expressed in three isoforms in striated muscle: two isoforms are expressed in skeletal muscle and one isoform in cardiac muscle (Dhoot *et al.*, 1978). It is a polar protein with a large number of positively charged residues. TnI transmits the calcium binding information to other units of the thin filament by a large-scale conformational change and calcium dependent interactions with other thin filament proteins (Van Eyk *et al.*, 1997; Solaro and Rarick, 1998; Stone *et al.*, 1998).

At present, the structure of TnI in isolation is poorly defined. It is proposed to have an open and extended conformation based on solvent accessibility (Dong *et al.*, 2000b). However, low- and high-resolution structures of TnI with other components of the Tn complex are determined. Three-dimensional structural information on the TnC/TnI complex based on SAXS, X-ray crystallography and neutron scattering results are published in several articles (Olah *et al.*, 1994; Olah and Trewhella, 1994; Stone *et al.*, 1998; Vassylyev *et al.*, 1998; Tung *et al.*, 2000). In the absence of regulatory calcium, the TnC/TnI complex is rigid but becomes more flexible upon calcium binding to the N-domain regulatory sites (Zhao *et al.*, 2000). TnC remains in its extended dumbbell conformation even when it is complexed with TnI (Kleerekoper *et al.*, 1995; Dong *et al.*, 2000a). The presence of TnI makes the flexible linker more rigid and limits the independent tumbling of the N- and C-domains of TnC (Abbott *et al.*, 2000b). A recent study of the $cTnC/cTnI/cTnT_{198\text{-}298}$ complex showed that cTnC domains are closer to each other within the Tn complex than in the crystal structure. However, they do not make physical contact with each other (Heller *et al.*, 2002). In contrast, the cTnI/cTnT in this complex remains in a long, extended form.

1.3.1 The interactions between troponin C and troponin I

TnI makes several points of contact with TnC (Fig. 8). They interact with each other in an antiparallel fashion, the N-terminus of TnI binds to the C-domain of TnC and *vice versa* (Sheng *et al.*, 1992; Farah *et al.*, 1994; Krudy *et al.*, 1994). The sequence of $cTnI_{32\text{-}70}$

($sTnI_{1-40}$) binds at the hydrophobic patch of cCTnC in a Ca^{2+}/Mg^{2+} dependent manner and stays bound throughout the entire muscle contraction cycle (Farah *et al.*, 1994; Krudy *et al.*, 1994; Pearlstone *et al.*, 1997; Vassylyev *et al.*, 1998; Gasmi-Seabrook *et al.*, 1999; Calvert *et al.*, 2000; Ferrieres *et al.*, 2000; Finley *et al.*, 2000; Mercier *et al.*, 2001; Ngai *et al.*, 2001). This interaction is responsible for keeping the TnC/TnI complex together during the relaxed state. The C-domain structure of cTnC slightly opens upon $cTnI_{33-80}$ binding whereas no significant structural opening has been reported for the respective skeletal complex (Gasmi-Seabrook *et al.*, 1999; Mercier *et al.*, 2001). The N-terminal region of TnI also includes a TnT-binding region (residues 54-79), which is proposed to be involved in the transmission of the Ca^{2+}-binding signal to the thin filament (Rarick *et al.*, 1999).

Fig. 8. A diagram showing cTnC-binding regions on cTnI sequence (Swiss-Prot ID P19429). Residues 1-32, the cardiac specific extension of cTnI contains two phosphorylation sites, S22 and S23, and has been proposed to interact with the N-domain of cTnC. Residues 33-70 interact with the C-domain of cTnC anchoring TnC and TnI together. The inhibitory region, residues 128-147, interact with actin and cTnC according to the phase of the contraction cycle and is modulated by the calcium dependent binding of the regulatory region $cTnI_{148-166}$.

The regulatory region of $cTnI_{148-166}$ ($sTnI_{116-131}$) interacts with the N-domain of TnC in a calcium dependent manner. It modulates the interaction between the inhibitory region of TnI, TnC, and actin filament during the contraction-relaxation cycle (McKay *et al.*, 1997; Tripet *et al.*, 1997; McKay *et al.*, 1998; Dong *et al.*, 1999; Li *et al.*, 1999; Ferrieres *et al.*, 2000). The regulatory region of cTnI binds to cNTnC in the presence of calcium and pulls the inhibitory region away from the actin filament (Solaro and Rarick, 1998; Dong *et al.*, 2001). In the resting phase, the interaction between TnI and actin/Tm is stronger than between TnI and TnC. However, upon calcium binding to TnC, the interaction between TnI and TnC becomes stronger whereas the TnI-binding to actin/Tm becomes weaker. The TnI oscilliates back and forth between these two states in synchronisation with the contraction-relaxation cycle (Perry, 1999). The binding of calcium to sTnC is enough to open the N-domain conformation. Whereas, the cardiac isoform also requires cTnI, its 150-166 residues,

to bind to the calcium form of cNTnC before the full N-domain opening can occur (Dong *et al.*, 1999; Li *et al.*, 1999). N-domains adopt similar structures in isoform complexes of cTnC/$cTnI_{147-163}$ and sTnC/$sTnI_{115-131}$ (Li *et al.*, 1999). This tissue specific difference between cardiac and skeletal thin filament activation is explained by different thermodynamics and kinetics of the isoforms (McKay *et al.*, 2000; Pearlstone *et al.*, 2000). The difference could possibly be explained by their adaptation to different physiological requirements, i.e. control of action, the involuntary and voluntary action of contraction, and the speed of action.

The inhibitory region $cTnI_{128-147}$ ($sTnC_{96-115}$) switches between actin and cTnC according to the phase of the muscle contraction and the relaxation cycle (Farah *et al.*, 1994; Farah and Reinach, 1995; Kobayashi *et al.*, 2000). This region alone is enough for the full inhibition of actomyosin ATPase (Syska *et al.*, 1976). In the absence of calcium, the inhibitory region of TnI is bound to actin/Tm where it inhibits the ATPase activity (Schaub and Perry, 1969; Greaser and Gergely, 1971). The inhibition is removed upon calcium binding when the inhibitory region is pulled away from actin and on to TnC (Weeks and Perry, 1978; Van Eyk *et al.*, 1993; Solaro and Rarick, 1998). The exact binding site of the inhibitory peptide on TnC is under debate. The physiologically relevant binding site for $cTnI_{128-147}$ has been proposed to locate at the linker region (Li *et al.*, 1999; Abbott *et al.*, 2000a; Luo *et al.*, 2000; Tung *et al.*, 2000). Farah and coworkers (1994) suggested that the inhibitory region binds to both domains of TnC. The N-domain binding model proposes that the inhibitory region binds to the hydrophobic pocket of the calcium saturated regulatory domain of TnC (Leszyk *et al.*, 1990; McKay *et al.*, 1997; McKay *et al.*, 1998). Conversely, C-domain binding has been proposed (Slupsky *et al.*, 1992; Howarth *et al.*, 1995). In this model, $cTnI_{33-80}$ ($sTnI_{1-40}$) and $cTnI_{128-147}$ ($sTnI_{96-115}$) are assumed to share overlapping binding sites on the C-domain of TnC and either one of them occupies it depending on the phase of the contraction/relaxation cycle (Tripet *et al.*, 1997; McKay *et al.*, 1999; Mercier *et al.*, 2000).

The controversy about the binding site for the inhibitory region on TnC probably results from studies with short peptides that may show nonspecific binding. Furthermore, various isoforms and in different lengths of peptides have been used in for studying the role of various peptides in binding studies. Controversies may also result from different methods

used. Recent publications agree that the inhibitory region of TnI binds to the C-domain of TnC (Brown *et al.*, 2002; Tripet *et al.*, 2002; Dong *et al.*, 2003; Lindhout and Sykes, 2003). In these studies, the N-terminal portion of $cTnI_{127-147}$ has been thought to associate with the C-domain of cTnC, and the C-terminal part of the inhibitory region bridges the N- and C-domains of cTnC but do not have specific interaction with the D/E linker (Brown *et al.*, 2002; Dong *et al.*, 2003). Moreover, the residues 131-133 of TnI have been reported to bind to the hydrophobic pocket on the C-domain (Lindhout and Sykes, 2003). It has also been shown that the inhibitory region could not replace $cTnI_{33-80}$ in the C-domain of cTnC (Abbott *et al.*, 2000a). It was emphasised that $cTnI_{33-80}$ has a structural role and stays bound to the C-domain whereas $cTnI_{128-147}$ has a more dynamic functional role (Li *et al.*, 2002). Based on NMR and fluoresence resonance energy transfer (FRET) studies of the cardiac cTnC/cTnI complex a different structure of the inhibitory region to that of the skeletal isoform has been suggested (Abbott *et al.*, 2001; Dong *et al.*, 2001; Dong *et al.*, 2003). These authors suggest that the skeletal hair-pin loop does not exist in the cardiac complex. Instead, they suggest that the inhibitory region is in an extended conformation in the presence of regulatory calcium and does not experience a specific interaction with the D/E linker of the cTnC.

The cardiac isoform of TnI has an N-terminal extension of 32 residues, $cTnI_{1-32}$ that interacts with the regulatory domain of cTnC (Finley *et al.*, 1999; Gaponenko *et al.*, 1999). Its function in the myofilament is not entirely clear. The cardiac specific extension is highly flexible in the presence of cTnC suggesting that it is either not bound to cTnC or binds only loosely (Dong *et al.*, 2000b). It has also been suggested that this region switches between the N- and C-domains of cTnC depending on the phosphorylation state (Ferrieres *et al.*, 2000). It could therefore mediate signals from TnC domains to each other. The unphosphorylated form interacts with the N-domain of cTnC and modulates the equilibrium between open and closed states of Ca^{2+}-cNTnC (Finley *et al.*, 1999; Gaponenko *et al.*, 1999; Abbott *et al.*, 2000b; Abbott *et al.*, 2001; Ward *et al.*, 2002). Bisphosporylation of cTnI disrupts this interaction and reduces the calcium affinity of cTnC and thus the calcium sensitivity of the contraction (Robertson *et al.*, 1982; Zhang *et al.*, 1995; Abbott *et al.*, 2000b; Abbott *et al.*, 2001).

1.3.2 Phosphorylation of troponin I

The signaling process of myofilament activation can be modulated not only with free intracellular [Ca^{2+}] but also by phosphorylation of cTnI. This is characteristic of the cardiac isoform. Phosphorylation is known to affect cardiac contractility and different pathologies are known to affect cTnI phosphorylation. There are multiple protein kinase A (PKA) and protein kinase C (PKC) phosphorylation sites on cTnI. Ser22 and Ser23 can be phosphorylated by PKA in response to β-adrenergic stimulation (Moir *et al.*, 1980). Phosphorylation of these sites results in structural changes and affects their interactions with TnC (Zhang *et al.*, 1995; Chandra *et al.*, 1997; Dong *et al.*, 1997; Jaquet *et al.*, 1998). The bisphosphorylated cardiac specific extension interacts with cTnT and/or cTnI (Schmidtmann *et al.*, 2002). Phosphorylation of Ser22 and Ser23 reduces the apparent calcium sensitivity (Robertson *et al.*, 1982). Ser42 and Ser44 in the part of cTnI that binds to cCTnC and Thr142 in the inhibitory region are all substrates for PKC (Noland *et al.*, 1995). Phosphorylation by PKC decreases actomyosin ATPase activity. Phosphorylation of Ser42 and Ser44 influences the maximum tension of myofilaments whereas phosphorylation of Thr142 together with phosphorylation of Ser42 and Ser44 appears to regulate thin filament sliding speed (Burkart *et al.*, 2003). Recently it was reported that the phosphorylation of Thr142 of cardiac cTnI reduces the affinity of the inhibitory peptide for calcium saturated cCTnC (Lindhout *et al.*, 2002).

1.4 Calcium sensitisers

The binding of calcium ion to the N-domain of TnC initiates the contraction of the cardiac muscle and the impulse is further transmitted to other subunits of the contractile apparatus via TnC/TnI interaction. Therefore, the manipulation of this signaling pathway appears an effective and specific treatment strategy for cardiac insufficiency that results from decreased ventricular function such as heart failure. An increase in the intracellular calcium concentration enhances the efficiency of muscle contraction. However, high levels of intracellular calcium result in higher demands for oxygen. This is further exacerbated because the weak muscle has to work even harder. In addition, arrhythmias, which result from elevated intracellular calcium concentrations and which can subsequently lead to death

have been observed (De Mello, 1982; Kusuoka *et al.*, 1990). An alternative idea for calcium sensitisers was introduced over twenty years ago (Herzig *et al.*, 1980; Solaro and Rüegg, 1982). Calcium sensitisers are a heterogeneous group of molecules that enhance the response of myofilaments to calcium without an increase in the intracellular calcium concentration and thus have a positive inotropic effect on cardiac contractility (recent reviews by Lee and Allen (1997), Endoh (2001; 2003), and Arteaga *et al*. (2002)).

A heart contracts when calcium binds to cNTnC. Conversely, it relaxes when calcium dissociates from cNTnC. This feature makes the calcium saturated form of cTnC an ideal target for calcium sensitisers (Haikala and Linden, 1995). Therefore, an additional important feature of calcium sensitisers in order not to impair the relaxation of cardiac muscle is that it dissociates from cTnC when Ca^{2+} dissociates. It is essential that an efficacious calcium sensitiser must not prohibit or inhibit any protein-protein interactions required for muscle contraction and relaxation. Rational drug design and understanding of the effective mechanism of an efficacious drug candidate requires knowledge of the relationship between structure and function (structure to activity relationship, SAR). SAR gives information about protein molecular structure and how it changes during the contraction-relaxation cycle. Moreover, it provides data on how potential drugs affect the structure and interact with other proteins in the contractile apparatus. Results from SAR studies have been used to evaluate some potential candidate calcium sensitizing molecules including EMD57033, trifluoperazine (TFP), bepridil, and levosimendan. Before any accurate structural data from NMR or X-ray studies of cTnC were available, the sTnC isoform was used as a template for modeling of drug-protein complexes. The binding site for hydrophobic calcium sensitising compounds was thought to be located on the hydrophobic patch that includes helices B, C, and D in the regulatory domain of TnC (Ovaska and Taskinen, 1991; Kleerekoper *et al.*, 1998).

1.4.1 The N-domain pathway

One of the possible mechanisms to enhance the apparent calcium sensitivity of cardiac muscle involves the regulatory domain of cTnC. Enhanced calcium sensitivity could be achieved by stabilising the calcium dependent interaction between cNTnC and the

regulatory region of cTnI, or by increasing the calcium affinity of cNTnC. However, an increase in calcium binding affinity would result in impaired relaxation because Ca^{2+} would dissociate relatively slowly from cNTnC (for review see Teramura and Yamakado (1998)). A better alternative would be to modulate cTnC/cTnI interaction prolonging the lifetime of the active form. Both domains of TnC have hydrophobic regions that are essential for cTnC/cTnI interaction; stabilisation of the complex and regulation of cardiac contraction. There are a couple of hydrophobic patches on the N-domain of cTnC that could be considered as potential drug binding sites. The hydrophobic region of cTnC that includes Met81 is a binding site for cTnI and is therefore essential to the activity. This is not considered as an ideal binding site for calcium sensitisers (Lin *et al.*, 1996). Instead it was suggested that another hydrophobic region of the N-domain including Met45, Met60 and Met80 of the regulatory site II was a potential binding site for calcium sensitising ligands (Kleerekoper *et al.*, 1998). A pharmacological agent that binds to this patch and does not interfere with the binding of cTnI could enhance the apparent calcium sensitivity of the myofilaments. Agents that have been reported to have an effect by interacting with cTnC at this region are bepridil and TFP (Kleerekoper *et al.*, 1998).

Bepridil (racemate of 1-isobutoxy-2-pyrrolidino-3-(N-benzylanilino)propane from Sigma Fig. 9) has often been used as a model molecule to study the mechanism of calcium sensitising of myofibrils even though it has been shown to be unsuitable for clinical use (Kobayashi *et al.*, 2001; Stains and Gay, 2001). Bepridil increases actomyosin ATPase activity and affinity of Ca^{2+} for cTnC (Solaro *et al.*, 1986). Its calcium sensitising effect is thought to be derived from its calcium dependent binding to the regulatory domain of cTnC in the presence of cTnI and the stabilisation of the open conformation (MacLachlan *et al.*, 1990; Li *et al.*, 2000b; Abusamhadneh *et al.*, 2001). The complex structure shows that bepridil binding to cNTnC sterically hinders the closing of the conformation and enhances the affinity of TnC for cTnI and thus increases the activity of ATPase (Li *et al.*, 2000b; Wang *et al.*, 2002). Bepridil binding to the N-domain of cTnC causes the N-terminus of the B helix to extend by forming a side chain interaction between Glu40 and Ser37 and thus stabilises the defunct site I and enables the domain opening (Li *et al.*, 2000b).

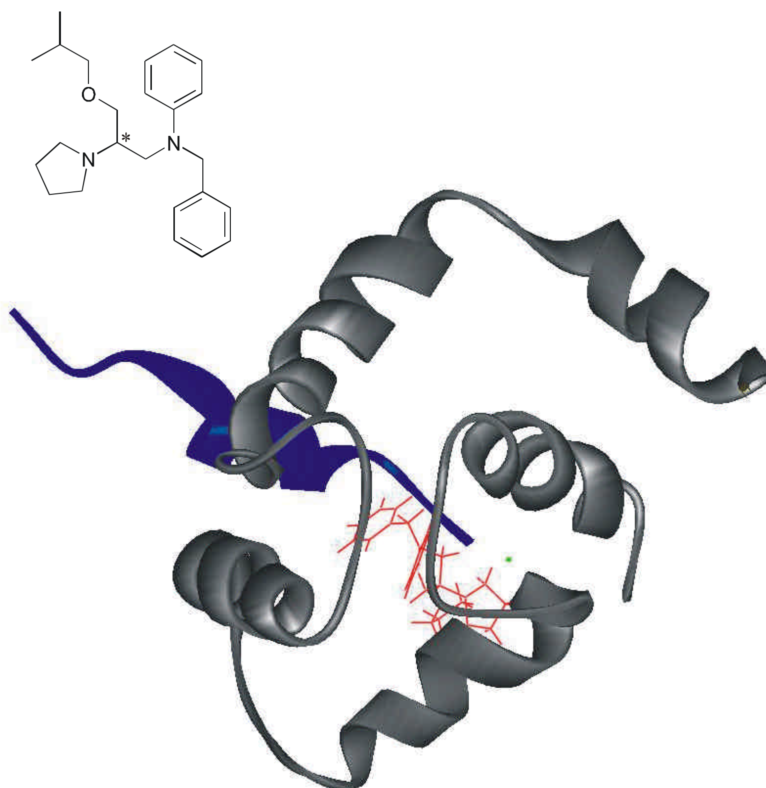

Fig. 9. **Chemical structure of the bepridil molecule and a schematic presentation of a 3D-structure of the complex cNTnC/cTnI$_{147-163}$ in the presence of bepridil (PDB entry 1LXF (Wang *et al.*, 2002).** The asterisk marks the chiral carbon atom on the bepridil molecule. The blue helix represents cTnI$_{147-163}$.

1.4.2 The C-domain pathway

A hydrophobic patch is located in the C-domain of cTnC. This is also reported to be the binding site for cTnI (Krudy *et al.*, 1994). Unlike the N-domain it is not considered to be a potential binding site for calcium sensitisers due to its structural role (Kleerekoper *et al.*, 1998). However, recently a modulatory role for the C-domain in thin filament activation has been proposed (Calvert *et al.*, 2000). EMD57033 (the R enantiomer of 5-[1-(3,4-dimethoxybenzoyl)-1,2,3,4-tetrahydro-6-quinolyl]-6-methyl-3,6-dihydro-2H-1,3,4-thiadiazin-2-one developed by Pharmaceutical Research, E. Merck, Darmstadt, Germany, Fig. 10) has

been reported to increase calcium sensitivity and force development in cardiac muscle (Beier *et al.*, 1991; Lues *et al.*, 1993; Solaro *et al.*, 1993). It interacts stereoselectively and calcium dependently with the C-domain of cTnC in the absence or presence of cTnI (Pan and Johnson, 1996; Li *et al.*, 2000a; Wang *et al.*, 2001). These authors suggested that EMD57033 binds to the structural domain of cTnC thus altering the interaction between cTnC and other thin filament proteins, especially cTnI, thereby enhancing the apparent calcium sensitivity of the myocytes.

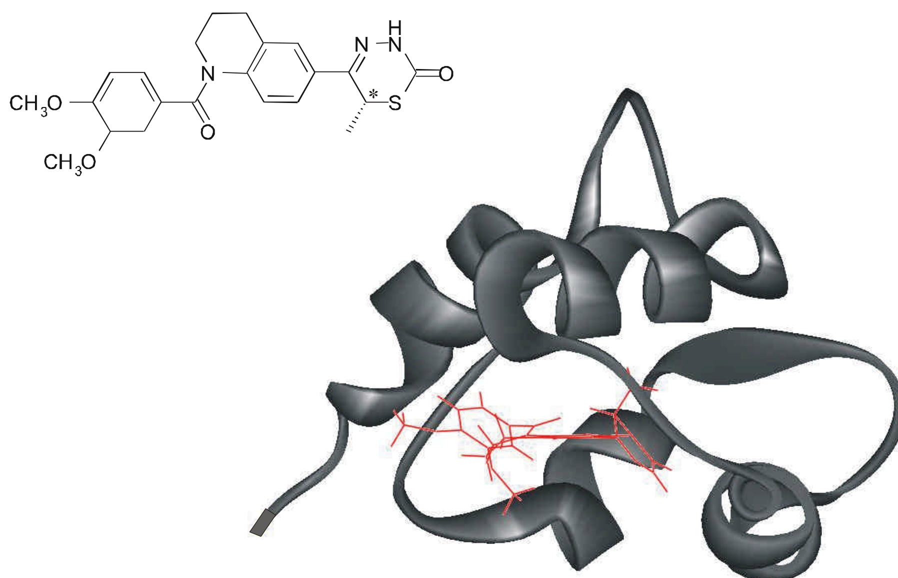

Fig. 10. Chemical structure of the EMD57033 molecule and a schematic presentation of a 3D-structure of cCTnC in the presence of EMD57033 (PDB entry 1IH0 by (Wang *et al.*, 2001). The asterisk marks the chiral carbon atom on the EMD57033 molecule.

1.4.3 Levosimendan

Levosimendan (the R enantiomer of {[4-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl) phenyl] hydrazono} propanedinitrile from Orion Pharma, Espoo, Finland, Fig. 11) is an inodilator designed for the treatment of acute cardiac failure. It has been shown to act as a calcium sensitiser in demembranated cardiomyocytes (Szilágyi *et al.*, 2003), isolated intact cardiomyocytes (Lancaster and Cook, 1997), guinea pig cardiac muscle skinned fibers

(Edes *et al.*, 1995; Haikala *et al.*, 1995a), and in human heart failure skinned fibers (Hasenfuss *et al.*, 1998). The positive inotropic effect of levosimendan is a result of an increase in calcium sensitivity of the contractile proteins and not an increase of the calcium influx (Hasenfuss *et al.*, 1998). Levosimendan induced tension increase is concentration-dependent and reversible (Edes *et al.*, 1995; Haikala *et al.*, 1995b). Levosimendan is accepted in clinical use as a calcium sensitiser in congestive heart failure and it has been reported to be a specific and well tolerated treatment (Kivikko *et al.*, 2002).

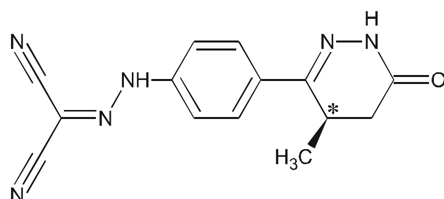

Fig. 11. Chemical structure of the levosimendan molecule. The asterisk marks the chiral carbon atom on the levosimendan molecule.

It has been suggested that cTnC is the target protein for levosimendan (Haikala *et al.*, 1995a). Levosimendan has a calcium-dependent affinity for the cardiac Tn complex as well as for isolated cTnC. It is able to bind to both domains of cTnC (Haikala *et al.*, 1995a). Thus it has been suggested that the calcium sensitising effect of levosimendan is mediated by its calcium-dependent binding to cTnC (Levijoki *et al.*, 2000).

The binding site for levosimendan on cTnC was hypothesised based on the sTnC structure to locate at the hydrophobic pocket of the N-domain of cTnC near the linker region (Ovaska and Taskinen, 1991). Preliminary NMR studies on the isolated N-terminal domain of cTnC revealed a spatial proximity of levosimendan and Met81, Met85 and Phe77 in the drug–protein complex (Pollesello *et al.*, 1994). Moreover, experimental data of several point mutated cTnC samples support this hypothesis. The mutation of residues Cys84, Asp87, or Asp88 in cTnC has no effect on TnC's calcium binding properties but have an effect on the calcium sensitization of myocytes in the presence, or absence of levosimendan (Levijoki *et al.*, 2000). However, conflicting results about the affinity of levosimendan to cTnC have been presented. Kleerekoper and Putkey (1999) studied several potential calcium sensitising

agents and their affinities to cTnC. They were not able to detect any evidence of reversible levosimendan binding to calcium saturated cTnC and suggested the calcium sensitising action of levosimendan was derived from the interaction with some other target protein than cTnC (Kleerekoper and Putkey, 1999).

1.5 Aims and focus of the thesis

The aim of this thesis is to study the interaction between levosimendan and cTnC by NMR in order to elucidate the molecular mechanism of levosimendan on its target protein. First, the structural changes induced on cTnC by the binding of calcium and of cTnI were studied to understand better their influence on the regulation of cardiac contraction (original publication I). Second, the interaction of levosimendan was studied on isolated cTnC (original publications II and III). Finally, a more complete Tn model was prepared by complexing cTnI peptides with cTnC. The interaction of levosimendan with such a cTnC/cTnI complex was studied to understand further the structure to activity relationship of this drug, and its resulting function in the regulation of the muscle contraction. These data should expand the knowledge of the mechanism of calcium sensitisers, of which levosimendan is the first to have reached the market (original publications III and IV).

2 MATERIALS AND METHODS

The detailed information and description of used samples, sample preparation, experiments, and conditions can be found in the original publications I to IV.

2.1 Cardiac troponin C samples

In this study three different recombinant cardiac TnC molecules were used. Recombinant human cardiac N-domain of TnC (cNTnC residues 1 to 91, Swiss-Prot ID P02590) included both cysteine residues in its sequence (I). Recombinant full-length chicken $cTnC_{CS}$ (Swiss-Prot ID P09860) lacks Met1 and includes mutations of Asp2Ala and Cys35Ser (II-IV), and full-length chicken $cTnC_{A\text{-}Cys}$ includes an additional mutation of Cys84Ser (II). The mutations of Cys35Ser and Cys84Ser have no significant effect on calcium sensitivity of calcium binding site II in cTnC and have no effect on the activation on myofibril ATPase activity (Putkey *et al.*, 1993; Dong *et al.*, 1996).

Unlabeled and uniformly ^{15}N and $^{15}N/^{13}C$ labeled cNTnC was over expressed and purified as described by Pääkkönen and coworkers (1998) (I). Unlabeled and uniformly ^{15}N and uniformly $^{15}N/^{13}C$ labeled $cTnC_{CS}$ and $cTnC_{A\text{-}Cys}$ were produced and purified as previously described by Krudy *et al.* (1994) and Finley *et al.* (1999) (II-IV). In total, about 20 mg of ^{15}N-labeled cNTnC, 80 mg of ^{15}N-labeled $cTnC_{CS}$, 10 mg of ^{15}N-labeled $cTnC_{A\text{-}Cys}$, 15 mg of $^{15}N/^{13}C$-labeled cNTnC, and 15 mg of $^{15}N/^{13}C$-labeled $cTnC_{CS}$ were used to carry out the experiments in this study.

2.2 Cardiac troponin I samples

Human cardiac TnI peptides of 32-79, 128-147, 148-163, 147-172, and 128-180 (Swiss-Prot ID P19429) were used in this study (I, III, IV). The cTnI peptides were synthesised with an automated solid-phase 433 A peptide synthesiser using 9-fluorenylmethoxycarbonyl (Fmoc) amino acids (Fields and Noble, 1990). cTnI peptides were chosen in respect to the essential cTnC binding regions for the regulation of the contraction.

Stock solutions of lyophilised pure peptides were prepared by dissolving in H_2O (Milli Q purity).

2.3 Characterisation of protein samples

The purity of the final protein samples was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970). Detection of protein bands was achieved by Coomassie Brilliant Blue staining (Wilson, 1979). Characterisation of purified samples was performed by reversed-phase (RP) chromatography and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. Protein quantities were determined according to Bradford (1976) using bovine serum albumin (BSA) as a standard.

2.4 Drug samples

Levosimendan and dextrosimendan, the R and S enantiomers of {[4-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl) phenyl] hydrazono} propanedinitrile [CAS registry number 141505-33-1], were synthesised at Orion Pharma, Espoo, Finland. In addition, levosimendan with ^{13}C-labeled aromatic ring was used in the original publication II. Freshly made levosimendan solutions (30 mM) in 30 mM potassium carbonate were used for each titration point (II). In the studies described in the original publications of III and IV, levosimendan and dextrosimendan were dissolved in dimethyl sulfoxide (DMSO) at concentrations up to 60 mM. These stock solutions were light protected and stored at room temperature. The stability of the stock solution was confirmed by one-dimensional proton NMR spectroscopy.

2.5 Liquid crystalline medium

Filamentous Pf1 phages for dilute liquid crystalline medium were produced and purified as previously described by Hansen and coworkers (1998) (I). The purity of phages were determined by SDS-PAGE (Laemmli, 1970) and Coomassie Brilliant Blue staining (Wilson, 1979). The concentration of the Pf1 solution was determined by spectrophotometrically at wavelength 270 nm according to Kostrikis and coworkers (Kostrikis *et al.*, 1994).

2.6 Protein ligand interaction studies by NMR

Most of the NMR spectra were acquired with Varian Inova 500, 600 or 800 MHz spectrometers. The 750 MHz spectral data were recorded at the SON NMR Large Scale Facility in Utrecht.

For assignment of isolated $cTnC_{CS}$ three-dimensional triple resonance HNCACB, CBCA[CO]NH, and HN[CO]CA spectra were acquired and for the assignment of the backbone of $cTnC_{CS}/cTnI_{32\text{-}79}/cTnI_{128\text{-}180}$ HN[CO]CA and HN[CO]CACB were recorded (Wittekind and Mueller, 1993; Kay *et al.*, 1994; Muhandiram and Kay, 1994; Yamazaki *et al.*, 1994).

The complex formation and interaction between levosimendan and cTnC were followed by two-dimensional ^{1}H-^{15}N-heteronuclear single quantum correlation spectra (^{1}H-^{15}N-HSQC) (Mori *et al.*, 1995) (II-IV). Two-dimensional ^{1}H-^{13}C-heteronuclear single quantum correlation spectra (^{1}H-^{13}C-HSQC) were acquired to follow levosimendan-induced changes in methionine residues (Santoro and King, 1992) (II). ^{13}C-edited NOESY spectra of selectively labeled levosimendan in the absence and presence of $cTnC_{CS}$ were acquired to detect intermolecular NOEs (Muhandiram *et al.*, 1993) (II). Heteronuclear ^{1}H-^{15}N residual dipolar couplings (RDCs) were measured from α/β half-filtered ^{1}H-^{15}N correlation spectra according to Cordier (1999) to study calcium and cTnI induced changes in the N-domain of cTnC (I).

2.7 Processing of NMR data

Acquired NMR data were processed by Felix 97.0 and Felix 2000 software programs (Biosym Technologies, Inc.) (I and II). NMRPipe (Delaglio *et al.*, 1995), NMRView (Johnson and Blevins, 1994), and SPARKY (Goddard T. D. and Kneller D. G., University of California, San Francisco, USA) software were used to process and analyse the NMR data for the original publications III and IV.

2.8 Small angle X-ray scattering

A fine focus copper X-ray tube in-line-focusing mode was used for small angle X-ray scattering (SAXS) measurements. The distance distribution function was calculated by the indirect Fourier transform method with the program Gnom (Svergun *et al.*, 1988) (II).

3 RESULTS AND DISCUSSION

In this section the central results of the study are presented and discussed regarding of the levosimendan effect on cTnC. The interaction is compared to two other potent calcium sensitiser molecules, bepridil and EMD57033, that are known to bind to cTnC and cause positive inotropy.

3.1 Calcium and cardiac troponin I binding induced changes on the regulatory domain of cardiac troponin C

In skeletal muscle, calcium binding to sNTnC opens the domain structure for sTnI binding, which eventually leads to muscle contraction (Gergely *et al.*, 1993; Gagné *et al.*, 1994; Gagné *et al.*, 1995; Slupsky and Sykes, 1995). The activation mechanism of cardiac muscle differs from skeletal muscle. Calcium binding to TnC is the initiator in both muscle types but Ca^{2+} induced structural changes and energetics of the activation differ.

The activation sequence of cTnC upon calcium and cTnI binding was studied by measuring NH RDCs. A dilute liquid crystalline medium was formed of filamentous Pf1 phages wherein protein molecules aligned partially (Hansen *et al.*, 1998). Partial alignment of protein molecules results in measurable RDCs. They give angular information about structural motifs, especially α-helices and β-sheets (Bax and Tjandra, 1997; Tjandra and Bax, 1997; Tjandra *et al.*, 1997). The NH RDCs were measured using uniformly ^{15}N-labeled cNTnC samples in apo and calcium saturated forms and also in complex with cTnI. The experimental values were compared with RDC-values calculated from the respective PDB-structures. The measured values for NH RDCs correlated well with the calculated values of corresponding structures of TnC. In the apo-form (PDB entry 1SPY), the N-domain was closed and in the presence of calcium and cTnI (PDB entry 1MXL) the domain structure was fully open. However, the calcium form of cNTnC gave a poor quality factor and correlation between measured and calculated RDC-values. This can be explained by the conformational isomerism present in the Ca^{2+}-form of cTnC between open and closed conformations (Pääkkönen *et al.*, 1998). It has been shown that the closed state is energetically more favored than the open state and therefore it dominates (Li *et al.*, 1997; Li *et al.*, 1999) (Fig.

12). Calcium binding primes the N-domain for cTnI binding which brings in energy that is sufficient to overcome the energy barrier and fully opens the N-domain of cTnC (Li *et al.*, 1999). In this open conformation, the skeletal and cardiac isoform structures closely resemble each other. The populations of open and closed states in the liquid crystalline phase are altered when compared to those of the liquid phase. The difference in populations can explain the poor correlation between measured and calculated values for Ca^{2+}-cNTnC (I).

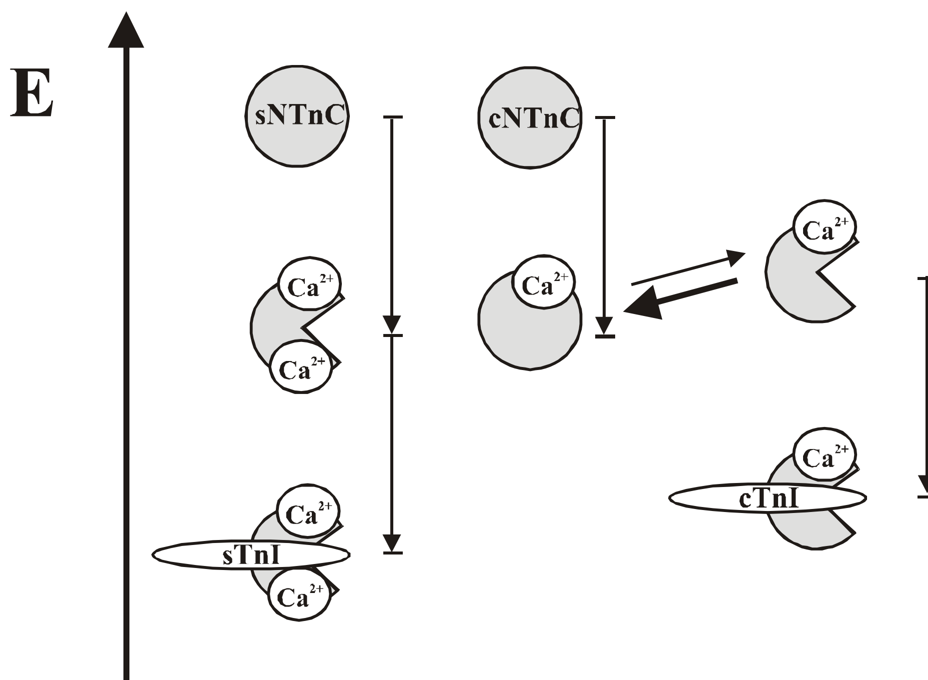

Fig. 12. **Energy diagram for sTnC and cTnC in different states of the contraction cycle.** Upon calcium binding to cNTnC an equilibrium between open and closed conformation exists in the cardiac isoform whereas the skeletal isoform is open showing no such conformational equilibrium (I) and (McKay *et al.*, 2000).

3.2 Stability of drug samples during the NMR experiments

Two cysteine residues, Cys35 and Cys84, in the cardiac troponin C sequence are able to form intra- and intermolecular disulfide bonds, *in vitro*, under mildly oxidizing conditions (Putkey *et al.*, 1993). At higher concentrations, above 0.5 mM and at neutral pH, it has been shown that N-domains interact with each other forming dimers (Slupsky *et al.*, 1995).

Therefore, a reducing agent dithiothreitol (DTT) was added to the cTnC samples to prevent disulfide bond formation. In addition to DTT, a bacterial inhibitor, sodium azide (NaN_3), was added to the cTnC samples to prevent bacterial growth and prolong the lifetime of expensive, isotopically labeled protein samples. However, these compounds were found to react with levosimendan destroying the experimental set up (Fig. 13). DTT reacted rapidly with levosimendan and modified it. In contrast, the reaction of levosimendan with sodium azide took days at 40°C to react completely with levosimendan transforming it into a soluble azide adduct. It is important to note that the modification of levosimendan in the presence of DTT is one of the factors that explains the discrepancies between the results for levosimendan binding to cTnC of various research groups.

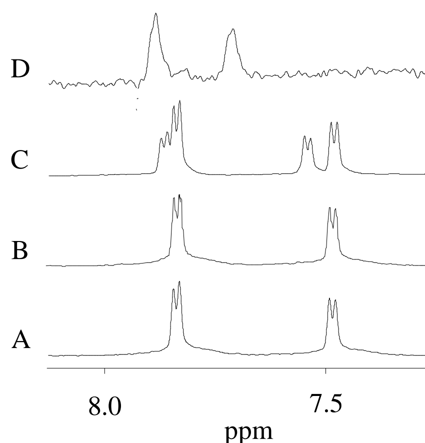

Fig. 13. An expansion of the aromatic region of the ^1H -NMR spectrum of levosimendan in the presence of DTT and NaN_3. A) A freshly prepared levosimendan solution in 20 mM Bis-Tris/ 10 mM $CaCl_2$, pH 6.8 at 40°C. B) The same sample after 24 hrs at 40°C. C) Sodium azide was added to a levosimendan sample in 20 mM Bis-Tris/ 10 mM $CaCl_2$, pH 6.8 at 40°C. The complete transformation to the azide adduct took days. D) DTT was added to a levosimendan sample in 20 mM Bis-Tris/ 10 mM $CaCl_2$, pH 6.8 at 40°C. Modification of levosimendan occured within minutes. (II)

No additives to the Bis-Tris/$CaCl_2$ buffer were added to the final protein samples to study the interaction of levosimendan with cTnC. However, DTT was included in the buffer during the purification steps though afterwards it was thoroughly washed-out with DTT free buffer on an ultra filtration membrane. The DTT, NaN_3, and protease inhibitor free cTnC samples were stable for weeks or even months and no indications of dimerization of cTnC

samples during the NMR or small-angle X-ray scattering (SAXS) experiments (Fig. 14) were detected. The determined radius of gyration of $cTnC_{CS}$, 20.2 ± 0.5 Å, is in good agreement with the previously published result of Fujisawa and co-workers (1987). The Guinier plot (Guinier, A. and Fournet, G. (1955) Small-Angle Scattering of X-rays, John Wiley & Sons, Inc., New York) shows no aggregation of $cTnC_{CS}$ at 0.3 mM concentration under NMR conditions.

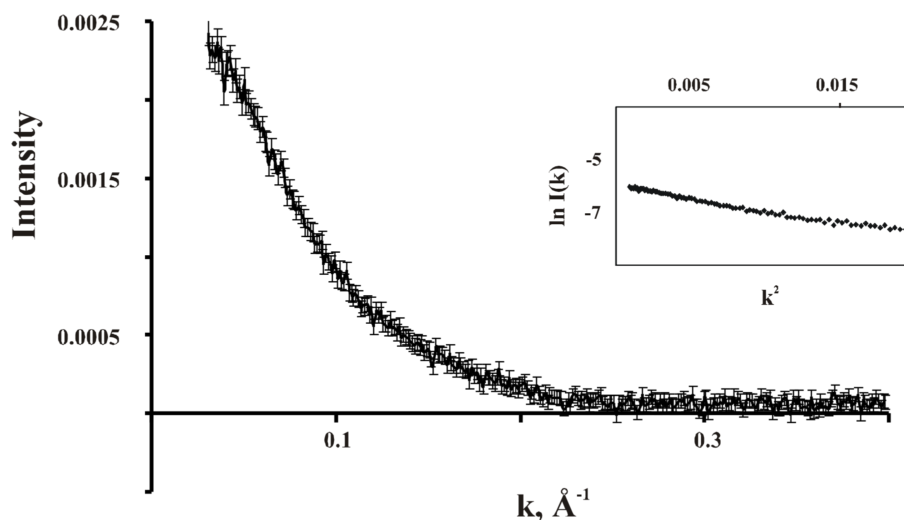

Fig. 14. **Guinier plot of $cTnC_{CS}$ without DTT show no evidence of dimerization or aggregation of protein judged by the Guinier law at the smallest *k* values.** (II)

3.3 Levosimendan interaction with cardiac troponin C

3.3.1 Binding of levosimendan to isolated cardiac troponin C

The interaction of levosimendan with the isolated $cTnC_{CS}$ was studied by following chemical shift changes in the ^{1}H-^{15}N-HSQC spectrum upon addition of the drug. This method is fast, has a high sensitivity and has been widely used for detecting ligand-protein interactions. The major drawback of the method is that it detects changes in the backbone of the protein and most of the side chains cannot be followed. Based on chemical shift changes in the ^{1}H-^{15}N-HSQC spectrum, interaction between levosimendan and cTnC was observed

on isolated $(Ca^{2+})_3$-$cTnC_{CS}$ (Fig. 15). Levosimendan interacted with both domains of the isolated cTnC as was previously shown by Haikala and co-workers (Haikala *et al.*, 1995a).

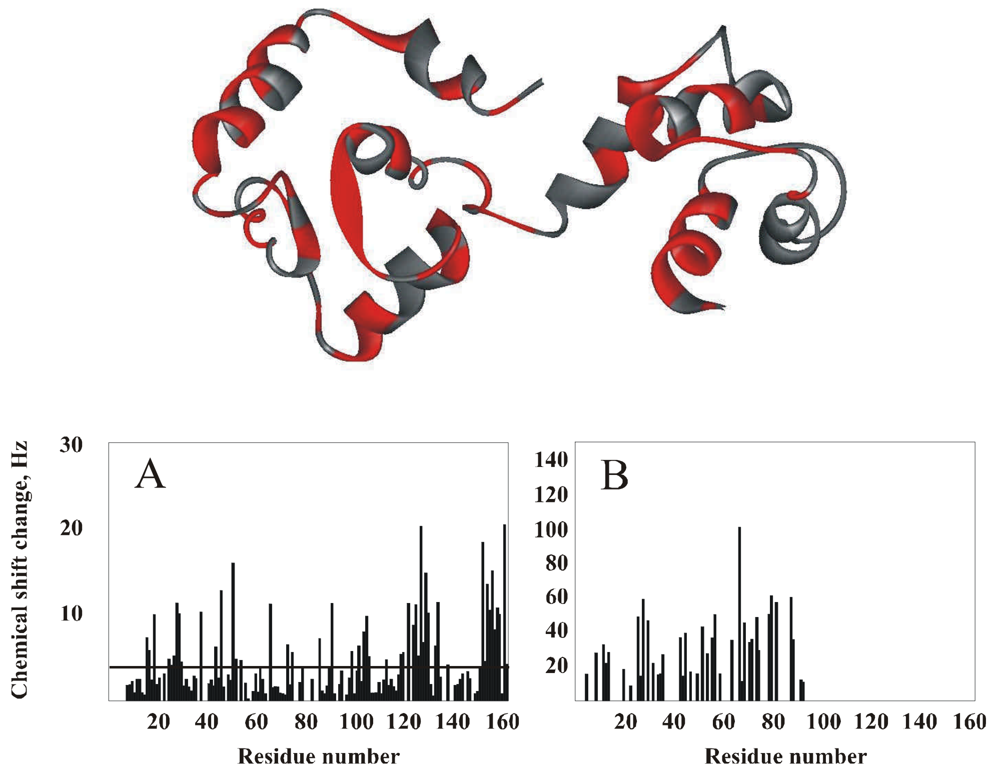

Fig. 15. **Levosimendan induced chemical shift changes of $cTnC_{CS}$ followed by ^{1}H-^{15}N HSQC spectrum at 800 MHz.** Chemical shift changes larger than 4 Hz (black line) and all the residues showing a slow exchange process are plotted on a schematic 3D structure of cTnC in red (PDB entry 1AJ4). Chemical shift changes are also shown as a function of amino acid sequence. A) Chemical shift perturbations in the fast exchange. B) Chemical shift changes of slow exchange timescale resulting in two sets of resonances. Chemical shift changes are shown as a distance in Hz from the original peak, $\Delta \nu = \sqrt{\Delta \nu_H^2 + \Delta \nu_N^2}$.

Levosimendan induced chemical shift changes in a slow exchange process ($k_{ex} < 50$ s^{-1}) in the N-domain. In addition, small chemical shift perturbations in fast time scale exchange were also induced by levosimendan in the N-domain. There are two observable signals for many of the residues all over the regulatory domain. The affinity of levosimendan to cNTnC, K_d being about 10 μM, was estimated from the change in the integrated volume

of disappearing peaks and newly appearing peaks along the levosimendan titration curve. No conclusions regards levosimendan binding site(s) in the N-domain were possible. However, by comparing levosimendan-binding results from various samples, an estimate for the binding region could be made. There were some clear differences between the behaviour of levosimendan binding to various cTnC samples that pointed to a hypothesised binding site.

First, isolated Ca^{2+}-cNTnC results did not reveal any evidence of the slow timescale conformational change caused by levosimendan (II). The second observation was that no slow timescale exchange of the binding of levosimendan to the N-domain of the full-length $(Ca^{2+})_3$-$cTnC_{A\text{-}Cys}$ occured though the C-domain interaction was clearly observable (II). However, the full-length $(Ca^{2+})_3$-$cTnC_{CS}$ unambiguously showed a levosimendan interaction with the N-domain which occured simultaneous with the C-domain binding (II and IV). It was clear from these results that levosimendan also binds to the N-domain of cTnC. The binding region most probably locates at the hydrophobic region of cTnC close to the D/E linker. This has been modelled to be the interaction site for hydrophobic molecules (Ovaska and Taskinen, 1991). Similarly, the prerequisite for full-length cTnC including Cys84 for the interaction between levosimendan and the regulatory domain of cTnC to result in the structural change as was also reported by Levijoki et al. (2000). Therefore, Cys84 is either essential for levosimendan binding, or its mutation to serine slightly alters the conformation of the binding region and levosimendan binding is no longer possible. This suggests that the binding takes place close to the flexible linker. Consequently, small differences in this region due to the missing C-domain were able to prevent levosimendan from binding to its target protein.

The *P*(r) function of calcium saturated $cTnC_{CS}$ shows that it remains in its elongated form in the presence of levosimendan (Fig. 16). Levosimendan binding does not bring the domains in closer spatial proximity to each other as has been shown to be the case for bepridil (Li *et al.*, 2000b). Interestingly, levosimendan binding increases the radius of gyration from 20.2 ± 0.5 Å to 21.7 ± 0.6 Å and slightly longer distances appear in the *P*(r) function in the presence of levosimendan. Similarly, a slight increase in the mean distance was observed when cTnI binding to cTnC reduced the linker region flexibility (Dong *et al.*, 1999). Thus these observations support the hypothesis that the levosimendan binding site to

be located close to the D/E linker making the flexible linker region more rigid thus limiting the degrees of freedom of the domains.

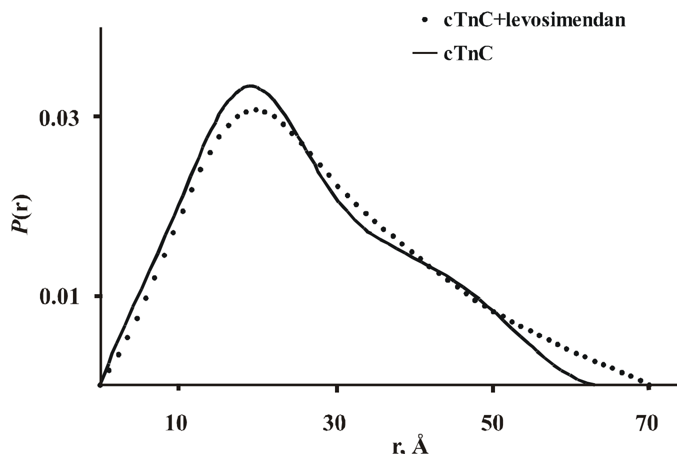

Fig. 16. ***P*(r) function of $cTnC_{CS}$ with and without levosimendan.** Drug binding to calcium saturated $cTnC_{CS}$ does not pull the domains together. The radius of gyration is slightly larger for $cTnC_{CS}$/levosimendan than cTnC alone and longer distances are also seen in the presence of levosimendan indicating drug binding close the linker region. (II)

The interaction of bepridil with cTnC is shown to take place at the hydrophobic region of the N-domain and stabilise the open conformation (Abusamhadneh *et al.*, 2001; Wang *et al.*, 2002). A competitive binding study between levosimendan and bepridil was attempted to determine whether the binding site of both calcium sensitisers is the same. Unfortunately, there were several isoforms of bepridil present under used conditions and no competition experiment could be completed.

The binding of levosimendan to the C-domain of cTnC, resulted in small chemical shift changes assigning two potential binding sites in the structural domain. This interaction occured as a fast exchange process ($k_{ex} > 10^4$ s^{-1}). These C-domain binding sites appear to locate on opposite sides of the domain (Fig. 15). Levosimendan induced chemical shift changes were compared with racemate EMD53998 induced changes and found to be very similar. However, EMD57033 has been shown to have one binding site on the C-domain of cTnC (Li *et al.*, 2000a; Wang *et al.*, 2001). The chemical shift changes were too small to allow accurate determination of the affinity of levosimendan to cCTnC sites (II).

3.3.2 Binding of levosimendan to cardiac troponin C complexed with cardiac troponin I

Levosimendan was shown to be able to interact with both domains of cTnC (Haikala *et al.*, 1995a) (II), but the interaction site that is responsible for the calcium sensitising effect and the mechanism were not known. Therefore, a more complete but still rather limited model of the contractile apparatus was prepared to study the levosimendan interaction with cTnC. Two peptides of human cTnI, consisting of residues 32-79 and 128-180, were used to make the complex with $(Ca^{2+})_3$-$cTnC_{CS}$. The $cTnI_{32-79}$ comprises the C-domain binding region. In contrast, the $cTnI_{128-180}$ entity includes the inhibitory region together with the regulatory region $cTnI_{147-166}$ that interacts with the N-domain of cTnC. The full-length cTnI is rich in positively charged amino acids such as lysine and arginine, therefore nonspecific interactions between levosimendan and isolated cTnI would be expected to take place to some extent. These peptides were chosen because they included all the essential cTnC binding regions for the regulation of the cardiac contraction and in order to minimise nonspecific interactions.

Both cTnI peptides behaved as expected under the used NMR conditions. A 1:1:1 complex of $cTnC_{CS}$:$cTnI_{32-79}$:$cTnI_{128-180}$ was formed as indicated by the observed chemical shift changes in ^{1}H-^{15}N-HSQC spectra. Our assignments agreed well with previously reported spectra and assignments of cTnC/cTnI (Abbott *et al.*, 2001). Thus indicating that the same structure of cTnC was formed with TnI peptides as when using the full-length cTnI. Therefore the complex used in our study was most likely to be in functionally relevant conformation.

Levosimendan induced changes to the $cTnC_{CS}$/$cTnI_{32-79}$/$cTnI_{128-180}$ complex was followed by ^{1}H-^{15}N-HSQC spectra. Binding of levosimendan to this complex resulted in chemical shift changes and the significant chemical shift perturbations are located only in the N-domain of $cTnC_{CS}$ (Fig.17). This is an unambiguous indication of levosimendan binding only to the regulatory domain of $cTnC_{CS}$ in the presence of cTnI. The C-domain binding sites detected with the isolated $cTnC_{CS}$ are blocked by cTnI and thus are not a prerequisite for the calcium sensitising effect of levosimendan to occure. Kleerekoper and co-workers

(1998) have suggested that the hydrophobic region in the C-domain would not be an ideal binding site for calcium sensitisers because cTnI may block the drug binding sites.

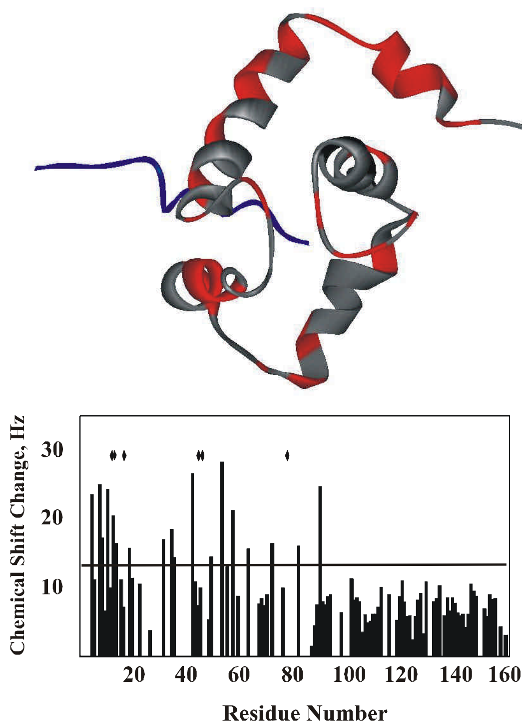

Fig. 17. **Levosimendan induced chemical shift changes on cTnC$_{CS}$ complexed with cTnI$_{32\text{-}79}$ and cTnI$_{128\text{-}180}$ determined by ^{1}H-^{15}N HSQC spectrum at 750 MHz.** Chemical shift changes are shown as the distance in Hz from the original peak, $\Delta n = \sqrt{\Delta n_H^2 + \Delta n_N^2}$. Chemical shift changes larger than 13 Hz (black line) are plotted on a schematic 3D structure of cNTnC/cTnI$_{128\text{-}147}$ (PDB entry 1MXL). (IV)

Levosimendan binding in the presence of cTnI causes numerous chemical shift changes in the ^{1}H-^{15}N-HSQC spectrum of cTnC, similar to those of the isolated cTnC. No exact binding site could be identified based on the spectral changes. It is possible that the structural change caused by the drug binding, covers the chemical shift changes resulting from the closeness of the bound drug and therefore the exact binding site indicated by the changes on the NMR spectrum could not be determined. As the calcium binding sites are structurally coupled, Ca^{2+} or ligand binding to one part of the domain may also cause

observable changes in the other parts of the domain (Li *et al.*, 1997). A quite unexpected result was the significant chemical shift perturbations in the N-helix upon levosimendan binding. Gulati and co-workers (1992) have shown that the N-helix, together with the inactive Ca^{2+} site I, is required for the full activity of cTnC (Gulati *et al.*, 1992). It has been suggested that the N-helix is important for the calcium binding signal transmission within the Tn complex to occur (Fredricksen and Swenson, 1996; Smith *et al.*, 1999). Therefore, it is intuitively understandable that the binding of a calcium sensitiser will also affect this helix even though its precise function and binding sites are not known.

3.3.3 Stereoselective interaction of levosimendan with cardiac troponin C

Levosimendan and dextrosimendan are the R and S enantiomers of simendan. The effects of levosimendan and dextrosimendan as calcium sensitisers were compared, *in vitro*, using skinned fibers (III). It was clear from these studies that both stereoisomers have a calcium sensitising effect on myocytes. However, the effects of dextrosimendan appeared at concentrations about 30 times higher than those of levosimendan. The difference in the potency of the calcium sensitising effect between the two stereoisomers was used to investigate the calcium sensitising interaction site of levosimendan by NMR (III). The interaction of simendan stereoisomers on cTnC was studied in the absence, or presence of cTnI peptides.

In the absence of cTnI, both stereoisomers interacted with both domains of $cTnC_{CS}$. The interaction with the N-domain resulted in chemical shift changes and revealed two different timescale exchange processes, fast exchange and slow exchange. Levosimendan was found to have an order of magnitude higher affinity for cNTnC than dextrosimendan in the isolated cTnC. It also induced a 25 % more definite resonance doublings resulting from the slow exchange process than those caused by dextrosimendan. In addition, each stereoisomer caused some of the doubled peaks and chemical shift changes to shift in different directions (Fig. 18).

In the C-domain of isolated $cTnC_{CS}$, both stereoisomers have two distinct binding sites and their respective interactions occur in fast exchange. However, a significant

difference in their binding behaviour was observed. Dextrosimendan was found to have the strongest binding site in the C-domain of $cTnC_{CS}$ in the absence of cTnI. In contrast, levosimendan has a lower affinity to the same C-terminal site (residues 150-160). However, the chemical shift changes are very small and therefore no accurate determination for the binding constants of levosimendan to the C-domain was possible. The stereoselective binding of the ligands in the C-domain is clear in the absence of cTnI (Fig. 18). This was an interesting finding because EMD57033 results in an apparent calcium sensitisation of the myocytes and this effect has been proposed to occur via the stereoselective binding to the C-domain of cTnC (Li *et al.*, 2000a). Dextrosimendan has the same chirality as EMD57033.

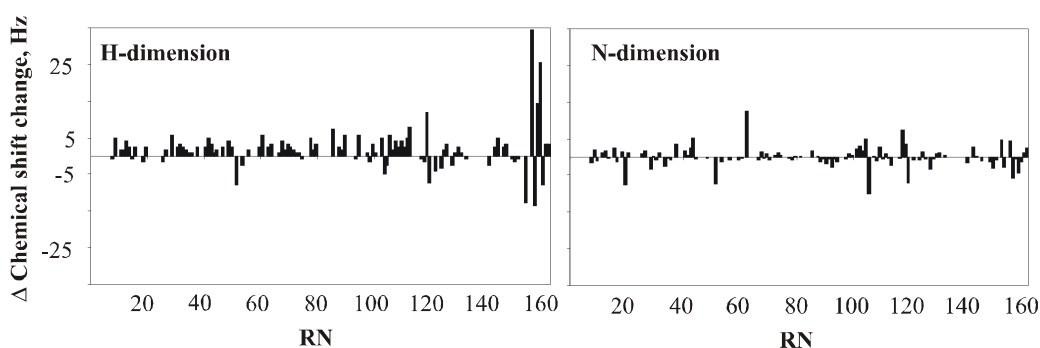

Fig. 18. **Chemical shift change differences on $cTnC_{CS}$ induced by levosimendan and dextrosimendan.** Differences are calculated as $\Delta\Delta n = \Delta n_L - \Delta n_D$, where L indicates levosimendan and D is for dextrosimendan.

In the presence of cTnC binding regions of cTnI, $cTnI_{32-79}$ and $cTnI_{128-180}$, the C-domain was blocked from levosimendan and dextrosimendan binding (III, IV). The interaction with the N-domain must therefore be significant in respect of the calcium sensitising effect of levosimendan. The stereoisomers of simendan induced different chemical shift changes in the NMR spectra of this protein model. In Fig. 19, the differences between the stereoisomers induced chemical shift changes on cTnC are shown. No significant differences between the stereosiomers were detected in the C-domain interaction. On the other hand, the differences between the stereosiomers on the N-domain were numerous and no exact binding site could be determined. It seems possible that the

suggested conformational isomerism of cTnC masks the chemical shift changes resulting from the change in chemical environment adjacent to the bound drug. However, it is possible that the chemical shift differences observed between the stereoisomers for the N-domain binding were due to the chirality of this molecule and thus a stereospecific interaction.

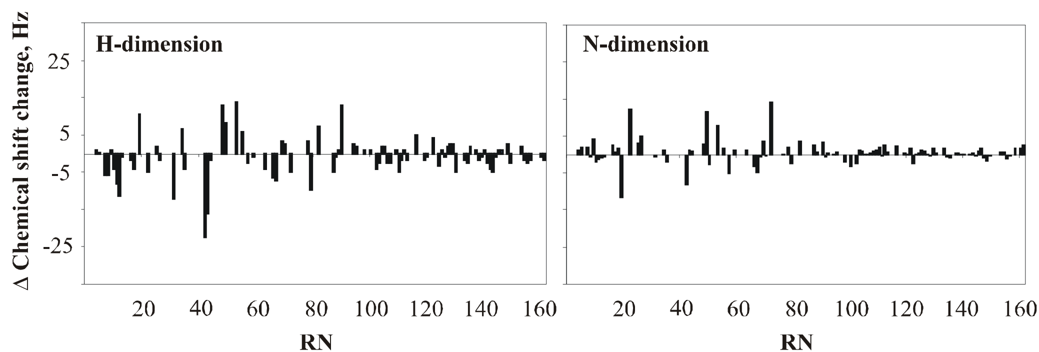

Fig. 19. **Chemical shift change differences on the complex of $cTnC_{CS}/cTnI_{32\text{-}79}/cTnI_{128\text{-}180}$ induced by levosimendan and dextrosimendan.** Differences are calculated as was described in the figure legend for Fig. 18.

4 SUMMARY AND CONCLUSIONS

Structural information of protein complexes combined with the knowledge of their functions elucidates the understanding of cellular mechanisms. Furthermore, knowledge about a protein's structure-activity relationship is especially useful when new, effective treatments with specific mode of action against different diseases are designed. Detailed information of muscle contraction, roles of each protein of the contractile apparatus and understanding of their interactions in various stages of the contraction cycle is essential for the design of the specific treatment for cardiac insufficiency. In recent years, cardiac troponin C has been in the focus of this research because of its central regulatory role in the contraction of cardiac muscle. Several molecules have been suggested as candidates as calcium sensitisers since they modulate the response of the contractile apparatus to a calcium signal without an increase in intracellular calcium concentration. However, many of them have unwanted side effects or are nonspecific and therefore they are unsuitable for clinical use.

On the other hand, levosimendan is used as a calcium sensitiser in congestive heart failure and it has been reported to be a specific and well tolerated treatment. It undergoes stereoselective interaction with the N-domain of calcium saturated cardiac troponin C in the presence of cardiac troponin I peptides of 32-79 and 128-180. Unfortunately, the exact binding site could not be determined at this point. Upon levosimendan binding to the N-domain of cTnC, numerous chemical shift changes throughout the entire N-domain were seen in the ^{1}H-^{15}N-HSQC spectrum. No NOEs could be assigned and the short lifetime of the cTnC-levosimendan complex limited the useable NMR experiments. Furthermore, the stability and function of levosimendan samples was adversely affected when DTT was observed to react with levosimendan. As a consequence, the isolated cNTnC domain and Cys84Ser mutated cTnC were unable to interact with levosimendan, resulting in a slow exchange process. Therefore it is suggested that the results of levosimendan interaction with cTnC reported by some other groups, which used the same reducing agent and have been the subject of controversy have been obtained from unsuitable experimental conditions.

Several hydrophobic calcium sensitisers have been reported to bind to the hydrophobic region in the N-domain of cTnC. Even though, no exact binding site for the

calcium sensitiser levosimendan on cTnC could be determined at this point, there are indications that the aforementioned region is also the binding site for levosimendan. The conformation of the N-domain of cTnC in the proximity of the D/E linker region and the presence of Cys84 were found to be essential for the binding of levosimendan.

To function properly, it is important that a calcium sensitiser whose effects are mediated via cTnC does not prevent the interaction between cTnI and cTnC. A potent calcium sensitiser will prime the cNTnC for cTnI binding calcium dependently. One of the likely mechanisms to achieve this is to shift the equilibrium between open and closed states of the N-domain of cTnC, towards the open conformation (Fig. 20). It would enhance the cTnI binding to cTnC by lowering the required energy level for the interaction to take place. It may also expand the lifetime of the cTnC/cTnI complex, consequently the active conformation.

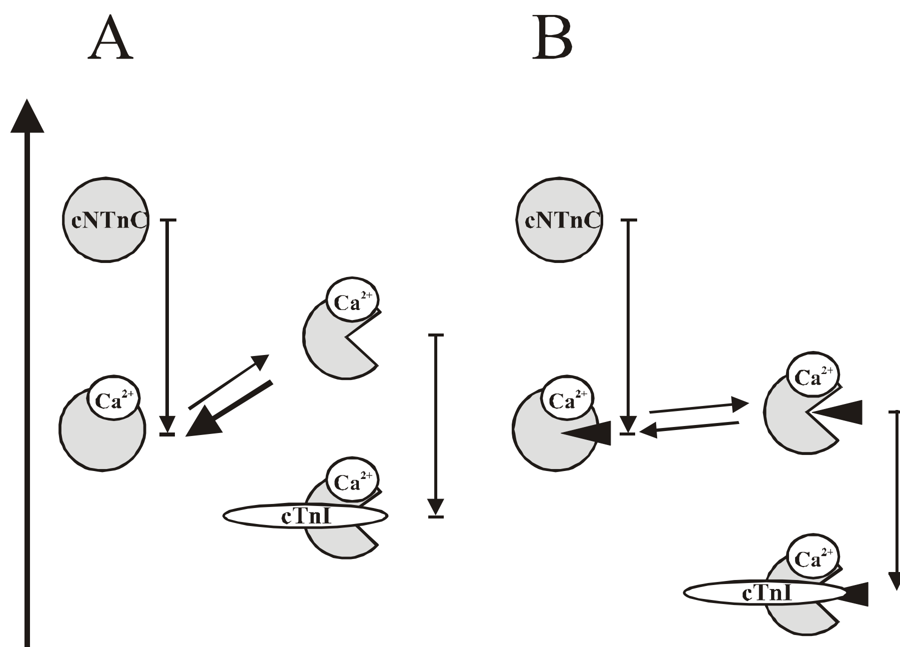

Fig. 20. **An energy diagram of an effect of a potential calcium sensitiser.** A) Normal energy levels during excitation cycle in cardiac muscle (original publication I and (McKay *et al.*, 2000). B) Calcium sensitiser such as levosimendan possibly shifts the equilibrium from closed towards the open state and thus primes the N-domain for cTnI binding. Levosimendan in the complex of cTnC/cTnI may also prolong the lifetime of the complex and thus enhance the contraction.

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6 APPENDICES

Scientific publications from I to IV.