Solution Structure and Main Chain Dynamics of the Regulatory Domain (Residues 1–91) of Human Cardiac Troponin C*

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The three-dimensional structure of calcium-loaded regulatory, i.e. N-terminal, domain (1–91) of human cardiac troponin C (cTnC) was determined by NMR in water/trifluoroethanol (91:9 v/v) solution. The single-calcium-loaded cardiac regulatory domain is in a “closed” conformation with comparatively little exposed hydrophobic surface. Difference distance matrices computed from the families of Ca2+-cTnC, the apo and two-calcium forms of the skeletal TnC (sTnC) structures reveal similar relative orientations for the N, A, and D helices. The B and C helices are closer to the NAD frame- work in Ca2+-cTnC and in apo-sTnC than in 2Ca2+- sTnC. However, there is an indication of a conformational exchange based on broad 15N resonances for several amino acids measured at several temperatures. A majority of the amides in the B and C helices and in the calcium binding loop exhibit very fast motions with comparatively small amplitudes according to the Lipari- Szabo model. A few residues at the N and C termini are flexible. Data were recorded from nonlabeled and 15N-labeled samples, and backbone dynamics was investigated by 15N T1, T2, and heteronuclear nuclear Overhauser effect as well as by relaxation interference measurements.

Troponin C (TnC) is the calcium-binding protein in the thin filament of skeletal and cardiac muscle and belongs to the EF-hand family of proteins. The structure of TnC closely resembles that of calmodulin, a ubiquitous calcium sensor, with an N-terminal and a C-terminal domain connected by a long central α-helix. Under physiological conditions the C-terminal domain of TnC is always occupied by Ca2+ or Mg2+. In the N-terminal domain the low affinity sites of TnC bind calcium ions when they are released from the sarcoplasmic reticulum of skeletal or cardiac muscle myocytes. The conformational changes brought about by the calcium binding to the N-terminal domain of skeletal troponin C (sTnC) have been followed by NMR (1–4). The data confirm the early hypothesis of an open and a closed conformation (5–7). According to the paradigm the Ca2+-induced conformational changes of the N-terminal domain are transmitted to the other components of the troponin complex and then to tropomyosin, triggering the muscle contraction (8–12).

The sequence of the cardiac TnC (cTnC) is 70% identical to that of sTnC, whose structure in the calcium-saturated form has been determined by x-ray crystallography (7, 13) and by NMR (14). However, while the N-terminal domain of sTnC contains two calcium-binding sites, the N-terminal domain of cTnC has only one intact Ca2+-binding site. The other site, often referred as a defunct site, does not bind calcium. The functional meaning of this difference between the cardiac and skeletal troponin C has not been explained in structural terms.

Even if there is no direct structural proof that the molecular mechanism of action of cTnC is the same as for sTnC, evidence has been given that also in cTnC the N-terminal domain is responsible for the regulation. It is assumed that the C-terminal domain plays primarily a structural role, since its calcium-binding sites are occupied even when the intracellular calcium concentration drops to micromolar values (15, 16).

Recently, Sia et al. (17) working on cysteine (Cys-35 and Cys-84) to serine-mutated cTnC, showed that, in its calcium-saturated form, the N-terminal domain is in the closed conformation. This result implies that there is a much smaller conformational change to be expected upon calcium binding than in sTnC as proved by the very recent study by Spyracopoulos et al. (18). We aimed to confirm the existence of the closed form of the N-terminal domain for the human cTnC. Since the closed form of the N-terminal domain of cTnC was an unexpected result, we wanted to assess the structural integrity by dynamics measurements to get insight into a plausible conformational exchange between the closed and open states.

Experimental Procedures
Cloning and Expression of Recombinant Human Cardiac Troponin C N-terminal Fragment—The coding sequence of human cTnC was cloned by using the reverse transcription-polymerase chain reaction technique and human heart poly(A)+ RNA as a template and primers (20). The amplified DNA fragment was digested, purified, and subcloned to the TGM3-vector (Promega). For protein expression the subcloned insert was isolated and ligated to the glutathione S-transferase fusion protein vector pGEX-2T (Pharmacia PL-Biochemicals) (19). The bacterial expression for production of human cTnC glutathione S-transferase fusion protein was carried out in Escherichia coli DH5 α-cells. The cells were grown at 37 °C overnight in minimum medium according to Janson et al. (20), using ammonium chloride instead of ammonium sulfate. The culture was diluted 1:25 in minimum medium containing 15N-labeled ammonium chloride (1 g/liter) and grown at 37 °C to the middle of the growth phase, prior to induction with isopropyl β-D-thiogalacto- pyranoside (0.5 mM) for 4 h. The cells were harvested by centrifugation, and an aliquot of the collected cells was analyzed for estimation of the
amount of cTnC N-terminal fragment by SDS-polyacrylamide gel electrophoresis.

**Purification of Recombinant Human N-Labeled cTnC N-terminal Fragment**—The collected bacterial paste of the 5-liter culture was diluted in 50 ml of 16 mM Na2HPO4, 4 mM NaH2PO4, 150 mM NaCl, 1% Triton-X-100, pH 7.5. The final concentration of Triton-X-100 was 0.25% phenylmethylsulfonyl fluoride and 25 µl of benzotriazole. The cells were disrupted by sonication on ice. The suspension was clarified by centrifugation and used as the starting material for purification on the glutathione Sepharose (10 ml; Pharmacia) affinity column. The recombinant human cTnC was further purified by HPLC anion exchange chromatography (Mono-Q HR 5/5) as described by Pollesello et al. (19). The free calcium was removed using Chelex-100 affinity chromatography (30 ml; Bio-Rad) equilibrated in water/ammonia solution, pH 8.0, and the eluted purified protein was freeze-dried. The amount of protein was estimated according to Bradford (21). The purity of the purified human cTnC fragment was analyzed by reversed phase HPLC (C18 TSK TMS 250 column, 0.46 x 4 cm) (19), and by SDS-polyacrylamide gel electrophoresis followed by Coomassie Brilliant Blue staining (22). The reversed-phase chromatography-purified protein peak was further analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

**NMR Spectroscopy**—Samples of the freeze-dried N-terminal fragment of cTnC (10 mg) were dissolved in 500 µl of a solution containing 7.7 mM dithiothreitol in H2O. Dithiothreitol was added to prevent potential dimerization of cTnC due to a possible disulfide formation between Cys-35 or Cys-84. The pH was adjusted at 6.0 with the addition of microliters of NaOH 0.5 M. Thereafter, CaCl2 was added to a final concentration of 3.9 mM. Finally, D2O and perdeuterated trifluoroethanol (TFA) were added in order to obtain 750 µl of a 1.3 mM protein solution in 82% H2O, 9% D2O, 9% perdeuterated TFA, and pH 6.0 ± 0.1 (not corrected for the deuteron effect).15N-Labeled sample (1.3 mM) was prepared to 300 µl in a Shigemi microcell.

NMR spectra were acquired mostly with a 600-MHzVarian Unity NMR spectrometer, and certain spectra were taken with a 500-MHz Varian Unity and a 400-MHz ARX Bruker spectrometer. For the nonlabeled sample homonuclear two-dimensional spectra, correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY) with mixing times of 50, 100, and 200 ms), were collected at 30 °C and at 40 and 50 °C (with mixing times 150 and 200 ms, respectively). For the 15N-labeled sample, heteronuclear single quantum correlation spectroscopy spectra were taken at temperatures from 20 to 50 °C with 2 °C increments. 1H-NMR spectra were recorded at 50 and 90 MHz as well as 13C (with relaxation delays 42, 168, 336, 589, 841, 1114, 1514, 2102, and 2691 ms), T2 (16, 17, 64, 95, 127, 159, 223, 286, and 382 ms) and heteronuclear NOE spectra were recorded at 30 °C. In the CPMG spin echo pulse sequence the delay (2τ) was 900 µs (23). The 15N spin lock strength in T,ρ measurements (4, 8, 15, 30, 46, 61, 76, 106, and 122 ms) was limited to 1.7 kHz due to experimental restrictions. The cross-correlation rate (ξ) was determined by the relaxation interference measurement (24). The relaxation delay (2τ) was 70 ms. The data were processed with Felix 95 software.

**Structure Generation**—Spin systems were assigned from the through bond correlation spectra and sequence specific assignments were deduced from the NOEs between the adjacent spin systems. The spectral overlap was partly unraveled by 1H-N editing and exploiting temperature dependence of HN resonances. Distance restraints were extracted from the homonuclear NOE data by fitting a second-order polynomial to integrated cross-peak volumes (I) of the NOE series. Additional distance restraints were extracted from NOEs observable in the 15N-labeled sample and from NOE cross-peaks in the 1H-15N spectra at 20 °C. The distance between residues was calculated from the NOE cross-peaks with a program modified to compute DDMs for families of structures. The relaxation and heteronuclear NOE data were analyzed in terms of the commonly used Lipari-Szabo model-free spectral density (28) with the programs by Palmer (27). The overall correlation time τc was estimated from R/R0 ratios (28). The cross-correlation rate between the dipole dipole and chemical shift anisotropy (29) was measured by a modified HSDC 1H-15N correlation experiment with a relaxation period prior to the 15N evolution time, as described by Tjandra et al. (24). During the relaxation period (2τ) the two 15N doublets relax with rates proportional to the square of the sum and difference of doublet dipole and chemical shift anisotropy interactions. The in-and anti-phase components are subsequently chosen separately for the detection yielding two spectra. The cross-correlation rate (η) is obtained from the ratio of the signal intensities (Ia and Ib) of the two spectra by η = (1/2) tanh−1(Ib/Ia) (24).

**RESULTS**

**Structure**—The structure of the calcium-loaded form of the N-terminal domain of cTnC (Fig. 1) comprises five helices, i.e. N (residues 4–10), A (14–29), B (43–47), C (57–64), and D (74–89). The A and B helices and the C and D helices form a pair of EF-hands connected by the central loop between the B...
and C helices. The defunct site is between the A and B helices and the calcium binding site between the C and D helices. The two sites are spatially close, and there are interstrand hydrogen bonds between the backbone amide protons and carbonyl oxygens (residues 36 and 72). The spatially adjacent N, A, and D helices are approximately orthogonal to each other, and they form a compact structural unit, the NAD-unit (18).

The structural statistics are given in Table I. The overall fold is defined by 224 long range NOEs. The backbone heavy atom RMSD is 1.1 ± 0.2 Å. The helices and the calcium binding site are well defined. Three residues at each termini, the defunct site, and the central loop have larger RMSD (>2 Å). The all heavy atom RMSD is 1.8 ± 0.2 Å. The extensive spectral overlap in the aliphatic region prevented us from deriving stereospecific assignments for side chain methylene groups. The RMSD per residue reflects approximately the distribution of the number of restraints (on the average 19) per residue.

The orientation of the A and B helices is based on the observation of 20 NOEs. These NOEs are primarily between aliphatic protons. For example, we find an NOE between Phe-20 and Met-45, which are in the middle of the A helix and the B helix, respectively. In an open conformation these distances would be clearly longer, and NOEs except in the vicinity of the loop would be beyond detection. However, the uncertainty in the side chain to side chain distance bounds involving particularly the pseudo atoms of methyl groups do not allow us to determine the interhelical angles precisely. Due to the overlap of in the two-dimensional homonuclear spectra, few NOEs between the A and B as well as between the C and D helices were not assigned. The loop between the A and B helices, i.e. the defunct site, is quite irregular and not particularly well defined. We identify 17 NOEs from the defunct loop. In the central loop between the B and C helices the prolines 52 and 54 are both in the trans configurations. We did not find any evidence for cis-trans isomerism.

**Dynamics**—The rate constants of the $^{15}$N longitudinal relaxation ($R_1$) are nearly constant (1.5 ± 0.2 s$^{-1}$) over the entire sequence. Only for Gly-91, the $R_1$ is substantially smaller. The variation in the rate constant of the transverse relaxation ($R_2$) is larger. For the helices and the calcium binding site the $R_2$ values are 10 ± 2 s$^{-1}$ and toward the N- and C termini the $R_2$ values drop for a few residues. Furthermore, for residues Val-28, Ser-37, Thr-38, Lys-39, Ile-61, and Val-64 the transverse relaxation proceeds so fast that the rate constants (>15 s$^{-1}$) could not be determined reliably. The values of $R_{lP}$ for residues 37–40 were smaller than the corresponding $R_2$ values but owing to the off-resonance effects (30) quantitative comparisons were not made. The $^{15}$N line widths for these residues measured from the HSQC spectrum recorded at 30 °C are indeed larger than on the average (Fig. 2). Whereas at increased temperature (40 and 50 °C), the line widths are only 1–2 Hz wider than on the average. At lower temperatures (10 and 20 °C), also the lines for residues Ile-36, Leu-41 and Gly-42 become broader than the average (Fig. 3). The rate of the $^{15}$N cross-correlation ($\eta$) between the dipole and chemical shift anisotropy was insensitive to the conformational exchange. The comparison of $\eta$ with $R_2$ reveals residues experiencing fast transverse relaxation (Fig. 3). For the residues (Val-28, Thr-38, Lys-39, Ile-61, and Val-64) with very fast transverse relaxation, no values of $\eta$ were obtained due to the insensitivity of the cross-correlation experiment.

The heteronuclear Overhauser enhancement was nearly constant and close to the maximum value (+0.82) (28) for the helices and the calcium-binding site but decreased for a few residues at the N and C termini. Heteronuclear NOEs could not be determined reliably for residues Ser-37 and Thr-38 due to the line broadening.

The principle components of the inertia tensor computed for the cNTnC structure were (0.81:0.85:1), which implies that the overall rotational diffusion can be regarded approximately isotropic. Furthermore, the ratio of $R_2/R_1$ for the helices did not vary significantly, which also implies an approximately isotropic rotational diffusion characterized by $\tau_m = 7.7$ ns. Therefore we considered the analysis of relaxation data in terms of the commonly used Lipari-Szabo model-free spectral density (26) reasonable. First the simple model was used with the order parameter ($S^2$) as the only free parameter. Subsequently, the time constant $\tau_m$ for the fast internal motion was allowed to vary as well. In this way a statistically good fit was obtained for the majority of the residues. For the residues in the helices and in the calcium binding site $S^2$ is approximately 0.9. The extended model in which the internal motion is divided into two components (31) resulted for the residues at the N and C termini (2–8 and 86–91) in a statistically good fit. At the N and C termini $S^2$ drops rapidly (Fig. 4). For the remaining residues

TABLE I

| Characteristics of the structures of Ca$^{2+}$-cNTnC |
|-----------------------------------------------|
| Distance restraints                          |
| Total                                         | 1356 |
| Intraresidue                                  | 474  |
| Interresidue                                  | 882  |
| Sequential                                    | 363  |
| Medium                                        | 295  |
| $i,i+2$                                       | 82   |
| $i,i+3$                                       | 155  |
| $i,i+4$                                       | 58   |
| Long                                          | 224  |
| Dihedral restraints $\phi$                    | 32   |
| Restraint violations/structure                |
| Distance of >0.5 Å                            | 0    |
| Distance of >0.3 Å                            | 1.4  |
| Distance of >0.1 Å                            | 43   |
| RMSD (Å)                                      |
| Backbone atoms (CA, CO, N)                    | 1.08 ± 0.24 |
| All atoms                                     | 1.75 ± 0.22 |
| $\phi$, $\psi$ in core or allowed regions    | 93.5% |

FIG. 2. $^{15}$N line widths color-coded from blue to red as the width of the ribbon. Residues with large $^{15}$N line widths are mostly at the defunct site. The broad red part of the ribbon represents residues with line broadening.
For comparisons to skeletal troponin C, the angle between helices A and B in cNTnC (135°) is approximately the same as that of apo-sNTnC (138°) but very different from that of 2Ca\textsuperscript{2+}-sNTnC (81°) (17). The angle between the C and D helices (130°) is more similar to the corresponding angle in apo-sNTnC (145°) than to the angle in 2Ca\textsuperscript{2+}-sNTnC (78°). Owing to the comparatively large distance bounds of the few NOEs in the calcium binding site the angle between the C and D helices is subject to uncertainty which does not allow us to prove unambiguously differences between cNTnC and sNTnC. The relative orientations of the N, A, and D helices of cNTnC are similar to the corresponding orientations in both apo- and 2Ca\textsuperscript{2+}-sNTnC. In this NAD frame of reference the orientation of the B and C helices of cNTnC resemble more those of the apo-form than the two-calcium form of sNTnC. This is obvious from the DDMs computed from the families of structures in order to obtain unbiased comparisons (Fig. 5). In cNTnC the B helix is closer to

![Figure 3](http://www.jbc.org/)  
**FIG. 3.** A, 15N line widths were measured at 20 (●), 30 (○), and 40 °C (●) versus residue. Residues 52 and 54 are prolines. Other missing data points are due to overlaps or extreme broadening beyond observation. B, transverse relaxation rate ($R_2$) (○) and cross-correlation rate ($r$) (●) versus residue.

![Figure 4](http://www.jbc.org/)  
**FIG. 4.** Generalized order parameter $S^2$ (●) versus residue. The order parameter at the Lipari-Szabo model-free spectral density was computed from longitudinal transverse relaxation and from heteronuclear NOE measurements.

![Figure 5](http://www.jbc.org/)  
**FIG. 5.** Difference distance matrices computed between the cNTnC and the two-calcium form (A) and apo form (B) of the skeletal troponin C. C, for the completeness the DDM is computed between the apo and two-calcium forms of the sNTnC. Distances are coded according to the color code bar on top.
hinge about which the B helix reorients with respect to the calcium binding loops. The strong chemical shifts depend on the concentration of TFE but we do not report for the avian cTnC are absent in our NOE spectra. Therefore, it is interesting to consider the possibility of an exchange between the closed form and a low population of the open state as an explanation for the line broadening. In particular because prior to the results by Sia et al. (17) a large conformational change similar to the one observed for sTnC was generally expected for cTnC. It was shown, for example, that upon a calcium titration the chemical shift of the methyl of Val-28 changes much (δm28 = 0.16). In retrospect this significant change is probably explained by small movements of the nearby phenyls, because the structures of apo- and Ca2+-cTnC are so similar (18). In the case of the mutant calmodulin two conformers were distinguished from a double set of mutually excluding NOEs. We find no clear evidence for a similar situation, which at least implies that the determined closed conformation of cTnC has a population above 80–90%. A small population of the minor conformation could still cause the rapid transverse relaxation provided that the chemical shifts for the two conformations would differ significantly for instance due to ring current effects. A study of the structures of the apo and holo states of cTnC in the presence of the binding peptide from TnI, now in progress, should settle if the presently known calcium-loaded closed conformation is truly the biologically relevant conformation of the regulatory domain of cardiac troponin C.

The time-limiting step in muscle relaxation is most likely the release of calcium from the N-terminal half of troponin C. For free troponin C and calmodulin the off-rate of the Ca2+ ions from the regulatory sites is about 500 s−1 (3). However, in the presence of a binding peptide or drug this rate is dramatically reduced. For skeletal muscle with sTnC and smooth muscle with calmodulin, even a 100-fold reduction is acceptable, but not so for heart muscle. For the heart muscle to relax to about 90% in half a second the calcium off-rate has to be about 7 s−1. Maybe the cardiac TnC has developed to its present sequence and putative structure to cope with the strict timing requirements, which can be more easily met with a somewhat weaker calcium binding.

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