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Comparative NMR Studies on Cardiac Troponin C and a Mutant Incapable of Binding Calcium at Site II†

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ABSTRACT: One- and two-dimensional NMR techniques were used to study both the influence of mutations on the structure of recombinant normal cardiac troponin C (cTnC3) and the conformational changes induced by Ca^{2+} binding to site II, the site responsible for triggering muscle contraction. Spin systems of the nine Phe and three Tyr residues were elucidated from DQF-COSY and NOESY spectra. Comparison of the pattern of NOE connectivities obtained from a NOESY spectrum of cTnC3 with a model of cTnC based on the crystal structure of skeletal TnC permitted sequence-specific assignment of all three Tyr residues, as well as Phe-101 and Phe-153. NOESY spectra and calcium titrations of cTnC3 monitoring the aromatic region of the ^1H NMR spectrum permitted localization of six of the nine Phe residues to either the N- or C-terminal domain of cTnC3. Analysis of the downfield-shifted CαH resonances permitted sequence-specific assignment of those residues involved in the β-strand structures which are part of the Ca^{2+}-binding loops in both the N- and C-terminal domains of cTnC3. The short β-strands in the N-terminal domain of cTnC3 were found to be present and in close proximity even in the absence of Ca^{2+} bound at site II. Using these assignments, we have examined the effects of mutating Asp-65 to Ala, CBM-IIA, a functionally inactive mutant which is incapable of binding Ca^{2+} at site II [Putkey, J. A., Sweeney, H. L., & Campbell, S. T. (1989) *J. Biol. Chem. 264*, 12370]. Comparison of the apo, Mg^{2+}-, and Ca^{2+}-bound forms of cTnC3 and CBM-IIA demonstrates that the inability of CBM-IIA to trigger muscle contraction is not due to global structural changes in the mutant protein but is a consequence of the inability of CBM-IIA to bind Ca^{2+} at site II. The pattern of NOEs between aromatic residues in the C-terminal domain is nearly identical in cTnC3 and CBM-IIA. Similar interresidue NOEs were also observed between Phe residues assigned to the N-terminal domain in the Ca^{2+}-saturated forms of both cTnC3 and CBM-IIA. However, chemical shift changes were observed for the N-terminal Phe residues in CBM-IIA. This suggests that binding of Ca^{2+} to site II alters the chemical environment of the residues in the N-terminal hydrophobic cluster without disrupting the spatial relationship between the Phe residues located in helices A and D.

Muscle contraction is triggered by the binding of Ca^{2+} to the troponin C (TnC)[1] subunit of the troponin complex. Troponin C is a prominent member of a family of proteins which bind Ca^{2+} and modulate the action of other proteins. The members of this protein family have at least one helix–loop–helix or EF-hand Ca^{2+}-binding site originally observed in parvalbumin (Kretsinger & Nockolds, 1973) and also shown to be present in calmodulin (Babu et al., 1985, 1988), troponin C (Herzberg & James, 1985, 1988), and calbindin (Szebenyi & Moffat, 1986). There are two isoforms of TnC: one isoform is found in fast skeletal muscle (sTnC) whereas the other is found in cardiac and slow skeletal muscle (cTnC). Although both isoforms have four potential Ca^{2+}-binding sites, sites I–IV, site I in cTnC is inactive (Van Eerd & Takahashi, 1975, 1976; Collins et al., 1977).

The crystallographic structures of turkey and chicken sTnC revealed an overall protein structure which resembles a dumbbell, with two globular domains connected by a 31-residue α-helix (Herzberg et al., 1987; Herzberg & James, 1985, 1988; Sundaralingam et al., 1985; Satyshur et al., 1988). Both the N- and C-terminal domains contain a pair of helix–loop–helix Ca^{2+}-binding motifs. Calcium-binding sites III and IV, in the C-terminal domain of both skeletal and cardiac TnC, have high affinity for Ca^{2+} and also bind Mg^{2+} with lower affinity (Van Eerd & Takahashi, 1975; Potter & Gergely, 1977; Collins et al., 1977). Site II in cTnC and sites I and II in sTnC bind Ca^{2+} specifically but with lower affinity than sites III and IV (Potter & Gergely, 1977; Holroyde et

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[1] Abbreviations: TnC, cardiac or fast skeletal troponin C; cTnC, cardiac TnC; sTnC, skeletal TnC; cTnC3, bacterially synthesized cTnC(desM1,D2A); CBM-IIA, cTnC3(D65A); cTnCN1, mutant protein where the first 11 amino acids of cTnC3 (MADIYKAAVEQ) were replaced with the first 12 amino acids of calmodulin (MADQLTEEQIAE); TnI, troponin I; EDTA, ethylenediaminetetraacetic acid; CDTA, *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid; DTT, 1,4-dithiothreitol; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate; Tris-d_{11}, deuterated tris(hydroxymethyl)aminomethane; FID, free induction decay; NMR, nuclear magnetic resonance; DQF-COSY, double-quantum filtered two-dimensional correlated spectroscopy; TOCSY, two-dimensional total correlation spectroscopy, NOESY, two-dimensional proton nuclear Overhauser enhancement spectroscopy; NOE, nuclear Overhauser effect; COSY, two-dimensional correlated spectroscopy; TPPI, time-proportional phase incrementation.

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al., 1980; Johnson et al., 1980). Kinetic experiments have suggested that it is Ca^{2+} binding to the low-affinity sites, sites I and II of sTnC and site II in cTnC, which initiates muscle contraction (Johnson et al., 1979; Robertson et al., 1981).

Recently, the cDNA for chicken cTnC has been incorporated into a bacterial expression system for preparation of large scale quantities of protein and mutagenesis studies. Inactivation of Ca^{2+}-binding site II by conversion of Asp-65 to Ala directly demonstrated that site II in cTnC is responsible for triggering muscle contraction in skinned slow muscle fibers (Putkey et al., 1989). Activation by mutagenesis of site I in cTnC was found to confer Sr^{2+} and Ca^{2+} sensitivities similar to sTnC (Putkey et al., 1989; Sweeney et al., 1990). However, cTnC having an active site I and an inactive site II was unable to restore Ca^{2+}-dependent muscle contraction to TnC-depleted skinned slow muscle fibers (Sweeney et al., 1990). In a similar study it was recently shown that Ca^{2+}-binding sites I and II in sTnC are also responsible for triggering muscle contraction but that Ca^{2+} binding to both sites is required for full force generation in fast skeletal muscle fibers (Sheng et al., 1990).

Modeling studies, based on the crystal structure of turkey sTnC, have suggested that the binding of Ca^{2+} to sites I and II results in the movement of helices B and C away from helices N, A, and D, exposing previously buried hydrophobic residues (Herzberg et al., 1986a; compare Figure 4, middle and bottom panels). The movement of helices within the N-terminal domain and the resulting exposure of a hydrophobic surface has been postulated as the critical conformational change responsible for triggering muscle contraction. In two recent reports, the coupling of helices C and B to helix D by covalent or ionic bonds was found to inhibit the function of sTnC, providing experimental support for this model (Grabarek et al., 1990; Fujimori et al., 1990).

Nuclear magnetic resonance (NMR) has been used to probe metal-dependent conformational changes in sTnC (Tsuda et al., 1988, 1990), cTnC (Hincke et al., 1981; MacLachlan et al., 1990a), and tryptic fragments of sTnC (Drabikowski et al., 1985; Drakenberg et al., 1987), as well as the interaction of sTnC with peptides derived from the inhibitory region of TnI (Cachia et al., 1983; Campbell & Sykes, 1989) and the interaction of TnC with drugs (Drabikowski et al., 1985; MacLachlan et al., 1990b; Reid et al., 1990). One- and two-dimensional ^1H NMR spectroscopy has been used to assign aromatic residues within the hydrophobic clusters in the C- and N-terminal domains of sTnC (Drabikowski et al., 1985; Tsuda et al., 1988). The pattern of interresidue NOEs was found to be consistent with that predicted from known distances in the crystal structure of sTnC (Herzberg & James, 1985, 1988; Sundaralingam et al., 1985; Satyshur et al., 1988). Residues located in the short β-sheet formed between the two Ca^{2+}-binding loops in both the N- and C-terminal domains of sTnC were also partially assigned on the basis of the chemical shifts of downfield CαH protons, as well as COSY and NOESY spectra (Drabikowski et al., 1985; Tsuda et al., 1988).

Sequence-specific assignment of the three Tyr residues in cTnC were initially performed on the basis of Ca^{2+}-dependent chemical shifts, NOEs, and gadolinium broadening experiments (Hincke et al., 1981). Recently, additional assignments have been proposed using ring-current shift calculations, two-dimensional NMR techniques, and spectral simulation of NMR data using a model of cTnC based on the crystal structure of sTnC (MacLachlan et al., 1990a). This study utilized the Tyr assignments of Hincke et al. (1981) as a basis for further assignment of the aromatic and upfield-shifted methyl residues (MacLachlan et al., 1990a).

Here we report initial NMR studies using recombinant normal cTnC (cTnC3) and mutated derivatives. Sequence-specific assignment or localization to either the N- or C-terminal domain of 9 of the 12 aromatic residues has been accomplished using two-dimensional NMR and mutagenesis. These studies have resulted in the reassignment of Tyr-5 and Tyr-111 from previous work (Hincke et al., 1981; MacLachlan et al., 1990a). Using these assignments, we have examined the effects of mutating Asp-65 to Ala (CBM-IIA) in the apo, Mg^{2+}-, and Ca^{2+}-bound forms. Comparison of the apo, Mg^{2+}-, and Ca^{2+}-bound forms of cTnC3 and of CBM-IIA demonstrates that the inability of CBM-IIA to trigger muscle contraction is not due to global structural changes in the mutant protein but is a consequence of the inability of CBM-IIA to bind Ca^{2+} at site II. Analysis of conformational changes upon Ca^{2+} binding to site II showed them to be largely located in the N-terminal domain of cTnC3. Identification of the structural differences between the Ca^{2+}-saturated forms of cTnC3 and CBM-IIA will prove indispensable in elucidating the Ca^{2+}-dependent conformational changes which regulate muscle contraction in cardiac muscle.

Experimental Procedures

Materials. Deuterium oxide and Tris-d_{11} were purchased from Cambridge Isotope Labs. All other chemicals were of the highest purity available commercially.

Recombinant Proteins. Isolation of a cDNA for chicken cTnC and construction of bacterial expression plasmids for cTnC3 and CBM-IIA were described previously (Putkey et al., 1987a, 1989). The mutant cTnCN1 is encoded by an expression plasmid in which the first 11 codons of the cDNA for chicken cTnC in the plasmid pMC6B (Putkey et al., 1989) were replaced with the first 12 codons from an expression plasmid for chicken CaM. The plasmid pCaM23N (Putkey et al., 1987b) was first digested with *Eco*RI and blunt-ended with the Klenow fragment of DNA polymerase I. The plasmid was then digested with *Bam*HI and a 43 bp fragment was isolated by gel electrophoresis. This fragment was ligated into the plasmid called pTnC2-4a. This plasmid was then digested with *Eco*RI and *Nco*I, and a PL promoter was inserted as an *Eco*RI/*Nco*I fragment from the plasmid pCaMPL. The N-terminal sequence of cTnCN1 was confirmed by protein sequencing, and the mutant was shown to be functional in a myofibril ATPase assay (data not shown). In all bacterially synthesized cTnC proteins, the N-terminal initiator Met is removed by a posttranslational modification.

All proteins were isolated as described previously (Putkey et al., 1989). For NMR experiments, protein samples were extensively dialyzed against 50 mM $(NH_4)HCO_3$, pH 7.5, lyophilized, and dissolved in NMR buffer containing 20 mM Tris-d_{11} and 200 mM KCl in ^2H_2O at pH* 7.5.[2] Just prior to the NMR experiment the samples were made 10 mM in DTT. All buffer and reagent solutions used in the NMR studies were treated with Chelex 100 before use to remove trace metal contaminants. For preparation of apoproteins, samples were made 5–10 mM in both CDTA and EDTA, and approximately 1×10^5 cpm of $^{45}Ca^{2+}$ was added. The proteins were then desalted on a Bio-Gel P-6DG (Bio-Rad) column equilibrated in Chelex-treated 10 mM $(NH_4)HCO_3$ buffer, pH 7.5. Protein fractions devoid of $^{45}Ca^{2+}$ were pooled, lyophilized, and dissolved in the NMR buffer described above.

[2] pH* denotes a pH meter reading uncorrected for the deuterium isotope effect.

NMR Methods. ^{1}H NMR spectra were collected at 500 MHz. One-dimensional spectra were recorded with 512 transients, each with a spectral width of 7142 Hz, 8K data points, a 90° pulse, and a 2.5-s recycle time, at 40 °C. When necessary, resolution was enhanced by apodization of the FID by Gaussian multiplication and an exponential multiplication with a negative time constant. Proton chemical shifts are reported relative to the $^2H^1HO$ signal at 4.56 ppm.

Chemical shift changes in the aromatic region of the ^{1}H NMR spectra as a consequence of mutation were carefully compared by obtaining difference spectra between wild-type and mutant proteins. Protein samples were prepared in identical buffers and, as close as experimentally possible, at identical protein concentrations, DTT, and pH values. Difference spectra were obtained by subtraction of mutant CBM-IIA proteins from wild-type cTnC3 proteins so as to minimize the differences between the two proteins. As a control, subtraction of two different samples of either cTnC3 or CBM-IIA from each other were shown to give identical spectra in the aromatic region.

Two-dimensional DQF-COSY spectra (Piantini et al., 1982; Rance et al., 1983) were collected as 512 t_1 × 2048 t_2 complex data points. Phase-sensitive NOESY spectra (States et al., 1982; Macura et al., 1982) were collected with 100- and 150-ms mixing times as 512 t_1 × 2048 t_2 complex data points. The sweep width was 5555 Hz in both dimensions. Residual H_2O was suppressed by continuous irradiation during the relaxation delay. Two-dimensional NMR data were transferred to a MicroVax II and processed with the FTNMR software package, provided by Dr. Dennis Hare, Hare Research Inc. The spectra were apodized with a 35°-shifted sine bell squared function in t_2, and by a 45° shifted sine bell function in t_1. NOESY spectra were also alternatively processed in both dimensions with a Gaussian multiplication and an exponential multiplication with a negative time constant. TOCSY spectra were collected with a 40-ms spin lock using a combination of the States and TPPI methods (Marion et al., 1989) and processed using a 45°-shifted skewed sine bell in both dimensions.

Computer Molecular Modeling. The amino acid sequences of bovine cardiac TnC (Van Eerd & Takahashi, 1975; Putkey et al., 1987a,b) and chicken skeletal TnC (Wilkinson, 1976; Reinach & Karlsson, 1988) were aligned as in Figure 1 of Herzberg et al. (1986b). In order to construct a bovine cardiac model of TnC in the 2Ca^{2+} and Ca^{2+}-saturated forms, the sequence alignment and the computer program MUTATE (R. J. Read, unpublished) were used to replace the side chains of the 2Ca^{2+} turkey skeletal TnC crystal structure (Herzberg & James, 1988) and the 4Ca^{2+} skeletal TnC model (Herzberg et al., 1986a), respectively, with the homologous side chains of bovine cardiac TnC. This replacement was done by retaining atoms common to the native and mutated residues and then building any additional atoms required in the standard conformation for that particular amino acid residue. Insertion of the extra Gly residue into the aberrant calcium-binding loop I of bovine cTnC was done using the MMS suite of modeling programs on a Silicon Graphics (Iris) workstation. In order to relieve unacceptably close van der Waals contacts between atoms of the modeled cTnC and to idealize any inappropriate geometry introduced by the insertion of Gly-30, 300 cycles of conjugate gradient energy minimization were carried out on the cTnC models. The numbering system is based on that previously used for bacterially synthesized cTnC, cTnC3 (Putkey et al., 1989). The energy minimization was done using the GROMOS library of computer programs. The potential function used in this suite of programs is essentially that described by Van Gunsteren and Karplus (1982); the parameters were those of the set 37D of GROMOS with an 8-Å cutoff. All bond lengths and angles were optimized to fit the potential used in the energy minimization. The electrostatic charge contributions from the calcium ions were not considered in the calculations. Calcium-binding distances within loops II, III, and IV were idealized using the MMS suite of modeling programs on a Silicon Graphics (Iris) workstation.

Results

Inactivation of Ca^{2+}-binding site II in cardiac TnC through mutation of Asp-65 to Ala, CBM-IIA, has been shown to prevent the Ca^{2+}-dependent activation of muscle contraction by cTnC (Putkey et al., 1989). Mutation of Asp-65 to Ala not only results in the loss of a Ca^{2+}-coordinating ligand but may also disrupt a hydrogen-bonding network thought to be important in Ca^{2+} binding. From the crystal structure of sTnC, the first Ca^{2+}-coordinating ligand in a helix–loop–helix Ca^{2+}-binding site, Asp-65 in site II of cTnC, was shown to be involved in a hydrogen-bonding network responsible for maintaining the correct spatial orientation of the remaining Ca^{2+}-coordinating ligands within the site (Strynadka & James, 1989). The carboxyl side chain of the invariant Asp residue in the first position of the Ca^{2+}-binding loop contributes one oxygen atom to the Ca^{2+} ligand sphere and receives a hydrogen bond from the main-chain amide groups of the residues within the fourth and fifth positions of the loop. The other carboxyl side-chain oxygen atom of the invariant Asp residue is the recipient of a hydrogen bond from the main-chain NH of the invariant Gly in the sixth position of the Ca^{2+}-binding loop. Because of these interactions, no other amino acid can replace Asp as the first coordinating ligand. Consequently, mutation at the first Ca^{2+}-coordinating position may be responsible for the observed loss of Ca^{2+} binding at site II. The structural consequences resulting from the inability of this mutant to bind Ca^{2+} at site II and trigger Ca^{2+}-dependent muscle contraction have been studied here by one- and two-dimensional ^{1}H NMR spectroscopy. NMR provides a sensitive technique to detect conformational differences between recombinant normal cTnC (cTnC3) and the site II Ca^{2+}-binding mutant (CBM-IIA). cTnC3 is identical to tissue-derived bovine cTnC by all physical and functional criteria tested (Putkey et al., 1989; Sweeney et al., 1990). The conformational and structural differences detected in the Ca^{2+}-saturated forms of cTnC3 and CBM-IIA may reflect the structure/function relationships necessary for Ca^{2+}-activated muscle contraction.

Qualitative Comparison of the Aromatic ^{1}H NMR Region of cTnC3 and CBM-IIA. Conformational changes induced in cTnC3 and CBM-IIA upon metal binding were initially studied by comparison of the aromatic regions of their ^{1}H NMR spectra (Figure 1). From the near identity of the chemical shifts of the aromatic resonances, the apo forms of both cTnC3 and CBM-IIA were judged to be extremely similar (Figure 1A). This is more clearly seen in the difference spectrum of the two proteins, which shows only modest chemical shift differences in the aromatic region of the NMR spectra (Figure 1A). The similarities in chemical shifts demonstrate that conversion of Asp-65 to Ala in site II does not significantly alter the solution conformation of the N-terminal domain of the mutant protein, CBM-IIA, in the absence of metals.

In the presence of saturating concentrations of Mg^{2+}, both proteins were also found to have nearly identical aromatic residue chemical shifts (Figure 1B). This similarity is clearly demonstrated in the difference spectrum of the Mg^{2+}-saturated proteins. The addition of Mg^{2+} was found to produce a sig-

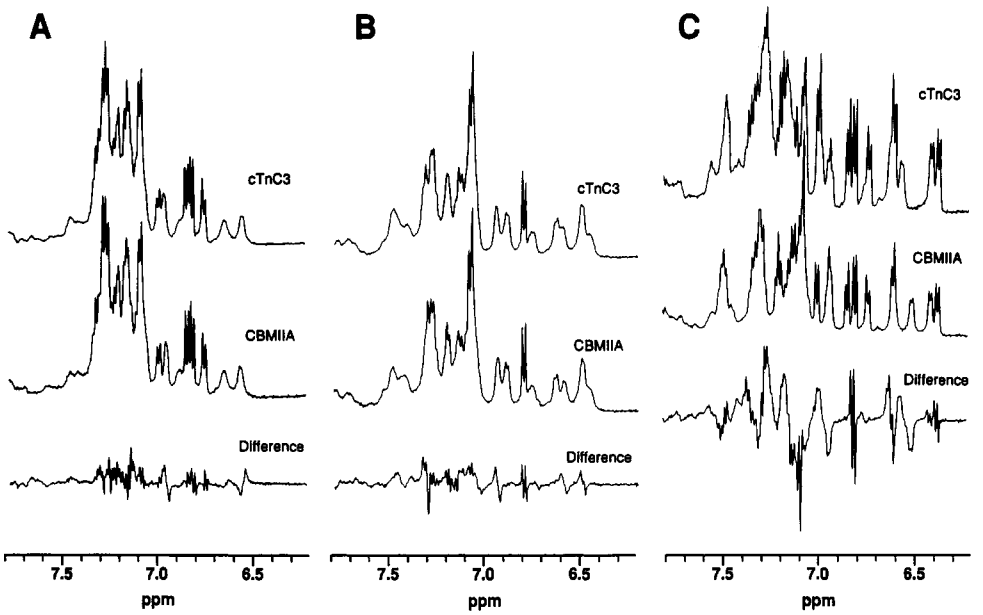

FIGURE 1: Comparison of the aromatic region of the ^1H NMR spectra of cTnC3 and CBM-IIA in absence of metals (A), in presence of saturating Mg^{2+} (B), and in presence of saturating Ca^{2+} (C). NMR spectra were recorded at 500 MHz and 40 °C, on 0.5 mM protein solutions having the following composition: 20 mM Tris-d_{11}, 200 mM KCl, and 5 mM DTT, pH* 7.5, in 99.96% ^2H_2O. Difference spectra, wild-type minus mutant, are shown at the bottom of each panel. Wild-type and mutant cTnC proteins were prepared in identical buffers and, as close as experimentally possible, at identical protein concentrations, DTT, and pH values.

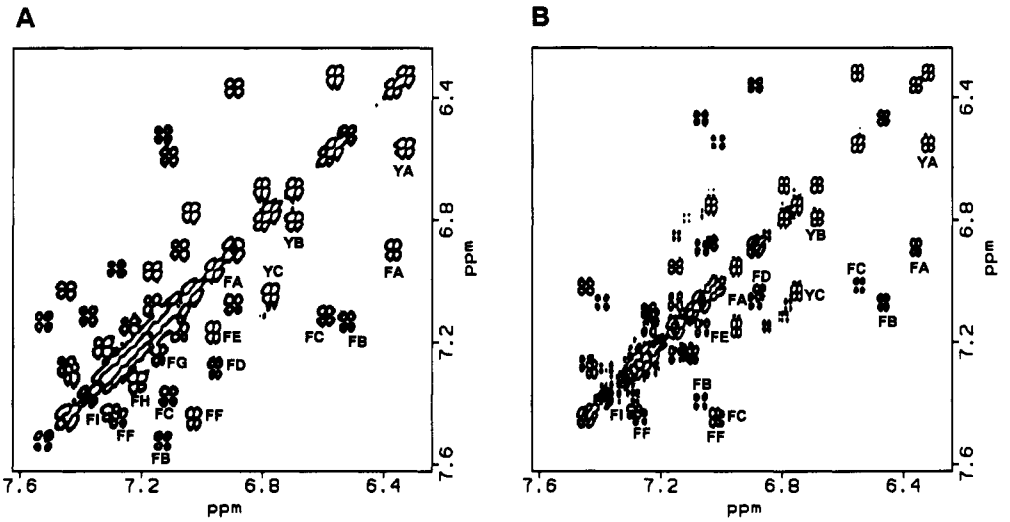

FIGURE 2: Aromatic region of the 500-MHz DQF-COSY spectra of Ca^{2+}-saturated cTnC3 (A) and CBM-IIA (B). The spectra were recorded in D_2O at 40 °C. The samples contained 1.5 mM protein, 20 mM Tris-d_{11}, 200 mM KCl, 10 mM DTT, and 6 mM $CaCl_2$ at pH* 7.5 in 99.96% ^2H_2O. Spin systems for all three Tyr and nine Phe residues were identified, and assignments are listed in Table I.

nificant line broadening of most aromatic resonances in both proteins (Figure 1B). An exception to this was Tyr-5. The line width of Tyr-5 remained unaltered from that observed in the apo forms of the proteins (Figure 1A,B). The increased line broadening in the presence of Mg^{2+} is indicative of exchange between the free and Mg^{2+}-bound forms of the protein. The similar tertiary structures of the Mg^{2+}-saturated forms of the two proteins, as judged by the near identity of the aromatic chemical shifts, was expected since inactivation of Ca^{2+}-binding site II in the N-terminal domain of cTnC3 by a single mutation should not alter the ability of the C-terminal domain to bind Mg^{2+}. Magnesium binds only to the high-affinity metal-binding sites III and IV (Potter & Gergely, 1975). The similarity between the Mg^{2+} forms of these two proteins suggests that if Mg^{2+} does bind at site II it either does not induce a conformational change or, less likely, it is coordinated in a similar fashion in both CBM-IIA and cTnC3.

The addition of Ca^{2+} to the Mg^{2+}-saturated proteins produced marked changes in the aromatic region of the ^1H NMR spectrum of both proteins as evidenced by both the decrease in line widths and chemical shift changes in the aromatic resonances (Figure 1C). The decrease in line width is likely due to replacement of Mg^{2+} bound at sites III and IV with Ca^{2+}. Large differences in chemical shifts were observed both in the one-dimensional spectra and in the difference spectrum obtained between the Ca^{2+}-saturated forms of cTnC3 and CBM-IIA (Figure 1C). These chemical shift differences were attributed to conformational changes occurring in cTnC upon Ca^{2+} binding to site II.

Spin System Identification and Partial Assignment in cTnC3 and CBM-IIA. The side chains of the aromatic amino acid residues were initially examined due to their relative ease of identification. In addition, aromatic amino acids are known to participate in the formation of the hydrophobic core in both the N- and C-terminal domains of TnC (Herzberg & James, 1985, 1988) and may be responsible in part for the critical interaction with TnI (Zot & Potter, 1987; Leavis & Gergely, 1984). Aromatic spin systems of the Ca^{2+}-saturated forms

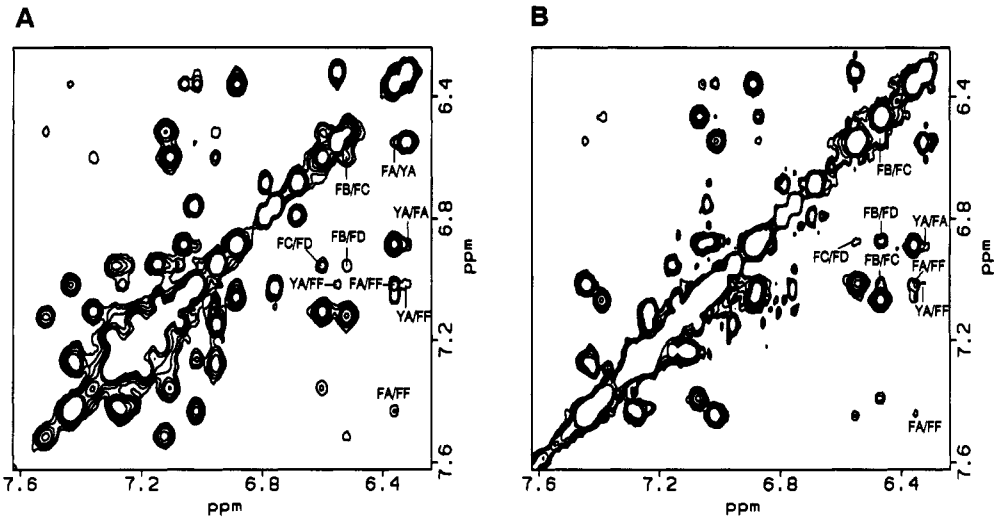

FIGURE 3: Aromatic region of the 500-MHz phase-sensitive NOESY spectra of Ca^{2+}-saturated cTnC3 (A) and CBM-IIA (B). The protein concentration was 4 mM for cTnC3 and 2 mM for CBM-IIA. Spectra were recorded with 150- and 100-ms mixing times, respectively, using sample conditions given in the legend to Figure 2. Interresidue NOEs are indicated.

of cTnC3 and CBM-IIA were assigned from the DQF-COSY spectra (Figure 2A,B). Both cTnC proteins contain nine Phe and three Tyr residues, but no His or Trp residues. The nine Phe residues are referred to as Phe-A to -I (FA to FI) and the three Tyr residues as Tyr-A to -C (YA to YC; see Table I). The spin systems of all three Tyr residues and six of the nine Phe residues were completely identified, and the three remaining Phe spin systems were partially identified in both proteins (Figure 2). Chemical shifts for Phe and Tyr spin systems of cTnC3 and CBM-IIA are given in Table I. At 40 °C, the magnetic equivalence between the C2H and C6H, and between the C3H and C5H resonances, indicates that all Phe and Tyr aromatic rings rotate rapidly with respect to the frequency differences in their chemical shifts. Multiple minor cross-peaks were observed in the DQF-COSY spectrum of CBM-IIA (Figure 2B) which were not seen in the spectrum of the wild-type protein (Figure 2A). These minor cross-peaks are suggestive of multiple species of CBM-IIA having minor conformational differences in solution. These minor conformational differences may partially result from the increased flexibility of CBM-IIA due to its inability to bind Ca^{2+} at site II.

Partial sequence-specific assignment of the aromatic spin systems in both cTnC proteins was accomplished by comparison of NOE connectivities observed in NOESY spectra and interresidue distances determined from the molecular model of cTnC, and by mutagenesis. Tyr-5 was assigned by comparison of the one-dimensional NMR spectra of the aromatic regions of cTnC3 and the mutant cTnCN1. In cTnCN1, Tyr-5 was removed by replacing the first 11 amino acids of cTnC with the first 12 amino acids from calmodulin. No other aromatic residues are altered by this mutation. The mutant cTnCN1 was found to be functional in both fast skeletal and cardiac myofibril ATPase assays (Putkey, unpublished). The resonance at 6.77 ppm, which is part of the Tyr-C spin system (Figure 2 and Table I), was completely eliminated from the spectrum of cTnCN1 (data not shown). Thus, Tyr-C was unequivocally assigned as Tyr-5 (Table I). Interestingly, Tyr-5, which is located in the N-terminal domain, was only slightly perturbed upon Ca^{2+} binding at site II as predicted from our model and the model of Herzberg et al. (1986) for sTnC (Figure 1 and Figure 4).

The resonances of Tyr-111 and -150, both located in the C-terminal domain of cTnC, were assigned by comparison of

Table I: Comparison of the Aromatic Residues Chemical Shifts in the Ca^{2+}-Saturated Forms of cTnC3 and the Mutant CBM-IIA

residue	assignment		C2,6H	C3,5H	C4H
Tyr-A	Tyr-150	wt	6.56	6.33	
		mutant	6.55	6.32	
Tyr-B	Tyr-111	wt	6.69	6.80	
		mutant	6.68	6.79	
Tyr-C	Tyr-5	wt	7.04	6.77	
		mutant	7.03	6.75	
Phe-A	Phe-101	wt	6.37	6.90	7.07
		mutant	6.36	6.89	7.07
Phe-B	N terminus	wt	6.52	7.13	7.52
		mutant	6.47	7.07	7.39
Phe-C	N terminus	wt	6.59	7.11	7.38
		mutant	6.54	7.02	7.45
Phe-D	N terminus	wt	6.96	7.29	
		mutant	6.87	7.04	
Phe-E	?	wt	6.96	7.17	7.07
		mutant	6.95	7.15	7.06
Phe-F	Phe-153	wt	7.03	7.45	7.28
		mutant	7.02	7.45	7.27
Phe-G	?	wt	7.14	7.24	
		mutant	7.15	7.24	
Phe-H	N terminus	wt	7.21	7.33	
		mutant	7.11	7.23	
Phe-I	?	wt	7.25	7.30	7.43
		mutant	7.23	7.29	7.43

observed NOE connectivities in the NOESY spectrum of cTnC3 with those predicted from the model of cTnC3 based on the crystal structure of sTnC (Herzberg & James, 1985, 1988) (Figure 4). Figure 3A,B shows the aromatic region of the NOESY spectra of cTnC3 and CBM-IIA. Strong intraresidue NOEs were observed within spin systems previously identified in the DQF-COSY spectra (Figure 2). Interresidue NOEs were also observed between Phe-A and Phe-F, Phe-F and Tyr-A, and Tyr-A and Phe-A in both cTnC3 and CBM-IIA (Figure 3A,B). A weak NOE, not observed at the contour level shown in Figure 3, was also consistently observed between Tyr-B and Phe-A. As shown in Figure 4A, this pattern of NOEs is remarkably consistent with the predicted pattern of interresidue NOEs, derived from the molecular model for the cluster of aromatic amino acids found in the C-terminal domain of cTnC. This cluster of aromatic amino acids has been found to be relatively conserved in the crystal structures of sTnC (Herzberg & James, 1985, 1988) and calmodulin (Babu et al., 1985, 1988). In addition, similar hydrophobic clusters have been identified using NOEs in parvalbumin (Levine et

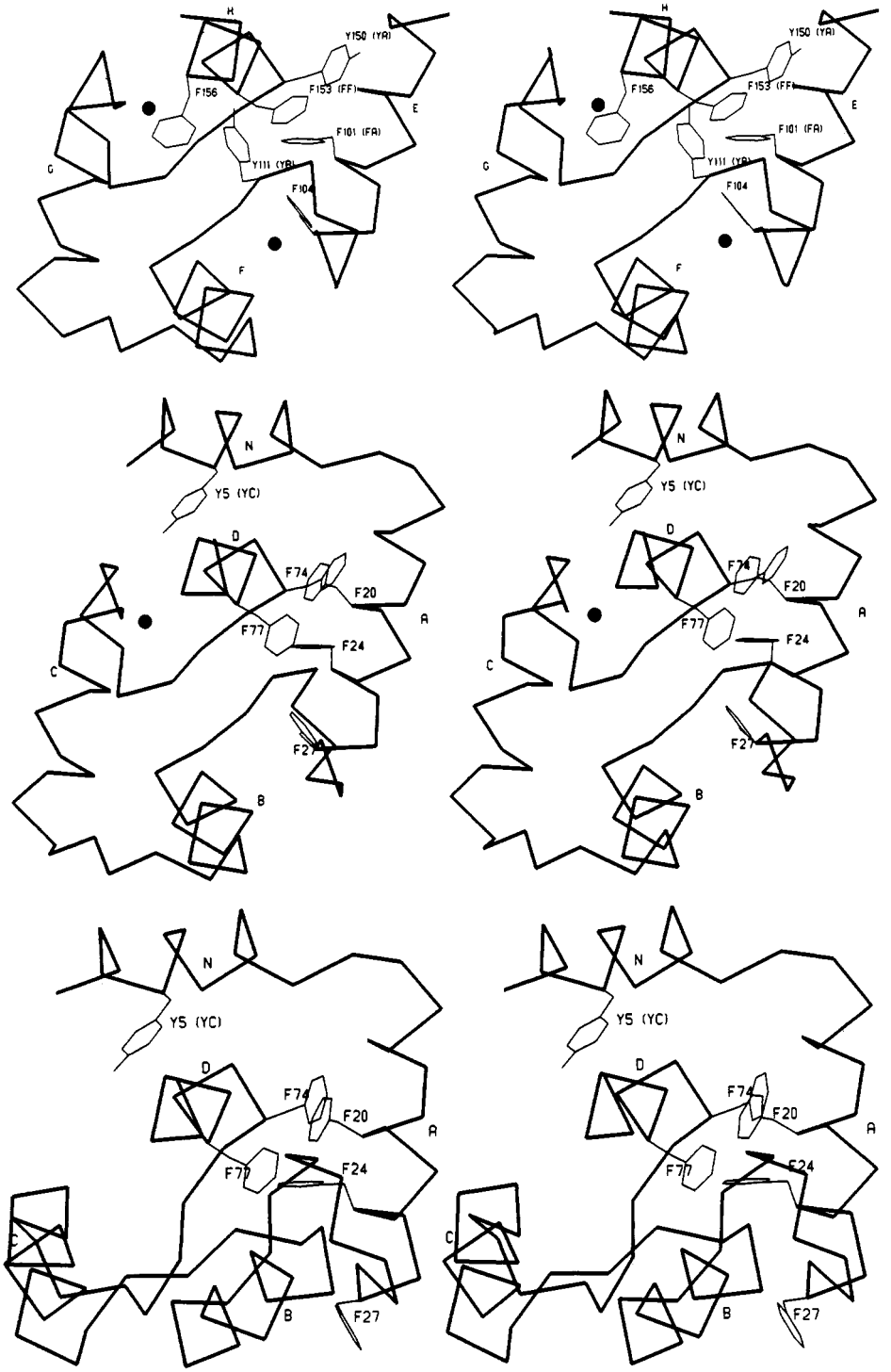

FIGURE 4: Stereographic views of the proposed molecular model of cTnC. Three stereographic views show the spatial relationships between aromatic side chains in (A, top) the C-terminal calcium bound form, (B, middle) the N-terminal calcium bound form, and (C, bottom) the N-terminal calcium-free form of cTnC3. All figures show the Cα backbone in thick lines and aromatic side chains in thin lines. Helices and side chains are labeled appropriately. Calcium ions are shown as black spheres.

al., 1983), calmodulin (Dalgarno et al., 1984), and sTnC (Drabikowski et al., 1985; Tsuda et al., 1988). On the basis of the similarity and consistency of the predicted and observed NOE patterns, Tyr-A and -B could be confidently assigned to Tyr-150 and -111, respectively, whereas Phe-A and Phe-F could be assigned to Phe-101 and -153, respectively (Figure 4A; Table I).

The assignment of Tyr residues reported here differs significantly from the assignments previously reported by Hincke et al. (1981), which were based on decoupling experiments, pH titrations, and gadolinium broadening experiments. Although the chemical shift assigned to Tyr-150 in Table I is consistent with this previous report, the assignments of Tyr-111 and Tyr-5 are inverted. Since Tyr-5 was unequivocally assigned here by mutagenesis, the assignment of Tyr-111 shown in Table I must be correct. Moreover, Ca^{2+} binding to high-affinity sites III and IV results in chemical shift changes in the Tyr-A and -B resonances (see below). This would be consistent with localization of Tyr-A and -B in the C-terminal domain and their assignment as Tyr-150 and -111, respectively.

A second set of interresidue NOEs (Figure 3A,B) was observed in both cTnC3 and CBM-IIA from Phe-B to Phe-C, Phe-D, and Phe-H; and from Phe-C to Phe-D. On the basis of the pattern of interresidue NOEs and comparison with

CBM-IIA, as well as Ca^{2+} titration experiments (see below), Phe-B, -C, -D, and -H could be confidently assigned to the N-terminal domain of cTnC. The pattern of internuclear NOEs was consistent with that predicted from the model, with the Phe residues forming part of the hydrophobic core within the N-terminal domain of both cTnC3 and CBM-IIA (Figure 4B, C). However, the pattern of interresidue NOEs was not sufficiently unique to permit sequential assignment of the N-terminal domain Phe residues. Spin systems assigned to Phe-E, -G, and -I could not be confidently assigned to either the N- or C-terminal domains of cTnC. Additionally, as expected there were no observable NOEs between aromatic residues assigned to the N-terminal domain with those assigned to the C-terminal domain.

Assignment or localization of the various aromatic resonances to the N- or C-terminal domain of cTnC and CBM-IIA was confirmed by monitoring chemical shift changes of the aromatic residues at various Ca^{2+}/protein molar ratio (data not shown). The addition of Ca^{2+} sufficient to fill either one or both high-affinity Ca^{2+}-binding sites resulted in increases in intensity of protons assigned to Tyr-111, Tyr-150, and Phe-101 in both cTnC3 and CBM-IIA.

Increasing the Ca^{2+}/protein molar ratio to 4 resulted in additional chemical shift changes of the aromatic resonances assigned of the N-terminal domain of cTnC3. However, in CBM-IIA, further chemical shift changes were not observed with the addition of Ca^{2+} sufficient to saturate site II in the wild-type protein. Thus, Ca^{2+}-dependent conformational changes resulting from Ca^{2+} binding at site II could clearly be distinguished from conformational changes induced by Ca^{2+} binding to the high-affinity sites III and IV. Chemical shifts of aromatic residues assigned to the C-terminal domain were not significantly altered upon Ca^{2+} binding at site II in wild-type cTnC3. The chemical shifts of Tyr-5 in cTnC3 and CBM-IIA were also not significantly altered upon the binding of Ca^{2+} (Figure 1). Thus, the N-terminal helix as judged by the aromatic resonances of Tyr-5 does not appear to be directly involved in the conformational changes that occur upon Ca^{2+} binding to monomeric cTnC proteins. This finding was predicted from the model of the Ca^{2+}-induced conformational change (Herzberg et al., 1986, 1987) in that the relative disposition of helices N, A, and D would not be changed in the calcium-free and calcium-bound forms of the molecule (compare Figure 4, panels B and C).

Effect of Ca^{2+} on β-Strands. Titration with Ca^{2+} also produced changes in the downfield-shifted CαH region, 4.9–5.4 ppm, of both cTnC3 and CBM-IIA (Figure 5). Low-field-shifted CαH resonances have been suggested to derive from residues involved in β-sheet structures (Dalgarno et al., 1983; Pardi et al., 1983) and in sTnC have been assigned to residues located in the short β-sheet between Ca^{2+}-binding sites III and IV (Drabikowski et al., 1985; Tsuda et al., 1988). Previously, differences in the downfield-shifted CαH resonances between the apo and Ca^{2+}-saturated forms of sTnC were attributed to the formation and/or stabilization of this β-sheet structure (Drabikowski et al., 1985).

To more closely examine Ca^{2+}-dependent differences in the β-strands located within the N- and C-terminal domains, TOCSY and NOESY spectra of the apo and Ca^{2+}-ligated forms of cTnC3, as well as Ca^{2+}-saturated CBM-IIA, were compared. On the basis of our model of cTnC and the crystal structure of sTnC, NOEs are expected between the CαH protons of Cys-35 and Asp-73, and Ser-37 and Thr-71 in the N-terminal domain, and between the CαH protons of Asp-113 and Arg-147, and Tyr-111 and Asp-149 in the C-terminal domain of cTnC3. From the TOCSY and NOESY spectra, several downfield-shifted CαH resonances in the various Ca^{2+}-ligated forms were assigned to the N- and C-terminal domains. The CαH protons of Tyr-111 and Asp-149 were assigned to 5.24 and 5.22 ppm on the basis of a CαH to CαH NOE as well as NOEs to the aromatic residues of Phe-101 and Tyr-111 (Figure 5C). Although these resonances could not be found in the apo form (FIgure 5A), no chemical shift changes in these resonances were observed between $2Ca^{2+}$/cTnC3 and the Ca^{2+}-saturated protein (Figure 5B,C; TOCSY spectra not shown). The CαH resonances at 5.21 and 5.11 ppm were assigned to Cys-35 and Asp-73 in Ca^{2+} saturated cTnC3 on the basis of an NOE between these CαH resonances and NOEs from both of these resonances to Phe-B (Figure 5C). Two other CαH to CαH NOEs, 4.75–4.81 ppm and 4.69–4.93 ppm, were assigned to the C- and N-terminal domain, respectively, on the basis of Ca^{2+} titrations and NOESY and TOCSY spectra. Thus, the pair of CαH resonances at 4.75 and 4.81 ppm belong to Asp-113 and Arg-147, and the pair at 4.69 and 4.93 ppm belong to Ser-37 and Thr-71. Similar NOE patterns were observed for the CαH resonances, 5.22–5.24 ppm and 4.74–4.81 ppm, located in the C-terminal domain β-strand structures of Ca^{2+}-saturated CBM-IIA (Figure 5D). NOE connectivities between the CαH resonances of Cys-35 and Asp-73, 5.34 and 5.30 ppm, and Ser-37 and Thr-71, 4.81 and 5.20 ppm, in Ca^{2+}-saturated CBM-IIA were observed despite the fact that the N-terminal domain of CBM-IIA can not bind Ca^{2+}. Thus, the β-strands in each of the two EF-hands in the N-terminal domain of CBM-IIA do exist and are spatially close even when both Ca^{2+}-binding sites are inactive (Figure 4C).

Discussion

Sequence-specific assignments of individual resonances are needed to compare in detail the solution conformations of proteins using NMR. Toward the goal of rapidly comparing conformational differences in a number of mutant cTnC proteins, we have initially chosen to study the aromatic and downfield-shifted CαH regions of the ^1H NMR spectrum of these proteins. We have used one- and two-dimensional NMR, molecular modeling, and site-specific mutagenesis for resonance assignments in these spectral regions. The sequence-specific assignments of Tyr-111 and Tyr-5 reported in Table I differ from those originally proposed by Hincke et al. (1981). The use of mutagenesis provides a compelling argument that the assignment of Tyr residues reported here are correct. The current assignments are consistent with the model for cTnC which predicts that binding of Ca^{2+} to the high-affinity sites would alter the chemical shifts of only Tyr-150 and Tyr-111 and not the chemical shifts of Tyr-5. More recently, the incorrect assignments of Hincke et al. (1981) were used to propose a model for the Ca^{2+}-saturated N-terminal domain of cTnC and to suggest a mode of binding for calcium-sensitizing drugs such as bepridil (MacLachlan et al., 1990a,b). This model must now be considered questionable since incorrect assignments were used as a basis for further sequence-specific resonance assignment and to obtain distance constraints for structure determination.

Differences in the tertiary structures of the Ca^{2+}-saturated forms of cTnC3 and CBM-IIA were studied using the assigned aromatic and CαH resonances. Comparison of the aromatic residue chemical shifts (Table I) reveals that only Phe residues assigned to the N-terminal domain show appreciable changes in chemical shift between the Ca^{2+}-saturated forms of cTnC3 and CBM-IIA. The similarity of the aromatic region of cTnC3 and CBM-IIA in the apo and Mg^{2+}-saturated forms

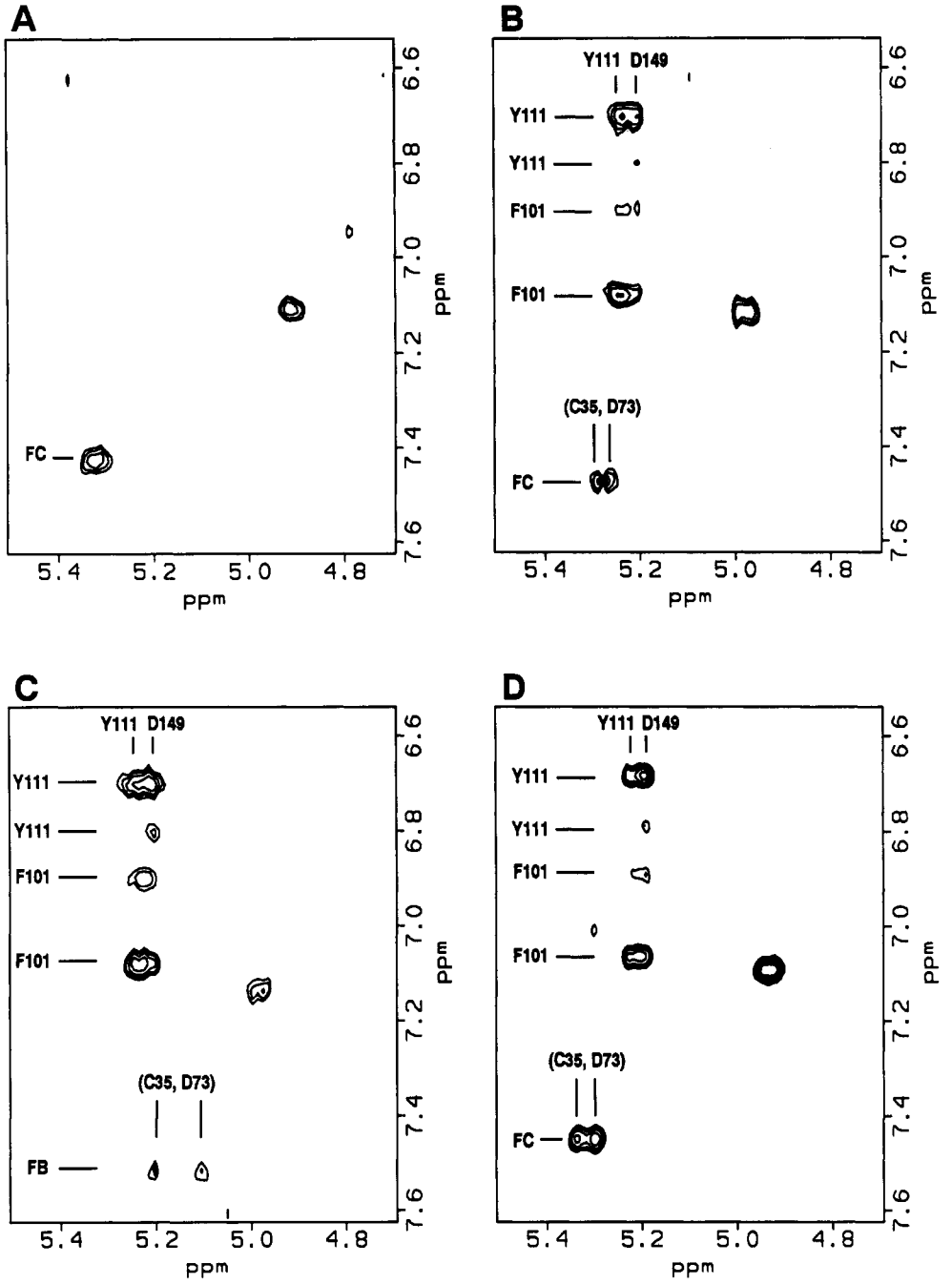

FIGURE 5: Portion of the 500-MHz phase-sensitive NOESY spectra of cTnC3 in absence of Ca^{2+} (A) and after addition of 1.8 (B) and 4 equiv of Ca^{2+} (C). Spectra were recorded with a 150-ms mixing time. The protein concentration was 0.6 mM, and other sample conditions were identical to those given in the legend to Figure 1. Also shown (D) is an equivalent region of the NOESY spectrum of Ca^{2+}-saturated CBMIIA obtained using the conditions given in the legend to Figure 3.

(Figure 1) as well as in Ca^{2+} titrations up to Ca^{2+}/protein ratios of 2 suggest that chemical shift differences observed in the Ca^{2+}-saturated forms of these two proteins result from Ca^{2+} binding at site II in cTnC3. The pattern of interresidue NOEs for the aromatic residues in the C-terminal domain are nearly identical in cTnC3 and CBM-IIA, further supporting the similarity of the C-terminal domain in both proteins. The orientation of the C-terminal domain aromatic cluster, deduced from NOEs, is in good agreement with the model of cTnC constructed from the crystal structure of sTnC.

In both cTnC3 and CBM-IIA, similar patterns of interresidue NOEs were observed between the aromatic rings of Phe-B, Phe-C, and Phe-D, which are assigned to the N-terminal domain hydrophobic core (Table I). Although this suggests that the spatial orientation of Phe-B, -C, and -D is not greatly altered by binding of Ca^{2+} to site II, structural changes in the N-terminal domain do occur as judged by the Ca^{2+} dependence of the chemical shifts of these aromatic residues. However, this does not appear to be associated with gross conformational changes and could result from a slight spatial reorientation of the aromatic residues within the hydrophobic core of the N-terminal domain as the positions of helices B and C change relative to helices A and D when Ca^{2+} binds at site II. The binding of Ca^{2+} to apocalbindin has been suggested to induce only subtle structural changes in the tertiary structure of the protein (Skelton et al., 1990). The absence of a major calcium-induced conformational change in calbindin 9K may not be surprising from both a structural and biochemical perspective. The three-dimensional structure of calbindin 9K (Szebenyi & Moffat, 1986; Skelton et al.,

1990) shows that unlike the calcium-bound domains of TnC and CaM there are no exposed hydrophobic surfaces in calbindin 9K. This is a result of the different interhelical angles and the larger size and conformation of the interhelical linker in calbindin 9K as compared to TnC and CaM (Strynadka & James, 1989). Furthermore, in keeping with the observed lack of an exposed hydrophobic surface in the calcium bound form of calbindin 9K and contrary to observations made in the TnC and CaM systems, there is no biophysical or biochemical evidence that calbindin 9K interacts with or modulates the activity of a target protein or that hydrophobic surfaces are exposed in calbindin 9K upon calcium uptake. Therefore, extrapolation of results monitoring the very subtle calcium-induced conformational change in calbindin 9K are probably not relevant to those expected in TnC and CaM.

The similar orientation of aromatic residues in the N-terminal domain of both Ca^{2+}-saturated cTnC3 and CBM-IIA, as well as the observed chemical shift changes in the aromatic residues, are consistent with the proposed model for the Ca^{2+}-induced conformational change (Herzberg et al., 1986; Strynadka & James, 1989; see Figure 4B,C). This model predicts a reorientation of the residues within the Ca^{2+}-binding loop upon Ca^{2+} binding and thus is consistent with the observed chemical shift changes in residues assigned to the N-terminus. In the N-terminal domain of cTnC, Phe-20, Phe-24, and Phe-27 are located in helix A while Phe-74 and Phe-77 are located in helix D. There are no aromatic residues in helices B and C. The proposed model for Ca^{2+} binding requires the movement of helices B and C and the linker peptide between them by up to 14 Å, while the N-terminal helix and helices A and D retain their relative positions (Herzberg et al., 1986; Strynadka & James, 1989). Thus, Ca^{2+} binding would not be expected to alter significantly the spatial orientation and hence the interresidue NOEs among aromatic residues located in helices A and D. However, the proposed large movement of helices B and C would be expected to produce significant chemical shift changes as a consequence of changes in the magnetic environment of amino acid residues located in the N-terminal domain. As there are no aromatic groups on helices B and C (Figure 4) this movement cannot be directly monitored in this study.

The short β-strand structures derived from the helix–loop–helix Ca^{2+}-binding loops in the N-terminal domain of cTnC3 and CBM-IIA are present regardless of the ability of the binding loops to coordinate Ca^{2+}. Short β-sheet structures formed between helix–loop–helix units have been identified in the crystal structures of parvalbumin (Moews & Kretsinger, 1975), calmodulin (Babu et al., 1988), calbindin (Szebenyi & Moffat, 1986; Skelton et al., 1990), and sTnC (Herzberg & James, 1988; Satyshur et al., 1988). In the crystal structure of sTnC which does not have Ca^{2+} bound at sites I and II and in the apo form of calbindin, these short β-sheet structures are still present between the two Ca^{2+}-binding sites. Additionally, as a consequence of the unique CαH chemical shifts of residues involved in β-strands, NMR has been extensively used to study these structures in calmodulin (Ikura et al., 1987), parvalbumin (Padilla et al., 1988), calbindin (Drakenberg et al., 1989; Skelton et al., 1990), and the C-terminal domain of sTnC (Tsuda et al., 1988). Using the assignments of CαH resonances located in the β-strands of cTnC3 and CBM-IIA, conformational changes within these structures as a consequence of Ca^{2+} binding were evaluated. The nearly identical chemical shifts and pattern of NOEs observed for the CαH resonances of Tyr-111, Asp-149, Arg-147, and Asp-113 in both cTnC3 and CBM-IIA (Figure 5) suggest that the β-strands in the C-terminal domain of the Ca^{2+}-saturated forms of the two proteins are nearly identical. Although chemical shift differences exist between cTnC3 and CBM-IIA for residues located in the N-terminal domain, careful evaluation of the CαH chemical shifts and NOEs for Cys-35, Asp-73, Ser-37, and Thr-71 shows that the β-strand in each one of the helix–loop–helix units is still present in CBM-IIA and these β-strands are spatially close (Figures 4 and 5). Thus, even in the absence of an active Ca^{2+}-binding site in the N-terminal domain of CBM-IIA, the short β-strand structures in each one of the EF-hands are formed and they are spatially close.

Thus, our results suggest that a conformational change occurs in the N-terminal domain upon Ca^{2+} binding but that aromatic residues within the hydrophobic core are not grossly affected. At this time, we can not yet determine if these differences reflect changes such as reorientation of the helices upon Ca^{2+} binding as proposed by Herzberg et al. (1986) and Strynadka and James (1989). In addition, differences in the flexibility and dynamics of the N-terminal helix in the presence and absence of Ca^{2+} may also play an important role. The availability of a cTnC3 mutant which can not bind Ca^{2+} at site II will facilitate future studies aimed at understanding at an atomic level the molecular events responsible for triggering muscle contraction. Sequential resonance assignment of cTnC3 using stable isotope enrichment and multidimensional NMR is currently in progress. Finally, the NMR results presented here demonstrate that the inability of CBM-IIA to trigger muscle contraction is the result of its inability to bind Ca^{2+} at site II and not the result of the specific mutation on the conformation or stability of the protein.

Registry No. Phe, 63-91-2; Tyr, 60-18-4; Asp, 56-84-8; Ca, 7440-70-2; Mg, 7439-95-4.

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