Cardiac Troponin I Induced Conformational Changes in Cardiac Troponin C As Monitored by NMR Using Site-Directed Spin and Isotope Labeling†

Quinn Kleerekoper,‡ Jack W. Howarth,‡ Xiaodu Guo,§ R. John. Solaro,§ and Paul R. Rosevear*,‡

Department of Biochemistry and Molecular Biology, University of Texas Medical School-Houston, Houston, Texas 77225, and Department of Physiology and Biophysics, College of Medicine, University of Illinois-Chicago, Chicago, Illinois 60612-7342.

Received May 5, 1995; Revised Manuscript Received August 14, 1995⊗

ABSTRACT: Conformational changes in both free cardiac troponin C (cTnC) and in complex with a recombinant troponin I protein [cTnI(33−211), cTnI(33−80), or cTnI (86−211)] were observed by means of a combination of selective carbon-13 and spin labeling. The paramagnetic effect from the nitroxide spin label, MTSSL, attached to cTnC(C35S) at Cys 84 allowed measurement of the relative distances to the ^{13}C-methyl groups of the 10 methionines of cTnC in the monomer or complex. All 10 ^{1}H−^{13}C correlations in the heteronuclear single- and multiple-quantum coherence (HSMQC) spectrum of [^{13}C-*methyl*]Met cTnC in the complex with cTnI(33−211) were previously assigned [Krudy, G. A., Kleerekoper, Q., Guo, X., Howarth, J. W., Solaro, R. J., & Rosevear, P. R. (1994) *J. Biol. Chem. 269*, 23731−23735]. In the presence of oxidized spin label, nine of the 10 Met methyl ^{1}H−^{13}C correlations of cTnC were significantly broadened in the cTnC(C35S) monomer. This suggests flexibility within the central helix, or interdomain D/E helical linker, bringing the N- and C-terminal domains in closer proximity than predicted from the crystallographic structure of TnC. In the spin-labeled cTnC(C35S)·cTnI(33−211) complex only N-terminal Met methyl ^{1}H−^{13}C correlations of cTnC(C35S) were paramagnetically broadened beyond detection, whereas correlations for Met residues (103, 120, 137, and 157) in the C-terminal domain were not. Thus, complex formation with cTnI decreases interdomain flexibility and maintains cTnC in an extended conformation. This agrees with the recently published study suggesting that sTnC is extended when bound to sTnI [Olah, G. A., & Trewhella, J. (1994) *Biochemistry 33*, 12800−12806]. The recombinant N-terminal domain of cTnI, cTnI(33−80), gave similar results as observed with cTnI(33−211) when complexed with spin-labeled cTnC(C35S). However, complex formation with the C-terminal fragment, cTnI(86−211), which contains the inhibitory sequence, is insufficient to maintain cTnC extended to the amount observed with either cTnI(33−211) or cTnI(33−80); although compared to that observed in free cTnC, it does cause decreased flexibility in the interdomain linker. In the absence of the N-terminal domain of cTnI, there is a decrease in flexibility within the N-terminal domain of cTnC. Interestingly, the N-terminal domain of cTnC in the reduced spin-labeled complex with cTnI(86−211), in the presence of ascorbate, showed two distinct conformations which were not seen in the complex with cTnI(33−211). Taken together, this suggests that both domains of cTnI modulate the flexibility within the interdomain linker of cTnC and may play important roles in the response of the cardiac contractile apparatus to a variety of external stimuli.

The troponin complex plays the major role in regulating excitation−contraction coupling in cardiac and skeletal muscle (Ebashi & Kodama, 1966). Within the complex, there are three subunits, troponin C (TnC),[1] troponin I (TnI), and troponin T (TnT), each of which contributes to the regulation (Zot & Potter, 1987). TnC undergoes a Ca^{2+}-dependent conformational change which modulates interactions with TnI and TnT. TnI inhibits actomyosin ATPase activity, while TnT, which is also required for this inhibition, binds the complex to tropomyosin. The interaction of the cardiac isoforms of TnI and TnC is the subject of this study.

From the crystallographic structures of skeletal TnC, cTnC, is predicted to be a dumbbell shaped molecule with two globular domains separated by a long central helix, or interdomain D/E linker, of eight to nine turns (Herzberg & James, 1988; Satyshur et al., 1988). Solution studies of Ca^{2+}-saturated sTnC suggest flexibility between helix D and helix E (Slupsky et al., 1995). The N-terminal domain contains

† This work supported by NIH Grants HL-22231 and HL49934 (to R.J.S.), by American Heart Association AHA92015340 (to P.R.R.), and by a fellowship from the American Heart Association of Metropolitan Chicago (to X.G.).

* Author to whom correspondence should be addressed at Department of Biochemistry, Molecular Genetics and Microbiology, University of Cincinnati Medical School, P.O. Box 670524, Cincinnati, OH 45267-0524.

‡ University of Texas Medical School−Houston.

§ University of Illinois−Chicago.

⊗ Abstract published in *Advance ACS Abstracts,* October 1, 1995.

[1] Abbreviations: TnC, cardiac or fast skeletal troponin C; cTnC, cardiac TnC; sTnC, skeletal TnC; sTnC·sTnI, binary complex formed between sTnC and sTnI; cTnC·cTnI, binary complex formed between cTnI and cTnC; cTnC3, recombinant cTnC(desM1,D2A); [^{13}C]cTnC-(C35S), [^{13}C-*methyl*]Met labeled cTnC(desM1,D2A,C35S); [SL-^{13}C]-cTnC(C35S), [^{13}C-*methyl*]Met cTnC(desM1,D2A,C35S) labeled at Cys 84 with the sulfhydryl specific spin label MTSSL, cTnI, cardiac troponin I; cTnI(33−211), recombinant cardiac TnI(desM1-T32) lacking the cardiac specific NH_2-terminus; cTnI(33−80), recombinant N-terminal domain of cardiac troponin I corresponding to residues 33−80; cTnI-(86−211), recombinant C-terminal fragment of cardiac troponin I corresponding to residues 86−211; DTT, 1,4-dithiothreitol; HSMQC, heteronuclear single- and multiple-quantum coherence; MTSSL, 1-oxyl-2,2,5,5-tetramethyl-Δ^3-pyrroline-3-methyl)methanethiosulfonate spin label; PMSF, phenylmethanesulfonyl fluoride; SDSL, site-directed spin labeling; Tris-d_{11}, deuterated tris(hydroxymethyl)aminomethane; IAANS, 2-(4′-iodoacetamidoanilino) naphthalene-6-sulfonic acid.

0006-2960/95/0434-13343$09.00/0

Ca^{2+}-binding sites I/II, while the C-terminal domain contains Ca^{2+}/Mg^{2+}-binding sites III/IV. Cardiac TnC differs significantly from skeletal troponin C (sTnC) in that the first Ca^{2+}-binding site, site I, is inactivated by several critical amino acid substitutions (Collins et al., 1977; VanEerd & Takahashi, 1976). Site II of cTnC is the Ca^{2+}-binding site responsible for regulating muscle contraction (Holroyde et al., 1980; Putkey et al., 1989; Robertson et al., 1981; Sweeney et al., 1990). Modeling studies of the N-terminal domain of sTnC, based on the crystal structure of sTnC, suggest that Ca^{2+} binding to the regulatory sites increases exposure of a hydrophobic pocket which results in an enhanced interaction with TnI (Herzberg et al., 1986). In cTnC, the movement of helices A/D with respect to helices B/C upon Ca^{2+} binding to site II is consistent with this model (Brito et al., 1991). Nuclear magnetic resonance (NMR) studies on cTnC show that only Met 45 and Met 81, located in the proposed hydrophobic pocket, undergo a measurable increase in solvent accessibility upon binding Ca^{2+} at site II (Howarth et al., 1995). No changes are seen for the methionines located in the C-terminal domain. Unlike the N-terminal Ca^{2+}-binding sites I/II, the high affinity Ca^{2+}/Mg^{2+} sites III and IV in the C-terminal domain are largely responsible for maintaining the stability of the interaction between cTnC and cTnI within the troponin complex (Cox et al., 1981; Negele et al., 1992; Zot & Potter, 1982).

Reconstitution studies with the skeletal or cardiac isoforms of TnC and TnI show the formation of a binary complex with multiple contacts between TnI and TnC, which involve segments located some distance from each other in the primary sequences (Byers & Kay, 1982; Farah et al., 1994; McCubbin et al., 1974; Sheng et al., 1992; Syska et al., 1976). At least three interaction sites between TnI and TnC are postulated: (1) a metal-independent structural site; (2) a Ca^{2+}/Mg^{2+}-dependent site; (3) a Ca^{2+}-specific binding site (Farah et al., 1994; Sheng et al., 1992; Syska et al., 1976). A combination of biochemical and biophysical studies demonstrate that both skeletal and cardiac TnC·TnI proteins bind in a head to tail or antiparallel fashion (Sheng et al., 1992; Farah et al., 1994; Krudy et al., 1994).

The nature of the structure of the sTnC·sTnI complex in solution has recently been examined by small angle X-ray scattering (SAXS) and neutron scattering. The data on sTnC·sTnI are interpreted in terms of an extended "dumbbell-like" structure for sTnC with sTnI winding around sTnC and making contact with the proposed hydrophobic patches in both the N- and C-terminal domains (Olah et al., 1994; Olah & Trewhella, 1994). Such a model is consistent with the known antiparallel arrangement in the TnC·TnI complex. The sTnC·sTnI model is in marked contrast to the known structure of calmodulin when bound to a 26-residue MLCK target peptide from myosin light chain kinase. Calcium-saturated calmodulin wraps around the peptide to allow both the N- and C-terminal hydrophobic faces to interact with the amphipathic MLCK α-helix (Ikura et al., 1992; Meador et al., 1992). This requires the observed crystallographic central helix to unravel and wrap around the peptide substrate (Ikura et al., 1992).

To investigate structural changes in cTnC in both the free protein or in complex with a cTnI protein, we have utilized site-directed spin labeling (SDSL) to measure relative distances from a covalently bound nitroxide spin label at Cys 84 of cTnC to selectively isotopically labeled amino acids in the protein. Using the methyl resonances of the 10 methionines of cTnC as spectral probes, we can detect these changes by NMR. Recently, the assignments for the 10 methionines in cTnC have allowed the following: detection of Ca^{2+}-induced conformational changes in the free protein; measurement of solvent accessibility of the methionine residues in cTnC in the presence and absence of Ca^{2+} as well as in the presence of the troponin I inhibitory peptide; and the orientation of cTnC and cTnI in the Ca^{2+} saturated cTnC·cTnI complex (Howarth et al., 1995; Krudy et al., 1994; Lin et al., 1994). In this study, we used SDSL in combination with carbon-13 labeling of the methyl groups of the 10 methionines to detect flexibility in the central helix of cTnC in addition to conformational changes induced in cTnC when bound with a recombinant cTnI protein: cTnI-(33−211), which lacks the 32 amino acid cardiac specific NH_2-terminus (Guo et al., 1994); cTnI(33−80), a N-terminal domain of cTnI, cTnI(33−211); and a C-terminal fragment of cTnI, cTnI(86−211).

EXPERIMENTAL PROCEDURES

Materials. L-[*methyl*-^{13}C]Methionine, deuterium oxide, and Tris-d_{11} were obtained from Cambridge Isotope Laboratories. The spin label (MTSSL), 1-oxyl-2,2,5,5-tetramethyl-Δ^3-pyrroline-3-methyl)methanethiosulfonate, was obtained from Reanal (Budapest, Hungary). All other chemicals were of the highest purity available commercially.

Recombinant Proteins. To place the expression of the cTnC3(C35S) protein (Lin et al., 1994; Putkey et al., 1993) under T7 promoter control, the *Nco*I/*Hin*dIII fragment containing cTnC3(C35S) from pTnCP$_L$3(C35S) was ligated into *Nco*I/*Hin*dIII digested pET3d vector (Novagen). The protein was overexpressed in *Escherichia coli* BL21(DE3) (Novagen). To obtain [*methyl*-^{13}C]Met-labeled cTnC3-(C35S), bacteria were grown in an enriched defined medium (Krudy et al., 1994) supplemented with 0.25 g of [*methyl*-^{13}C]Met/L and 50 mg/L ampicillin. Routinely, 1 L of cells were grown at 37 °C in Luria Broth until the absorbance at 600 nm reached 0.6. Approximately 20 mL from this culture was used to innoculate each liter of enriched defined medium, and the cells were incubated at 37 °C until the absorbance at 600 nm was between 0.8 and 1.0. The culture was then induced with 0.4 mM IPTG. Cells were harvested 4 h after induction, collected by centrifugation, and stored at −70 °C until use. The [*methyl*-^{13}C]Met-labeled protein was purified to apparent homogeneity as described previously (Lin et al., 1994). The overexpression and purification of rat cTnI(33−211), previously designated cTnI/NH_2, has been described (Krudy et al., 1994).

Construction, Overexpression, and Purification of cTnI-(33−80) and cTnI86−211). The polymerase chain reaction (PCR) was used to generate DNA fragments encoding amino acids 33−80 and 86−211 of cTnI(33−211) from cTnI. For cTnI(33−80), the 5′ PCR primer (5′-CTGGGTTGAAG-GCTCTC-AAGGGC-3′) annealed at a position within the pET9d vector of pcTnI/NH_2 (Guo et al., 1994) approximately 410 bp upstream from the rat cTnI/NH_2 start codon. The 3′ primer (5′-CCGACGCGGATCTTATCAACGCGTG CT-CAGAAC-GCGGCC-3′) corresponded to amino acids 73−80 and also encoded two stop codons upstream of a *Bam*HI site.

To generate cTnI(86−211), a 5′ amplification primer (5′-GCCAGCCTTTGGCCA TGGCTGGCCTGGGCTTTGAA-

GAACTTC-3′) was designed to amplify the mouse cTnI cDNA sequence from the 86th amino acid (Leu) to the 3′ end of the coding sequence. (The mouse amino acid sequence for 86−211 is identical to the rat sequence.) Leu^{86} was converted to methionine, the first codon of the truncated cTnI(86−211) incorporating a *Nco*I restriction site. To maximize expression of cTnI(86−211), amino acid 87 was changed to Ala. The 3′ primer (5′-GTTATTGCTCAGCG-GTGG-3′) was complementary to pET3d vector (Novagen, downstream to the *Bam*HI cloning site), in which the mouse cTnI cDNA was cloned.

PCR reactions were carried out at 47 °C for annealing, 74 °C for extension, and 94 °C for denaturation; 30 cycles were completed. PCR fragments were gel purified, digested with *Nco*I and *Bam*HI, and ligated into *Nco*I/*Bam*HI digested pET3d expression vector, resulting in the plasmids pcTnI-(33−80) and pcTnI(86−211). Constructs were transformed into *E. coli* (DH5α). The nucleotide sequences were confirmed by the dideoxy chain termination method, and the constructs were transformed into BL21(DE3) for overexpression. Expression of cTnI(33−80) and cTnI(86−211) was tested by growing LB cultures with a 1:10 000 dilution of the frozen stock containing 200 μg/mL ampicillin (Guo et al., 1994). Cells were induced with 0.4 mM IPTG at an absorbance at 600 nm of between 0.8 and 1.0 and grown overnight. Pellets harvested after centrifugation of the overnight cultures were lysed in 2× SDS−polyacrylamide gel sample buffer and run on a 15% SDS−polyacrylamide gel. Recombinant cTnI(33−80) and cTnI(86−211) proteins were purified essentially as previously reported for cTnI-(33−211) (Krudy et al., 1994).

Spin Labeling of cTnC(C35S). The [*methyl*-^{13}C]Met-labeled cTnC(C35S) was extensively dialyzed against 20 mM Pipes, 200 mM KCl, and 10 mM DTT at pH 7.0. After complete reduction of Cys 84, the protein was concentrated by Amicon filtration and loaded onto a Pharmacia Biotech Sephacryl S-100 HR column equilibrated in 20 mM Pipes and 200 mM KCl at pH 7.0 to remove the excess DTT. For preparation of [SL-^{13}C]cTnC(C35S), fractions devoid of DTT were pooled, and freshly dissolved MTSSL in 100 μL of HPLC grade acetone was added to the protein solution in a 5:1 excess of spin label to protein. After 48 h at room temperature, the MTSSL-protein was concentrated and loaded onto the Pharmacia Biotech Sephacryl S-100 HR column equilibrated in 20 mM Pipes and 200 mM KCl at pH 7.0 to remove excess MTSSL. Spin-labeled protein fractions were pooled and concentrated. For NMR experiments, the MTSSL labeled [*methyl*-^{13}C]cTnC(C35S), [SL-^{13}C]cTnC(C35S) samples were extensively washed with 20 mM Tris-d_{11} and 200 mM KCl at pH 7.5 using an Amicon filtration cell containing a PM-10 membrane. For spin label studies on free [SL-^{13}C] cTnC(C35S), the protein was washed with 20 mM Tris-d_{11} and 200 mM KCl in ^2H_2O at pH 7.5. At the end of the NMR experiments, the nitroxide spin label was reduced using a 5-fold excess of L-ascorbic acid added directly to the NMR protein sample.

Cardiac Troponin C Complex Formation with Recombinant cTnI(33−211), cTnI(33−80), and cTnI(86211). Complex formation between [^{13}C]cTnC(C35S) or [SL-^{13}C]cTnC-(C35S) and recombinant cTnI proteins was performed as previously described (Krudy et al., 1994). All spin-labeled complexes were made in the absence of DTT. A Bradford assay (Bradford, 1976) on each complex showed that both, cTnC and cTnI, were present in approximately equimolar amounts assuming minimal loss of cTnC during complex formation. Complexes were visualized before and after NMR experiments on both 15% SDS and native polyacrylamide gels by staining with Coomassie Brilliant Blue.

NMR Methods. Heteronuclear single- and multiple-quantum coherence (HSMQC) (Zuiderweg, 1990) spectra of [^{13}C]cTnC(C35S) or [SL-^{13}C]cTnC(C35S) either free or in complex with a recombinant cTnI protein were typically collected with 1024 complex data points in the t_2 domain and 300 increments in t_1 at 40 °C. The ^{1}H and ^{13}C spectral widths were 5556 and 2000 Hz, respectively. The water resonance was suppressed by continuous irradiation during the relaxation delay. HSMQC spectra were processed with a 60°-shifted sine-bell squared function and zero-filled to 1024 points in both t_1 and t_2. ^{1}H and ^{13}C chemical shifts were reported relative to the HDO signal at 4.563 ppm and the [^{13}C-*methyl*]Met signal at 14.86 ppm, respectively. All spectra were processed using the FELIX 2.0 software package (BioSym). The ^{1}H and ^{13}C assignments for the methionine methyl groups in [^{13}C]cTnC(C35S) or [SL-^{13}C]-cTnC(C35S) were assigned by comparison of HSMQC spectra of a set of [^{13}C-*methyl*]Met-labeled recombinant proteins having known Met residues mutated to Leu (Lin et al., 1994). Also, all 10 assignments for [^{13}C]cTnC(C35S) when complexed with cTnI (33−211) have previously been reported (Krudy et al., 1994).

RESULTS AND DISCUSSION

As part of a general program to understand Ca^{2+}-dependent protein−protein interactions regulating contraction in cardiac muscle, we have initiated NMR studies on the cardiac TnC·TnI complex. However, obtaining structural information has been made difficult by the following five factors: the large molecular mass of this complex (40 kDa), the predicted high percentage of α-helical structure, the lack of structural information on cTnI, the known insolubility of cTnI, and the presence of four EF-hand Ca^{2+} binding motifs in cTnC. By using selective isotope labeling and isotope-edited NMR techniques, we have previously shown that individual Met methyl groups in both cTnC and cTnI(33−211) can be used to monitor conformational changes in both proteins and thereby define their relative orientation (Krudy et al., 1994).

Spin Labeling of cTnC(C35S). Site-directed spin labeling can be used to further define spatial relationships within the complex. By covalently attaching a small thiol specific nitroxide spin label, MTSSL, to Cys 84 of cTnC(C35S), distances from the paramagnetic probe to ^{13}C-enriched methionine residues in the cTnC·TnI complex can be monitored qualitatively. Introduction of a paramagnetic spin label will result in line broadening of ^{1}H−^{13}C correlations for those residues within ~15 Å from the paramagnetic center of MTSSL because of the influence of the large magnetic moment of the unpaired electron on the longitudinal and transverse relaxation rates of the ^{1}H and ^{13}C nuclei. This influence varies as r^{-6} from the oxidized spin label to the observed nuclei. In addition, line broadening is also influenced by both overall tumbling and local motions within the protein (Solomon & Bloembergen, 1956). Model building with MTSSL attached to Cys 84 of cTnC, based on our model of cTnC (Brito et al., 1991), suggests that all four

Table 1: Comparison of Proton and Carbon Methionine Methyl Chemical Shifts in Ca^{2+}-Saturated [^{13}C]cTnC(C35S) and [SL-^{13}C]cTnC(C35S) Free in Solution[a]

methionine residue	[^{13}C]cTnC(C35S)[b]		[SL-^{13}C]cTnC(C35S) oxidized		[SL-^{13}C]cTnC(C35S) reduced[c]		chemical shift differences[d]	
	^{1}H ppm	^{13}C ppm	^{1}H ppm	^{13}C ppm	^{1}H ppm	^{13}C ppm	^{1}H ppm	^{13}C ppm
45	1.83	16.6	–[e]	–	1.87	16.4	0.04	nc
47	2.20	15.5	–[e]	–	2.13	15.4	−0.07	nc
60	1.91	15.5	–	–	1.96	15.7	0.05	nc
80	1.80	16.4	–	–	1.78	15.9	nc	−0.5
81	1.35	15.5	–	–	1.41	16.6	0.06	1.1
85	2.09	15.1	–	–	2.07	15.2	nc	nc
103	1.90	15.0	–	–	1.89	14.9	nc	nc
120	1.87	15.8	(1.85	15.7)[f]	1.85	15.7	nc	nc
137	1.90	15.6	1.90	15.6	1.90	15.6	nc	nc
157	1.80	14.4	(1.79	14.3)[g]	1.79	14.3	nc	nc

[a] Proton chemical shifts are reported relative to ^{2}H^{1}HO signal at 4.563 ppm and are accurate to ±0.01 ppm. Carbon chemical shifts are reported relative to [*methyl*-^{13}C]methionine at 14.86 ppm and are accurate to ±0.1ppm. The spectra were acquired at 40 °C as described under Experimental Procedures. [b] Chemical shifts taken from Lin et al. (1994). [c] Sample reduced with 5 equiv of ascorbic acid. [d] Chemical shift differences between the reduced [SL-^{13}C]cTnC(C35S) and [^{13}C]cTnC(C35S) are derived by subtraction of the chemical shift values for [^{13}C]cTnC(C35S) from the chemical shift values of the reduced [SL-^{13}C]cTnC(C35S). The accuracy of these values is ±0.02 for ^{1}H and ±0.2 for ^{13}C. No significant change is indicated by "nc." [e] Met methyl ^{1}H−^{13}C correlation broadened by nitroxide probe beyond detection. [f] The Met methyl ^{1}H−^{13}C correlation only observed at a 4-fold lower contour level. [g] The Met methyl ^{1}H−^{13}C correlation only observed at a 8-fold lower contour level.

C-terminal Met methyl groups are at least 25 Å from the paramagnetic probe, if we assume that the central helix is extended as observed in the crystal structure of sTnC (Herzberg & James, 1988; Satyshur et al., 1988). In contrast, the distance between the N-terminal Met methyl groups and the paramagnetic probe can range between 2.5 and 20 Å depending on the precise position of the oxidized spin label at Cys 84.

Previous studies using a large flourescent group, IAANS, attached to Cys 35 and/or Cys 84 have demonstrated that covalent labeling at either position does not significantly affect the biological activity of cTnC when assayed for cross-bridge formation (Hannon et al., 1992).

Spin Label Studies on Free cTnC(C35S). Comparison of the Met methyl ^{1}H−^{13}C correlations of [^{13}C]cTnC(C35S) with those of the reduced [SL-^{13}C]cTnC(C35S) suggests that the presence of the spin label bound to Cys 84 has little effect on the overall tertiary structure of the protein (Table 1). Significant ^{1}H and ^{13}C chemical shift differences were observed only for Met 81, which is located on the same face of the helix as the attached spin label at Cys 84 (Table 1). Met 80 showed the only other ^{13}C chemical shift change (Table 1). Small ^{1}H chemical shift changes were observed for Met 45, 47, and 60, located in the N-terminal domain of cTnC. While small changes are detected between the N-terminal domains of the proteins, no chemical shift differences were observed between their C-terminal domains.

Figure 1A−C shows the paramagnetic effects on the Met methyl ^{1}H−^{13}C correlations in 2Ca^{2+}-loaded [SL-^{13}C]cTnC(C35S) at pH 7.5. Under these conditions, the low affinity regulatory site II in the N-terminal domain does not contain bound Ca^{2+}. Of the 10 ^{1}H−^{13}C correlations, nine are significantly broadened as indicated by a loss of intensity in the HSMQC spectrum (Table 1). Figure 1A shows that Met

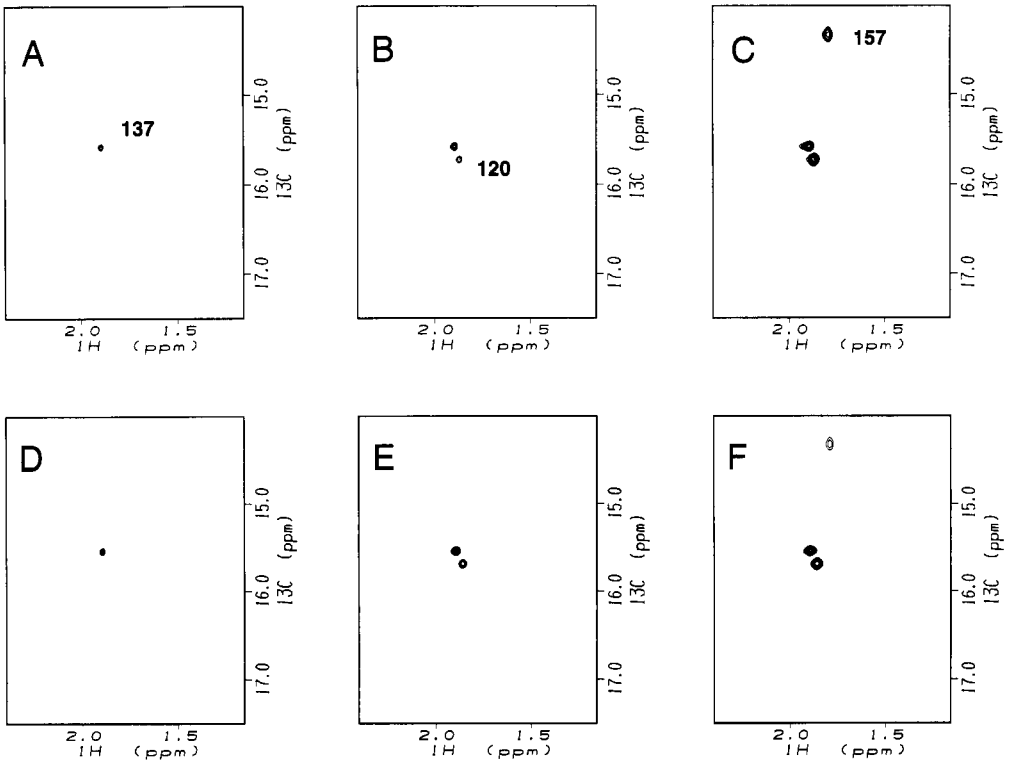

FIGURE 1: Two-dimensional HSMQC spectrum of 2Ca^{2+}-loaded [SL-^{13}C]cTnC(C35S) (A−C) and Ca^{2+}-saturated [SL-^{13}C]cTnC(C35S) (D−F). (A−C) Sample contained 1 mM [^{13}C]cTnC(C35S) and 2 mM Ca^{2+} in 20 mM Tris-d_{11}, 200 mM KCl, and 0.1 mM PMSF in $^{2}H_2O$ at pH 7.5. Spectrum taken at a (B) 4-fold and (C) 8-fold lower contour level. (D−F) Sample contained 1 mM [^{13}C]cTnC(C35S) and 4 mM Ca^{2+} in 20 mM Tris-d_{11}, 200 mM KCl, 0.1 mM PMSF, and $^{2}H_2O$ at pH 7.5. Spectrum taken at (E) 2-fold and (F) 6-fold lower contour level. Spectra were obtained at 500 MHz and 40 °C as described under Experimental Procedures.

137 is the only correlation observable, at a contour level identical to that used for observation of all 10 Met methyl $^1H-^{13}C$ correlations. Assuming an extended structure for cTnC, the methyl group of Met 137 is at the greatest distance, 32−41 Å , from the oxidized probe. Figure 1 panels B and C show that the paramagnetically broadened $^1H-^{13}C$ correlations for Met 120 and 157 could be observed at a 4- and 8-fold lower contour level, respectively (Table 1). The $^1H-^{13}C$ correlation for Met 103 was not observed at any contour level above the noise floor. Line broadening of $^1H-^{13}C$ correlations in both domains demonstrates that the central helix has considerable flexibility such that the N- and C-terminal domains can be brought into closer proximity than predicted by the crystal structures. Thus, the C-terminal Met methyl residues of 103, 120, and 157 are within sufficient distance, <15 Å, to be broadened by the oxidized probe. These data are consistent with the suggestion by Herzberg and James (1985) that the central helix, or interdomain D/E linker, is flexible in solution and, in cTnC, may bend around Gly 91. The SAXS study on Mg^{2+}-saturated sTnC is also consistent with a bent structure for sTnC (Heidorn & Trewella, 1988). Both SAXS data on sTnC and our paramagnetic studies on $2Ca^{2+}$-loaded cTnC reflect an "average conformation" in which considerable variability in the distances between the two domains is likely to exist. In addition, fluorescence energy transfer studies on sTnC at neutral pH also suggest a more compact structure than observed in the crystal (Wang & Leavis, 1990). Since multiple bending modes of the interdomain linker likely exist, a quantitative interpretation of the data in terms of distance measurements is not possible. However, we can correlate distances qualitatively from the C-terminal methionine residues to the probe as a result of line broadening. On the basis of the intensity of $^1H-^{13}C$ correlations, Met 103 was found to be the closest to the attached spin probe, followed by Met 157, 120, and 137 in $2Ca^{2+}$-loaded [SL-^{13}C]cTnC-(C35S) (Figure 1A−C; Table 1). The same qualitative order of distances from MTSSL to the C-terminal Met residues in Ca^{2+}-saturated [SL-^{13}C]cTnC(C35S) was also observed (Figure 1D−F; Table 1). These studies clearly demonstrate that the central helix is flexible in solution and that this flexibility can be detected using SDSL.

We also found no significant change in the paramagnetic effects on the Met methyl $^1H-^{13}C$ correlations when the pH was lowered to 5.8, which indicates that similar flexibility within the central helix exists between pH values 7.5 and 5.8. At pH values below 5.8, protein aggregation was observed. SAXS studies with sTnC and calmodulin at low pH were also prevented by protein aggregation in the presence of Ca^{2+} (Heidorn et al., 1988; Blechner et al., 1992).

Cardiac Troponin C(C35S) Complex Formation with Recombinant cTnI(33−211), cTnI(33−80), and cTnI(86−211). Analysis of HSMQC spectra of [^{13}C]cTnC(C35S) in complex with cTnI(33−211), cTnI(33−80), or cTnI(86−211) permitted assignment of the 10 $^1H-^{13}C$ correlations in cTnC. Table 2 lists ^1H and ^{13}C chemical shift differences between Ca^{2+}-saturated [^{13}C]cTnC(C35S) monomer or in complex with each cTnI protein. In the presence of cTnI(33−211), lacking the 32 amino acid cardiac specific NH_2-terminus, only chemical shifts of the $^1H-^{13}C$ correlations for Met 81, 120, and 157 are significantly altered. The recombinant N-terminal domain of cTnI, cTnI(33−80), was found to shift the $^1H-^{13}C$ correlations for Met 120 and 157 to the same extent as observed in intact cTnI(33−211). This is not surprising since both Met 120 and 157 are located on the same face in the C-terminal hydrophobic pocket of cTnC. Only a very minor effect on the ^1H chemical shift of Met 81 was observed (Table 2). These results further confirm the antiparallel arrangement of cTnC and cTnI in the complex.

Table 2: Proton and Carbon Methionine Methyl Chemical Shift Differences between Free [^{13}C]cTnC(C35S) and [^{13}C]cTnC(C35S) Complexed with cTnI Proteins[a]

methionine residue	[^{13}C]cTnC(C35S)·cTnI(33−211)		[^{13}C]cTnC(C35S)·cTnI(33−80)		[^{13}C]cTnC(C35S)·cTnI(86−211)	
	^1H ppm	^{13}C ppm	^1H ppm	^{13}C ppm	^1H ppm	^{13}C ppm
45	nc	nc[b]	nc	nc	nc	−0.03
47	nc	nc	nc	nc	−0.1	nc
60	nc	nc	nc	nc	nc	nc
80	−0.03	nc	nc	nc	−0.03	−0.4
81	0.49	0.7	0.03	nc	(0.06	0.7)[c]
85	−0.03	nc	nc	nc	nc	nc
103	−0.03	nc	−0.03	nc	−0.03	nc
120	0.11	0.9	0.12	0.9	−0.13	−0.5
137	nc	nc	nc	0.3	nc	nc
157	−0.74	−0.8	−0.74	−0.8	−0.05	nc

[a] Chemical shift differences are derived by subtraction of the chemical shift values of free [^{13}C]cTnC(C35S) from the chemical shift values for the various [^{13}C]cTnC(C35S) complexes with cTnI proteins. The accuracy of these values is ±0.02 ppm for ^1H and ±0.2 ppm for ^{13}C. [b] No significant change is indicated by "nc." [c] The Met methyl 81 $^1H-^{13}C$ correlation was found to be significantly less intense than the other $^1H-^{13}C$ correlations in this complex.

We also prepared the Ca^{2+}-saturated [^{13}C]cTnC(C35S)·cTnI-(86−211) complex using recombinant cTnI(86−211). This resulted in chemical shift changes for all methionines except 60 and 137 (Table 2). However, Met 157 showed a slight ^1H chemical shift of 0.05 ppm. The ^1H chemical shift of Met 47 was found to be shifted 0.10 ppm. Both ^1H and ^{13}C chemical shifts were observed for Met 80, 81, and 120. Chemical shift changes in both Met 80 and Met 81 require regions of cTnI between 86 and 211, although the magnitude of the chemical shift depends on the complex formed. Interestingly, the $^1H-^{13}C$ correlation for Met 120 was found to shift upfield in the complex, whereas a downfield chemical shift was observed for this residue in complexes with cTnI-(33−211) and cTnI(33−80) (Table 2). In contrast, the $^1H-^{13}C$ correlation for Met 157 changes significantly only upon complex formation with cTnI(33−211) or cTnI(33−80) (Table 2). The sensitivity of Met 120 to regions of cTnI between 86 and 211 as well as to cTnI(33−80) might be expected since cTnI(86−211) contains the well known inhibitory region. NMR studies have demonstrated that the inhibitory peptide derived from cTnI binds to the C-terminal domain of cTnC and protects both Met 120 and 157 from solvent (Howarth et al., 1995). Studies have suggested that the inhibitory region of TnI interacts with both the N- and C-terminal regions of cTnC (Farah et al., 1994; Kobayashi et al., 1994; Nagi & Hodges, 1992) and that the N-terminal domain of TnI may function as a negative effector of the inhibitory region binding to the C-terminal domain of cTnC (Nagi & Hodges, 1992).

The availability of methionine assignments in [^{13}C]cTnC-(C35S) complexed with cTnI(33−211), cTnI(33−80), or cTnI(86−211) permits the use of SDSL and isotope labeling to monitor conformational changes occurring in cTnC upon complex formation with the various cTnI proteins.

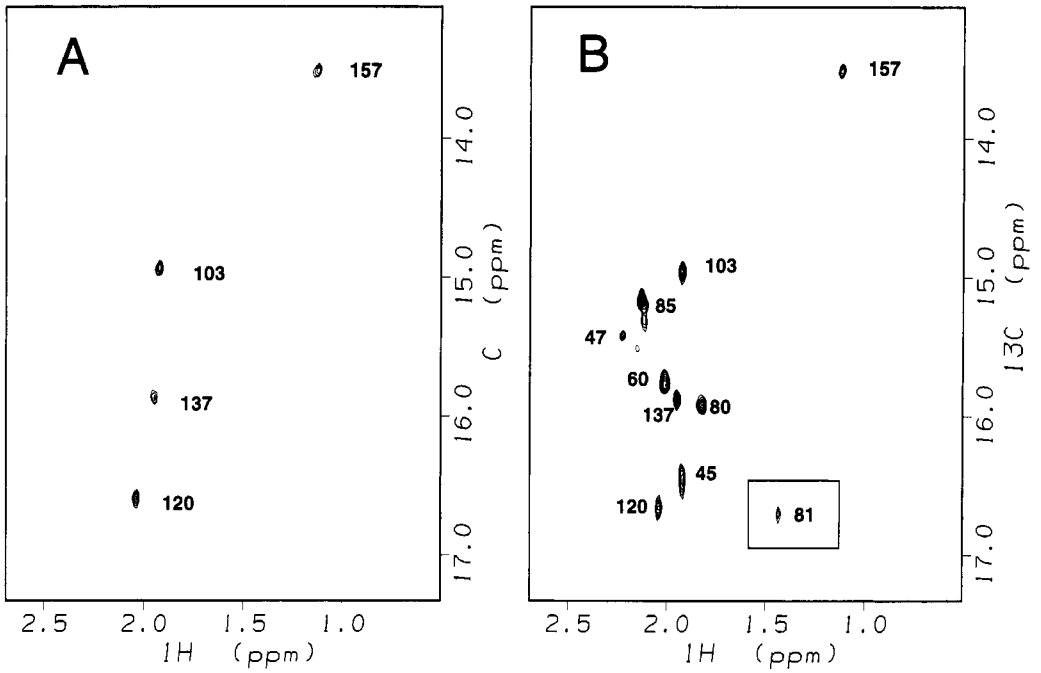

FIGURE 2: Methionine methyl region of the HSMQC spectrum of the Ca^{2+}-saturated [SL-^{13}C]cTnC(C35S)·cTnI(33−211) complex (A) and the ascorbate reduced Ca^{2+}-saturated [SL-^{13}C]cTnC(C35S)·cTnI(33−211) complex (B). Samples were approximately 1.5 mM [SL-^{13}C]-cTnC(C35S)·cTnI(33−211) complex and 6 mM Ca^{2+} in 20 mM Tris-d_{11}, 200 mM KCl, and 0.1 mM PMSF in ^2H_2O at pH 7.5. The nitroxide radical was reduced to the hydroxylamine with 5 equiv of ascorbate. The ^{1}H−^{13}C Met methyl correlation for 81, shown in the box, was only observable at a 2-fold lower contour level. Sample conditions were identical to those described in panel A. Spectra were obtained at 500 MHz and 40 °C as described under Experimental Procedures.

Site-Directed Spin and Isotope Labeling for Monitoring Conformational Changes in cTnC upon Complex Formation with Recombinant cTnC(33−211). Figure 2 shows the HSMQC spectrum of the Ca^{2+}-saturated [SL-^{13}C]cTnC·cTnI-(33−211) complex. In the presence of cTnI(33−211), four of the 10 Met methyl ^{1}H−^{13}C correlations were observed (Figure 2A). The ^{1}H−^{13}C correlations are assigned to Met 103, 120, 137, and 157, by comparing the known ^{1}H-^{13}C correlations for [^{13}C]cTnC(C35S)·cTnI(33−211) with the spectrum of the ascorbate reduced complex (Figure 2B and Table 3). These methionine residues are all located in the C-terminal domain of cTnC. In contrast, all of the N-terminal Met methyl ^{1}H−^{13}C correlations were paramagnetically broadened beyond detection. Upon reduction of MTSSL with ascorbate, all 10 ^{1}H−^{13}C correlations in the complex were detected (Figure 2B). Chemical shift changes in both Met 80 and 81 were detected in the reduced Ca^{2+}-saturated spin-labeled complex when compared to the Ca^{2+}-saturated complex (Table 3). These differences result from the spin probe, since minor chemical shift differences are also observed for these methionines in reduced free [SL-^{13}C]cTnC when compared to [^{13}C]cTnC (Table 1). Methionine 81 remains broad, observed only at a 2-fold lower contour level, in the spectrum of the reduced complex (Figure 2B). Chemical shift comparisons demonstrate that covalent attachment of the small nitroxide spin label, MTSSL, produces only local chemical shift changes near the site of attachment and does not significantly effect the overall tertiary structure of the cTnC·cTnI complex (Table 3). These observed changes most likely result from electronic effects or direct interaction with the spin label in the complex.

A combination of SDSL and selective isotope labeling of cTnC(C35S) demonstrates that complex formation with cTnI-(33−211) decreases flexibility of the central helix or inter-

Table 3: Proton and Carbon Methionine Methyl Chemical Shifts for Ca^{2+}-Saturated [^{13}C]cTnC(C35S)·cTnI(33−211) and [SL-^{13}C]cTnC(C35S)·cTnI(33−211)[a]

methionine residue	[^{13}C]cTnC·cTnI(33−211)[b]		[SL-^{13}C]cTnC(C35S)·cTnI(33−211) oxidized		[SL-^{13}C]cTnC(C35S)·cTnI(33−211) reduced		chemical shift differences Δ[c]	
	^{1}H ppm	^{13}C ppm	^{1}H ppm	^{13}C ppm	^{1}H ppm	^{13}C ppm	^{1}H ppm	^{13}C ppm
45	1.83	16.4	−[d]	−	1.91	16.3	0.08	nc
47	2.18	15.5	−	−	2.19	15.4	nc	nc
60	1.92	15.6	−	−	2.00	15.7	0.08	nc
80	1.77	16.2	−	−	1.83	15.9	0.06	−0.3
81	1.84	16.2	−	−	1.45[e]	16.7[e]	−0.39	0.5
85	2.06	15.2	−	−	2.08	15.3	nc	nc
103	1.87	15.0	1.90	14.9	1.90	14.9	0.03	nc
120	1.98	16.7	2.01	16.6	2.00	16.6	nc	nc
137	1.90	15.7	1.92	15.9	1.92	15.9	nc	nc
157	1.06	13.6	1.07	13.4	1.07	13.4	nc	nc

[a] Proton chemical shifts are reported relative to ^{2}H^{1}HO signal at 4.563 ppm and are accurate to ±0.01 ppm. Carbon chemical shifts are reported relative to [*methyl*-^{13}C] methionine at 14.86 ppm and are accurate to ±0.1 ppm. The spectra were acquired at 40 °C as described under Experimental Procedures. [b] Chemical shifts taken from Krudy et al. 1994. [c] Chemical shift differences between [SL-^{13}C]cTnC-(C35S)·cTnI(33−211) and [^{13}C]cTnC(C35S)·cTnI(33−211) are derived by subtraction of the chemical shift values for [^{13}C]cTnC(C35S)·cTnI(33−211) from the chemical shift values of the reduced [SL-^{13}C]cTnC-(C35S)·cTnI(33−211). The accuracy of these values is ±0.02 ppm for ^{1}H and ±0.2 ppm for ^{13}C. No significant change is indicated by "nc." [d] Met methyl ^{1}H−^{13}C correlation broadened beyond detection by nitroxide probe. [e] In the reduced [SL-^{13}C]cTnC(C35S)·cTnI(33−211) complex the Met methyl ^{1}H−^{13}C correlation shows significant broadening.

domain linker and suggests that cTnC is more extended in the cTnC·cTnI complex than observed in the monomer. Since Cys 84 is located in the N-terminal portion of the interdomain linker, all four C-terminal Met methyl groups must be at

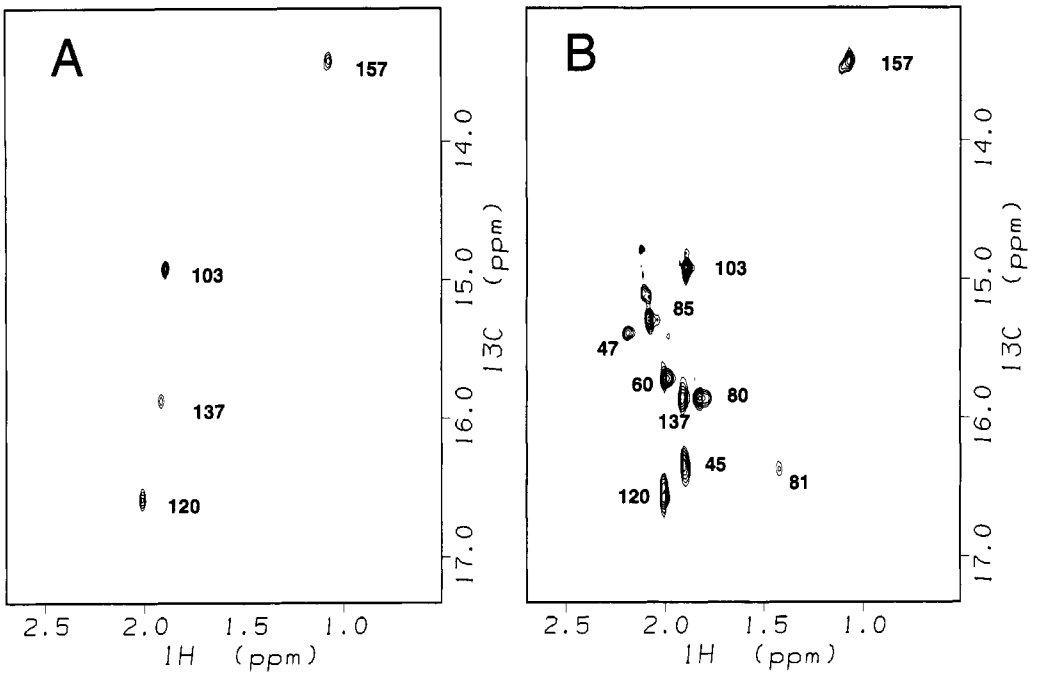

FIGURE 3: Methyl methionine region of the HSMQC spectrum of the Ca^{2+}-saturated [SL-^{13}C]cTnC(C35S)·cTnI(33−80) complex (A) and the ascorbate reduced Ca^{2+}-saturated [SL-^{13}C]cTnC(C35S)·cTnI(33−80) complex (B). Samples were approximately 1.5 mM [SL-^{13}C]-cTnC(C35S)·cTnI(33−80) complex and 6 mM Ca^{2+} in 20 mM Tris-d_{11}, 200 mM KCl, 0.1 mM PMSF, and in ^2H_2O at pH 7.5. The nitroxide radical was reduced to the hydroxylamine with 5 equiv of ascorbate. Sample conditions were identical to those described in panel A. Spectra were obtained at 500 MHz and 40 °C as described under Experimental Procedures.

least 15 Å from the attached spin label. On the basis of our model of cTnC, the N-terminal Met methyl residues range between 2.5 and 20 Å from the attached spin label depending on the precise position of the nitroxide radical. Thus, it is not surprising that these residues were all paramagnetically broadened beyond detection. Binding of cTnI(33−211) does not appear to cause a significant conformational change or a decrease in flexibility of this domain which would allow selective observation of several N-terminal methionines (Figure 2A and Table 3).

Site-Directed Spin and Isotope Labeling for Montoring Conformational Changes in cTnC upon Complex Formation with Recombinant cTnI(33−80). We have previously shown that the N-terminal domain of cTnI, cTnI(33−80) forms a proteolytically resistant and stable complex with cTnC (Krudy et al., 1994). Using a recombinant cTnI(33−80) protein, we have now demonstrated that it binds specifically to the C-terminal domain of cTnC in a manner identical to that found with native TnI (Table 2). Figure 3 shows the HSMQC spectra of the oxidized and reduced Ca^{2+}-saturated [SL-^{13}C]cTnC(C35S)·cTnI(33−80) complex. In the oxidized sample, only the C-terminal methionine residues, 103, 120, 137, and 157, were not paramagnetically broadened by MTSSL (Figure 3A; Table 4). As observed in the spin-labeled complex with cTnI(33−211), all N-terminal Met methyl ^{1}H−^{13}C correlations were broadened beyond detection (Figure 3A and Table 4). Reduction of MTSSL resulted in detection of all 10 ^{1}H−^{13}C correlations (Figure 3B and Table 4). Only small ^{1}H and ^{13}C chemical shifts in Met 45, 80, and 81 were found in the presence of the spin probe (Table 4). Similar changes were observed in Met 80 and 81 in the reduced free [SL-^{13}C]cTnC (Table 1). This clearly demonstrates that cTnI(33−80) is sufficient to decrease interdomain flexibility and maintain cTnC in a more extended conformation than observed in the monomer. The lack of

Table 4: Proton and Carbon Methionine Methyl Chemical Shifts for Ca^{2+}-Saturated [^{13}C]cTnC(C35S)·cTnI(33−80) and [SL-^{13}C]cTnC(C35S)·cTnI(33−80)[a]

methionine residue	[^{13}C]cTnC·cTnI(33−80)		[SL-^{13}C]cTnC(C35S)·cTnI(33−80) oxidized		[SL-^{13}C]cTnC(C35S)·cTnI(33−80) reduced		chemical shift differences Δ[b]	
	^{1}H ppm	^{13}C ppm	^{1}H ppm	^{13}C ppm	^{1}H ppm	^{13}C ppm	^{1}H ppm	^{13}C ppm
45	1.83	16.6	−[c]	−	1.91	16.3	0.08	−0.3
47	2.19	15.5	−	−	2.19	15.4	nc	nc
60	1.92	15.5	−	−	2.00	15.7	0.08	nc
80	1.79	16.3	−	−	1.83	15.9	0.04	−0.4
81	1.38	15.7	−	−	1.43	16.4	0.05	0.7
85	2.09	15.1	−	−	2.08	15.3	nc	nc
103	1.87	15.0	1.90	14.9	1.90	14.9	0.03	nc
120	1.99	16.7	2.01	16.6	2.00	16.6	nc	nc
137	1.90	15.9	1.92	15.9	1.92	15.9	nc	nc
157	1.06	13.6	1.07	13.4	1.07	13.4	nc	nc

[a] Proton chemical shifts are reported relative to ^{2}H^{1}HO signal at 4.563 ppm and are accurate to ±0.01 ppm. Carbon chemical shifts are reported relative to [*methyl*-^{13}C] methionine at 14.86 ppm and are accurate to ±0.1 ppm. The spectra were acquired at 40 °C as described under Experimental Procedures. [b] Chemical shift differences are derived by subtracting the chemical shift values of [^{13}C]cTnC·cTnI(33−80) from chemical shift values for the reduced [SL-^{13}C]cTnC(C35S)·cTnI(33−80). The accuracy of these values is ±0.2 ppm for ^{1}H and ±0.02 ppm for ^{13}C. No significant change is indicated by "nc" [c] Broadened beyond detection by the nitroxide probe.

observable paramagnetic effects on the C-terminal methionine residues sets a lower limit to the proximity of the N- and C-terminal domains when in complex with cTnI(33−80). If flexibility in the interdomain linker of cTnC exists in the cTnC·cTnI(33−80) complex, the C-terminal methionine residues must maintain a distance greater than 15 Å from the attached spin probe.

Site-Directed Spin and Isotope Labeling for Monitoring Conformational Changes in cTnC upon Complex Formation

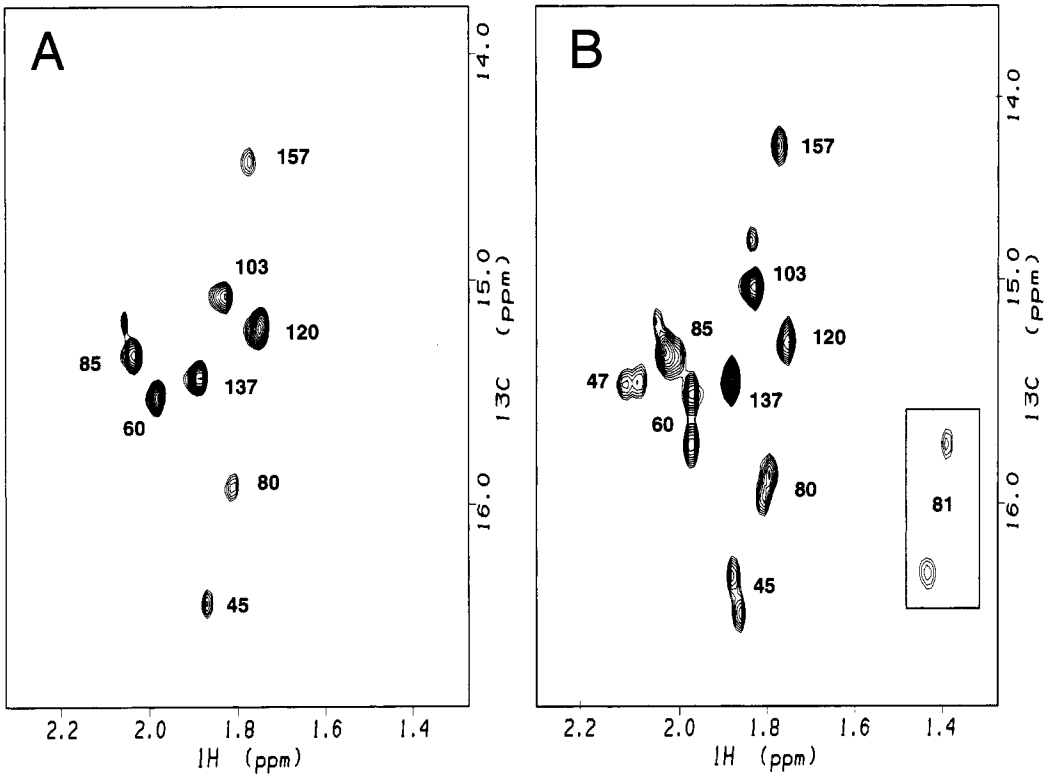

FIGURE 4: Methionine methyl region of the HSMQC spectrum of the Ca^{2+}-saturated [SL-^{13}C]cTnC(C35S)·cTnI(86−211) complex (A) and the ascorbate reduced Ca^{2+}-saturated [SL-^{13}C]cTnC(C35S)·cTnI(86−211) complex (B). Samples were approximately 1.5 mM [SL-^{13}C]-cTnC(C35S)·cTnI(86−211) complex and 6 mM Ca^{2+} in 20 mM Tris-d_{11}, 200 mM KCl, and 0.1 mM PMSF in ^2H_2O at pH 7.5. The nitroxide radical was reduced to the hydroxylamine with 5 equiv of ascorbate. The ^{1}H−^{13}C Met methyl correlation for 81, shown in the box, was only observable at a 6-fold lower contour level. Spectra were obtained at 500 MHz and 40 °C as described under Experimental Procedures.

with Recombinant cTnI(86−211). Flexibility within cTnC was also studied in the Ca^{2+}-saturated [SL-^{13}C]cTnC-(C35S)·cTnI(86−211) complex. We have already demonstrated that a stable complex can be formed between the C-terminal fragment of cTnI, cTnI(86−211), and Ca^{2+}-saturated [^{13}C]cTnC(C35S) (Table 2). Figure 4A shows the HSMQC spectrum of Ca^{2+}-saturated spin-labeled complex. In the presence of the oxidized spin label, eight of the 10 ^{1}H−^{13}C correlations can be observed in the Ca^{2+}-saturated complex. All Met methyl groups are detected upon addition of ascorbate (Figure 4B). In contrast to what is observed with cTnI(33−211), two distinct ^{1}H−^{13}C correlations are detected for the Met residues in the N-terminal domain. This observation is suggestive of two distinct conformations or decreased felixibility of this domain due to the presence of ascorbate. Upon removal of ascorbate, only one ^{1}H−^{13}C correlation was observed for each methionine (Table 5). To eliminate the possibility that nonspecific binding of ascorbate is involved, we added ascorbic acid to a Ca^{2+}-saturated [^{13}C]-cTnC(C35S)·cTnI(86−211) complex. This resulted in only one ^{1}H−^{13}C correlation for each Met (data not shown). Also, the lack of splitting of the ^{1}H−^{13}C correlations upon addition of free reduced MTSSL to the Ca^{2+}-saturated [^{13}C]cTnC-(C35S)·cTnI(86−211) complex rules out nonspecific binding of free MTSSL as the origin of the splitting seen in the covalently attached spin-labeled complex (data not shown). Since cTnC(C35S) containing MTSSL covalently attached at Cys 84 does not contain any free sulfhydryl groups, the origin of the two N- terminal conformations cannot result from cross-linking between site I and the D-helix in the linker region as observed in an activated form of cTnC containing an intramolecular disulfide bond (Hannon et al., 1993; Putkey et al., 1993). However, the different conformations of TnC depending on state of oxidation may be relevant to effects of oxidative stress on the myocardium, which has been shown to involve direct depression of myofilament response to Ca^{2+} (MacFarline & Miller, 1992).

In the presence of the oxidized spin label (Figure 4A), the intensities of the ^{1}H−^{13}C correlations vary with Met 60, 120, 137, and 103 being the most intense while those for 45, 80, 85, and 157 being visibly weaker. This pattern is strikingly different from what was observed in the Ca^{2+}-saturated spin-labeled complexes with cTnI(33−211) and cTnI(33−80). On the basis of the intensities of the ^{1}H−^{13}C correlations, Met 120 and 137 are unperturbed by the paramagnetic probe and are therefore at least 15 Å from the spin label. The ^{1}H−^{13}C correlations for Met 103 and 157 are only slightly perturbed by the nitroxide spin label (Figure 4A and Table 5). On the basis of the model of cTnC having an extended central helix, Met 120 and 137 are at the greatest distance from the paramagnetic probe, with Met 103 and 157 approximately 5−10 Å closer to the probe. Thus, cTnI-(86−211) is sufficient to decrease flexibility compared to that observed in free cTnC but is insufficient to maintain cTnC extended as has been observed in complexes with cTnI(33−211) and cTnI(33−80). This might have been predicted since cTnI(86−211) contains the Ca^{2+}-dependent regulatory interaction site while cTnI(33−80) contains the Ca^{2+}/Mg^{2+}-dependent interaction site responsible for maintaining stability of the cTnC·cTnI complex. In addition, the interaction of cTnI(86−211) with cTnC appears to decrease flexibility in the N-terminal domain of cTnC as judged by

Table 5: Proton and Carbon Methionine Methyl Chemical Shifts for Ca^{2+}-Saturated [^{13}C]cTnC(C35S)·cTnI(86−211) and [SL-^{13}C]cTnC(C35S)·cTnI(86−211)[a]

			[SL-^{13}C]cTnC(C35S)·cTnI(86−211)							
	[^{13}C]cTnC·cTnI(86−211)		oxidized		reduced[d] plus ascorbate		reduced[e] minus ascorbate		chemical shift differences Δ[b]	
methionine residue	^{1}H ppm	^{13}C ppm	^{1}H ppm	^{13}C ppm	^{1}H ppm	^{13}C ppm	^{1}H ppm	^{13}C ppm	^{1}H ppm	^{13}C ppm
45	1.84	16.3	1.87	16.4	1.86/1.88	16.3/16.5	1.87	16.5	0.03	nc
47	2.10	15.5	−[c]	−	2.10/2.13	15.5	2.05	15.3	−0.05	nc
60	1.92	15.5	1.98	15.5	1.97	15.5/15.8	1.98	15.5	0.06	nc
80	1.77	16.0	1.80	15.9	1.79/1.80	15.9/16.0	1.83	16.1	0.06	nc
81	1.41	16.2	−	−	1.44/1.39	16.30/15.75	1.38	15.5	−0.03	−0.6
85	2.07	15.2	2.03	15.3	2.03	15.3	2.05	15.3	nc	nc
103	1.87	15.1	1.83	15.1	1.83	15.0	1.88	15.0	nc	nc
120	1.74	15.3	1.75	15.2	1.75	15.3	1.84	15.6	0.10	−0.3
137	1.89	15.4	1.89	15.4	1.88	15.5	1.90	15.5	nc	nc
157	1.75	14.5	1.77	14.5	1.77	14.4	1.79	14.3	0.04	nc

[a] Proton chemical shifts are reported relative to ^{2}H^{1}HO signal at 4.563 ppm and are accurate to ±0.01 ppm. Carbon chemical shifts are reported relative to [methyl-^{13}C] methionine at 14.86 ppm and are accurate to ±0.1 ppm. The spectra were acquired at 40 °C as described under Experimental Procedures. [b] Chemical shift differences between [SL-^{13}C]cTnC(C35S)·cTnI(86−211) and [^{13}C]cTnC(C35S)·cTnI(86−211) are derived by subtraction of the chemical shift values for [^{13}C]cTnC(C35S)·cTnI(86−211) from the chemical shift value of [SL-^{13}C]cTnC(C35S)·cTnI(86−211), after removal of ascorbic acid. The accuracy of these values are ±0.02 ppm for ^{1}H and ±0.2 ppm for ^{13}C. No significant change is indicated by "nc" [c] Met methyl ^{1}H−^{13}C correlation broadened beyond detection by nitroxide probe. [d] Sample reduced with 5 equiv of ascorbic acid. [e] Chemical shifts for the reduced [SL-^{13}C]cTnC(C35S)·cTnI(86−211) complex after removal of the ascorbic acid.

the observation of ^{1}H−^{13}C correlations for Met 45, 60, 80, and 85 (Figure 4A and Table 5). Depending on the amount of flexibility, model building studies with Ca^{2-}-saturated cTnC suggest that these methionines can be within 5−15 Å from the paramagnetic probe. It is very likely that cTnI-(86−211), which lacks the N-terminal domain, is interacting with the hydrophobic pocket in a manner different from that of cTnI(33−211). This could account for the observation of several N-terminal Met methyl ^{1}H−^{13}C correlations. Methionines 47 and 81, which are not detected, are closest to the oxidized spin label (Figure 4). Therefore, binding of cTnI(86−211) must result in a minor N-terminal conformational change since, in the model, Met 47 is the furthest methionine residue from the spin label. Qualitatively, on the basis of broadening of the ^{1}H−^{13}C correlations, the Met residues 45, 80, and 85 are positioned approximately 7−15 Å from the nitroxide radical. Assuming no large tertiary change occurs in the C- terminal globular domain, the paramagnetic effects on the Met methyl correlations give evidence for a bent central helix with the distance between the two domains closer than predicted from the crystal structure or observed in cTnC complexes with cTnI(33−211) and cTnI(33−80). In addition, the data support a decrease in flexibility within the N-terminal domain of cTnC when complexed with cTnI(86−211).

CONCLUDING REMARKS

We have previously shown that cTnC and cTnI bind in an antiparallel fashion with the N-terminus of cTnI interacting with the C-terminus of cTnC (Krudy et al., 1994). We have also shown that the inhibitory peptide, cTnIp, binds to the C-terminal domain of cardiac troponin C on the basis of protection of the methyl groups of Met 120 and 157 from the soluble spin label HyTEMPO (Howarth et al., 1995). Using a combination of SDSL and selective isotope labeling of cTnC, we have extended our studies on free cTnC as well as cTnC bound to one of three recombinant cTnI proteins: cTnI(33−211); cTnI(33−80); or cTnI(86−211).

To study conformational change in cTnC, both free in solution and complexed with cTnI(33−211), cTnI(33−80), or cTnI(86−211), we utilized SDSL and selective isotope labeling to monitor the flexibility of the central helix in cTnC. We believe that the binding of cTnI does not disrupt the tertiary structures of the N-and C-terminal globular domains of cTnC to the extent that would prevent distances from the oxidized spin label to the Met residues in cTnC to be qualitatively measured as a function of flexibility in the central helix. This is supported by our previous NMR studies (Krudy et al., 1994) monitoring chemical shift changes in the Met methyl residues of cTnC in the binary complex (Table 2), SAXS data on the sTnC·sTnI complex (Olah et al., 1994; Olah & Trewhella, 1994), and high-resolution NMR studies of the cTnC(88−162)·cTnI(33−80) complex (Howarth, Rance, and Rosevear, unpublished). In both the cTnC·cTnI(33−211) and cTnC·cTnI(33−80) complexes, all of the C-terminal Met methyl residues of cTnC are at least 15 Å from the spin label (Figures 2 and 3). In the cTnC·cTnI(86−211) complex, Met 157 is significantly closer to the spin probe attached at Cys 84. As noted previously, quantitative interpretation and complete analysis of the intensity changes is complicated. However, selective paramagnetic effects on Met 157, and to a lesser extent on Met 103, demonstrate that the interdomain linker is more flexible permitting closer contact between the N- and C-terminal domains of cTnC when complexed only to the C-terminal domain of cTnI. Thus, the N-terminal domain of cTnI, cTnI-(33−80) may not only be responsible for helping to maintain stability of the cTnC·cTnI complex but also modulate interactions between the C-terminal region of cTnI and the N-terminal regulatory domain of cTnC. One mechanism by which this could occur is to alter the flexibility of the interdomain linker in a Ca^{2+}-dependent manner. This could also account for the known observation that phosphorylation of the cardiac specific N-terminus, residues 1−32, results in reduced sensitivity of the troponin complex to Ca^{2+} (Guo et al., 1994; Liao et al., 1994). Also, in the absence of the N-terminal domain of cTnI, the C-terminal fragment of cTnI, cTnI(86−211), appears to decrease the flexibility in the N-terminal domain of cTnC to a greater degree (Figure 4). Again, this suggests a modulatory or functional role for the

N-terminal domain of cTnI in addition to a structural role in maintaining the stability of the cTnC·cTnI complex. Our studies on Ca^{2+}-saturated cTnC·cTnI(33−211) agree with the model derived from SAXS data on sTnC·sTnI which shows that TnC is bound to TnI in an extended conformation (Olah et al., 1994; Olah & Trewhella, 1994). However, we believe this complex is more dynamic than may be suggested by either the crystallographic structures of sTnC or the model of the sTnC·sTnI complex derived from SAXS data. The Gly in the Lys-Gly-Lys sequence in TnC is responsible for considerable instability within the interdomain linker of TnC (Ding et al., 1994). Thus, while the interdomain linker is considerably less flexible in cTnI bound cTnC resulting in cTnC being more extended than free cTnC, flexibility within the interdomain linker appears to be modulated not only by the presence of bound Ca^{2+} at the regulatory site but also by the interaction of the N-terminal domain of cTnI with the C-terminal domain of cTnC. The ability of both cTnI domains to modulate flexibility within the interdomain linker of cTnC may play an important role in the response of the cardiac contractile apparatus to a variety of external stimuli which affect the N- and C-terminal domains of both proteins. This hypothesis is consistent with the suggestion that the N-terminal region of TnI functions as a negative effector of the binding of the inhibitory region of TnI (Nagi & Hodges, 1992).

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BI9510183