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Nuclear Magnetic Resonance Study on Rabbit Skeletal Troponin C: Calcium-Induced Conformational Change†

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ABSTRACT: Rabbit skeletal muscle troponin C (TnC) was investigated by means of ^{1}H NMR in the presence of dithiothreitol that prevents dimerization of the protein. Two-dimensional (2D) ^{1}H NMR spectra were observed in order to assign resonances to specific amino acids. One-dimensional ^{1}H NMR spectra were observed as a function of Ca^{2+} concentration. The Ca^{2+}-induced spectral change is categorized into two types: type 1 corresponds to the conformational change of the C-terminal-half domain (Ca^{2+} high-affinity sites) and type 2 to that of the N-terminal-half domain (Ca^{2+} low-affinity sites). From the 2D NMR spectra and Ca^{2+} titration data, it was suggested that (1) amide protons of Gly-108, Ile-110, Gly-144, and Ile-146 are hydrogen-bonded when the C-terminal-half domain binds 2 mol of Ca^{2+} and (2) hydrogen bonds of Gly-108, Ile-110, Gly-144, and Ile-146 are destroyed or weakened when the C-terminal-half domain releases 2 mol of Ca^{2+}. Nuclear Overhauser enhancement difference spectra as well as the Ca^{2+} titration data suggested that a hydrophobic cluster is formed in the C-terminal-half domain when the C-terminal-half domain binds 2 mol of Ca^{2+}. A hydrophobic cluster exists in the N-terminal-half domain without regard to Ca^{2+} binding to the N-terminal-half domain. The spectra of Tyr-10 showed both types of spectral change during the Ca^{2+} titration. The results suggested that Tyr-10 of apo-TnC interacts with the C-terminal-half domain.

Troponin C (TnC)[1] is a component of the troponin complex protein in thin filaments of myofibril, together with the other components TnI and TnT (Ebashi, 1974). The binding of Ca^{2+} to TnC induces a conformational change. This conformational change is transmitted to the other two components and results in a release of the inhibition of the actin–myosin interaction, which triggers the contractile activity (Gergely & Leavis, 1980).

Rabbit skeletal muscle TnC is a single polypeptide of 159 residues (Collins et al., 1975) and contains four calcium binding sites, two low-affinity Ca^{2+}-specific sites ($K_{Ca^{2+}} = 2 \times 10^5$ M^{-1}) and two high-affinity Ca^{2+} sites which can bind also Mg^{2+} ($K_{Ca^{2+}} = 2 \times 10^7$ M^{-1}, $K_{Mg^{2+}} = 5 \times 10^4$ M^{-1}) (Potter & Gergely, 1975). On the basis of the crystal structure of parvalbumin, Kretsinger and Barry predicted that the four Ca^{2+}-binding sites of TnC are in the "helix–loop–helix" conformation (Kretsinger & Barry, 1975). Sin and co-workers

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[1] Abbreviations: NMR, nuclear magnetic resonance; TnC, calcium binding component of troponin; TnI, inhibitory component of troponin; TnT, tropomyosin binding component of troponin; Ca_2TnC, TnC with 2 mol of calcium at high-affinity sites; Ca_4TnC, TnC with 4 mol of calcium at high- and low-affinity sites; CaM, calmodulin; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; β-Me, β-mercaptoethanol; 2D, two-dimensional; COSY, correlated spectroscopy; NOE, nuclear Overhauser enhancement effect; NOESY, nuclear Overhauser enhancement effect spectroscopy; RCT, relayed coherence transfer in COSY; COCONOSY, combined COSY/NOESY experiment; QD, quadrature detection; TSP-d_4, (trimethylsilyl)propionic-d_4 acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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suggested that sites I and II in the N-terminal half are the low-affinity sites and sites III and IV in the C-terminal half are the high-affinity sites (Sin et al., 1978).

NMR studies have been reported on the intact protein (Levine et al., 1977, 1978; Seamon et al., 1977; Andersson, et al., 1982; Teleman et al., 1983) and also on proteolytic fragments (Birnbaum & Sykes, 1978; Evans et al., 1980; Leavis et al., 1982; Drabikowski et al., 1985). These studies indicate that when Ca^{2+} is progressively added to apo-TnC, sites III and IV are filled with Ca^{2+} first and sites I and II second.

Margossian and Stafford showed that TnC undergoes Ca^{2+}-induced dimerization (Margossian & Stafford, 1982). Thus, it is likely that previous NMR studies were done for the dimer of TnC.

Recently, X-ray structure analyses at 2.8-Å resolution were reported of crystals of chicken and turkey skeletal muscle TnC's with two calcium ions at sites III and IV (Sundaralingam et al., 1985; Hertzberg et al., 1985a,b). It was shown that TnC with two calcium ions assumes a rather unique dumbbell-like structure.

It is our intention to examine ^1H NMR spectra of the TnC monomer and to clarify the conformational change of this protein accompanying Ca^{2+} binding in more detail.

Experimental Procedures

Rabbit skeletal muscle TnC was obtained by procedures reported previously (Szynkiewicz et al., 1984). The absence of the dimer in 1 mM TnC aqueous solution used in the present NMR work was examined by SDS and native polyacrylamide gel electrophoresis (PAGE) in the presence and the absence of reductant: β-mercaptoethanol (β-ME) or dithiothreitol (DTT).

^1H NMR spectra were obtained on JEOL JNM-GX400 and JNM-GX500 spectrometers operating at frequencies of 400 and 500 MHz, respectively. Two-dimensional NMR spectra of COSY, relayed COSY, and NOESY were observed with pulse sequences described previously (Bax & Freeman, 1981; Kumar et al., 1980; Eich et al., 1982). In most cases, COSY and NOESY spectra were simultaneously observed by the COCONOSY method (Haasnoot et al., 1984; Gurevich et al., 1984). The mixing time was 28 ms for relayed COSY and 185 ms for NOESY. A time domain data matrix of 256 × 2048 points was in most cases expanded to 1024 × 2048 points by "zero filling" in the t_1 dimension. Chemical shifts were measured from an internal standard of TSP-d_4. All spectra were recorded at a temperature of 40 °C.

Ca^{2+} titration was performed by the addition of aliquots of 80 mM $CaCl_2$ (pH 7.5) solution to a 1 mM aqueous solution of apo-TnC. The absence of calcium in apo-TnC was confirmed by a Hitachi 208 atomic absorption spectrometer. pH titration was carried out by the addition of 4% KOD or 4% DCl solution to the NMR samples. pH values quoted here are direct meter readings measured on a Horiba F7CL pH meter and are not corrected for the deuterium isotope effect.

Results

Effect of Reductant on Spectra. Figure 1 shows patterns of electrophoresis of apo-TnC as a function of the concentration of reductant. In the absence of reductant there appear two bands corresponding to the dimer and monomer. Upon addition of reductant, the dimer band disappears, and only the monomer band remains. Ca^{2+}-saturated TnC (Ca_4TnC) required a larger amount of reductant to give only the monomer band than apo-TnC. These findings suggest that a disulfide bridge at Cys-98 is formed between two molecules (rabbit

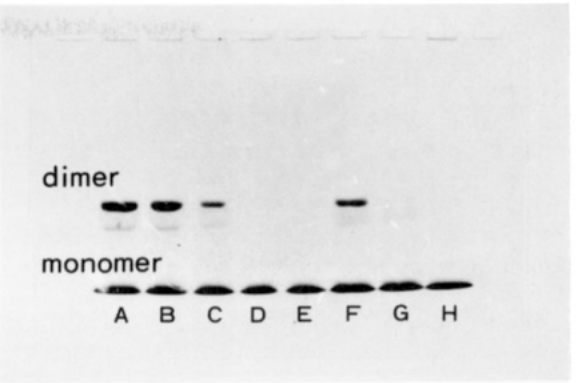

FIGURE 1: Electrophoretograms of the dimer and monomer of troponin C in 12.5% polyacrylamide gels. Lanes A, B, C, and F show dimer and monomer bands. Lanes D, E, G, and H show monomer band only. [TnC] = 1 mM. (A) No EGTA; (B) no EGTA, 1 mM β-ME; (C) 0.1 mM EGTA, 10 mM β-ME; (D) 0.1 mM EGTA, 20 mM β-ME; (E) 0.1 mM EGTA, 30 mM β-ME; (F) 0.1 mM EGTA, 3 mM DTT; (G) 0.1 mM EGTA, 7 mM DTT; (H) 0.1 mM EGTA 10 mM β-ME, 4 mM DTT.

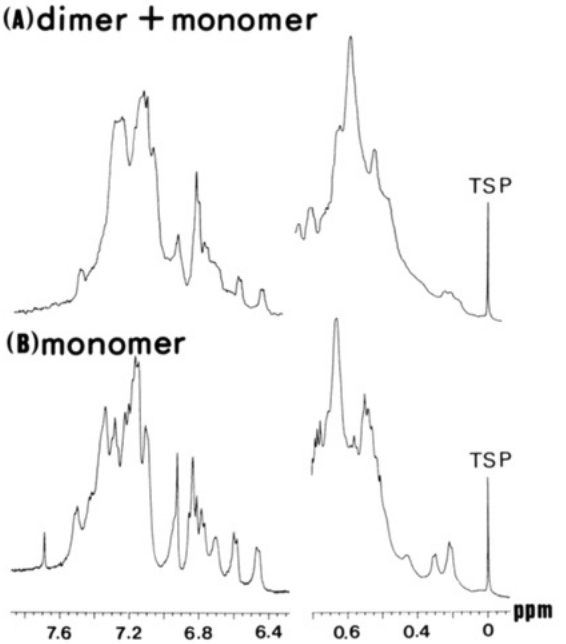

FIGURE 2: Aromatic and high-field methyl region of 400-MHz ^1H NMR spectra of Ca_4TnC in D_2O: [TnC] = 1 mM, 0.2 M KCl, pH 8.5, and 40 °C. (A) [DTT] = 0 mM; (B) [DTT] = 10 mM.

skeletal muscle TnC contains only one cysteine at the 98th position). In the presence of DTT, ^1H NMR spectra of TnC consisted of rather sharp and well-resolved peaks and were somewhat different from those in the absence of DTT (Figure 2).

Levine et al. (1977) and Seamon et al. (1977) studied the change of ^1H NMR spectra of TnC in the course of Ca^{2+} titration. We also carried out a Ca^{2+} titration experiment of NMR spectra for TnC dimer without reductant. Spectral changes observed here for the dimer were very similar to those observed by Levine et al. and by Seamon et al. In particular, the spectra of the Ca_4TnC dimer in the present study remarkably resemble those observed by them, suggesting that the previous studies were done for TnC dimer or the mixture of dimer and monomer. All NMR measurements in the following were done for TnC monomer solutions containing DTT.

Assignments of TnC Monomer Spectra. Analysis of amino acid spin systems was carried out by COSY, NOESY, and RCT in D_2O solution (all exchangeable hydrogens were re-

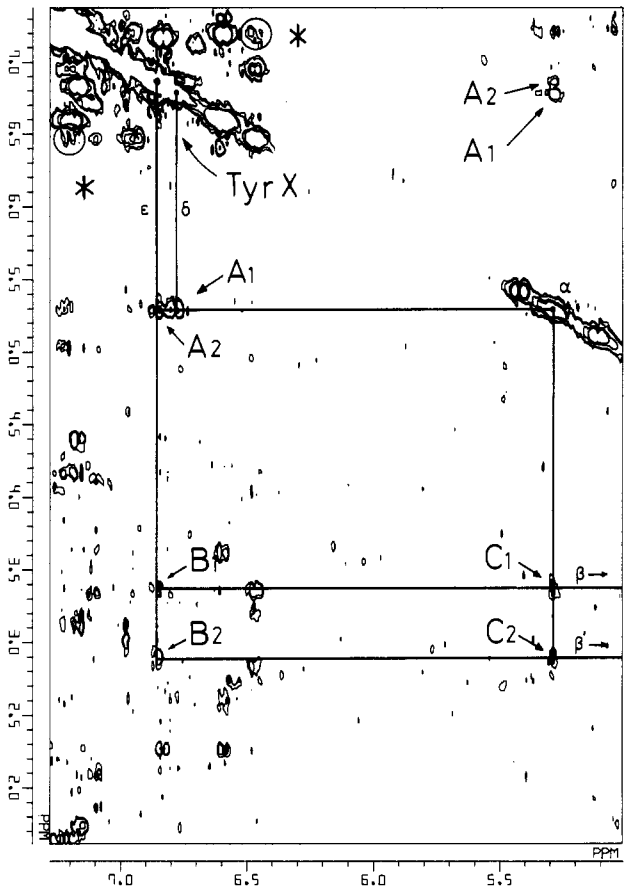

FIGURE 3: Expanded view of 400-MHz NOESY spectrum of Ca_4TnC in D_2O: [TnC] = 1.8 mM, [DTT] = 20 mM, 0.2 M KCl, pH 7.8, and 40 °C. A_1 and A_2 represent intraresidue NOE cross-peaks between ring protons and the α-proton of Tyr-X. B_1 and B_2 represent cross-peaks between the ϵ ring proton and β-protons of Tyr-X. C_1 and C_2 represent cross-peaks between the α-proton and β-protons of Tyr-X. NOE cross-peaks marked by (*) indicate the proximity of the δ-protons of Phe-99 and Phe-148.

placed with deuteriums). The following proton signals were identified by use of COSY from their unique spin–spin coupling connectivities: methine, methylene,and methyl protons of Gly, Ala, Thr, Val, Ile, Phe, His, Tyr, Ser, Cys, Asp, and Asn and aromatic ring protons of Phe, His, and Tyr (TnC has no Trp). Ser, Cys, Asp, and Asn belong to the same spin system of the so-called AMX type.

For Ca_4TnC, resonances of the following protons were assigned by the use of COSY: (1) ring protons of His-125 (TnC of rabbit muscle has only one His), Tyr-X, Tyr-Y, Phe-A, Phe-B, Phe-C, and Phe-D, (2) α-protons of Ala-A, Ile-A, Ile-B, eight Gly's, and 12 AMX spin systems, (3) methylene protons of Tyr-X, Tyr-Y, and 12 AMX spin systems, and (4) methyl protons of Ile-A, Ile-B, and six Met's.

NOESY was used to identify the through-space connectivity between self-closed spin systems such as aromatic ring protons and their α-methine and β-methylene protons. Figure 3 demonstrates an example of spectral analysis of NOESY. δ and ϵ ring protons of Tyr-X in Figure 3 were distinguished by pH titration [pK_a = 11.7 (Tyr-X), pK_a = 11.9 (Tyr-Y)]. A_1 and A_2 represent NOE cross-peaks between ring protons [6.76 ppm (δ), 6.86 ppm (ϵ)] and the α-methine proton (5.29 ppm) of Tyr-X. B_1 and B_2 represent intraresidue NOE cross-peaks between ϵ ring protons and β-methylene protons (2.90 and 3.35 ppm). Cross-peaks C_1 and C_2 which are also observed in COSY are due to intraresidue NOE connectivities among α- and β-protons of Tyr-X. Consequently, we can assign the spin system of Tyr-X.

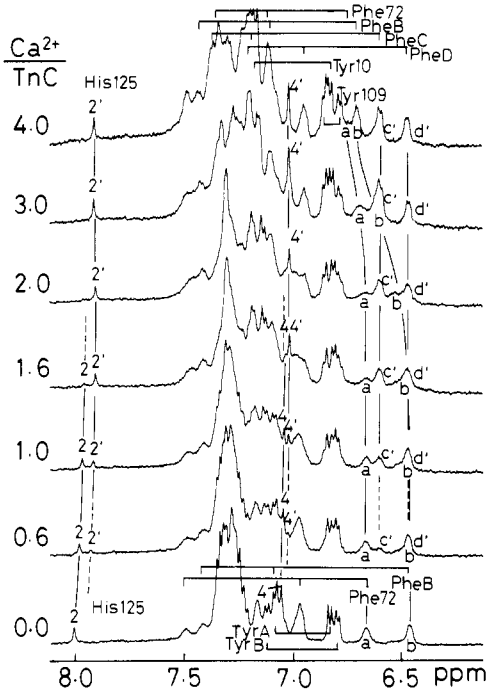

FIGURE 4: Aromatic regions of 500-MHz ^{1}H NMR spectra of rabbit skeletal muscle TnC in D_2O as a function of molar ratio of Ca^{2+}/TnC: [TnC] = 1 mM, [DTT] = 10 mM, 0.2 M KCl, pH 7.5, and 40 °C.

We assigned α-protons of Tyr-X, Tyr-Y, Phe-C, Phe-D, and His-125, methylene protons of Tyr-X, Tyr-Y, Phe-C, and Phe-D, and γ-methyl protons of Ile-C and Ile-D.

The sequential individual resonance assignment of backbone protons was carried out by a combined use of COSY and NOESY in H_2O solution. Two types of the NOE connectivity were detected: d_1 (from NH_{i+1} to CH_i) and d_2 (from NH_{i+1} to NH_i) connectivities as referred to in the notation used by Wüthrich et al. (1982).

For Ca_4TnC, the following two fragmental sequences were identified: xxx-Ala^A-xxx-Gly^A-Tyr^X-Ile^A-xxx (xxx's are any one of Asp, Asn, and Ser) and Gly^B-yyy-Ile^B (yyy is any one of Glu, Gln, Pro, Met, Arg, and Lys). On the basis of the amino acid sequence of TnC, the two sequences can be assigned to Asn^{105}-Ala^{106}-Asp^{107}-Gly^{108}-Tyr^{109}-Ile^{110}-Asp^{111} and Gly^{144}-Arg^{145}-Ile^{146}, respectively.

For apo-TnC, spectral analysis was done in a similar way. δ- and ϵ-protons of two tyrosines (Tyr-A, Tyr-B) were identified by pH titration (for both Tyr's, pK_a = 11.3). Rabbit skeletal muscle TnC has two tyrosines, Tyr-10 and Tyr-109. At this stage we could not identify of Tyr-10 and Tyr-109. A sequence of Thr^A-zzz-qqq-Phe^A (zzz is either of Ala, Thr, Val, Leu, or Ile and qqq is either of Asp, Asn, Ser, or Cys) was found. This sequence can be assigned to Thr^{69}-Ile^{70}-Asp^{71}-Phe^{72} by reference to the primary structure.

Ca^{2+}-Induced Spectral Change of TnC. ^{1}H NMR spectra of TnC were observed at various stages of Ca^{2+} binding. Figure 4 shows the aromatic parts of the spectra as a function of the molar ratio of Ca^{2+}:TnC. The spectrum of Ca_4TnC is shown at the top and that of apo-TnC at the bottom. Upon addition of calcium ion up to 2 mol per TnC, the His-125 C2 proton signal at 8.01 ppm of apo-TnC (peak 2) decreases in intensity and disappears at 2 mol of Ca^{2+} per TnC. Simultaneously, the C2 proton signal of TnC with two calciums in the C-terminal-half domain (Ca_2TnC) (peak 2′) appears at 7.91 ppm and grows in intensity with increasing Ca^{2+} content. The His-125 C4 proton signal at 7.06 ppm of apo-TnC (peak 4) also decreases in intensity, and the signal of Ca_2TnC (peak

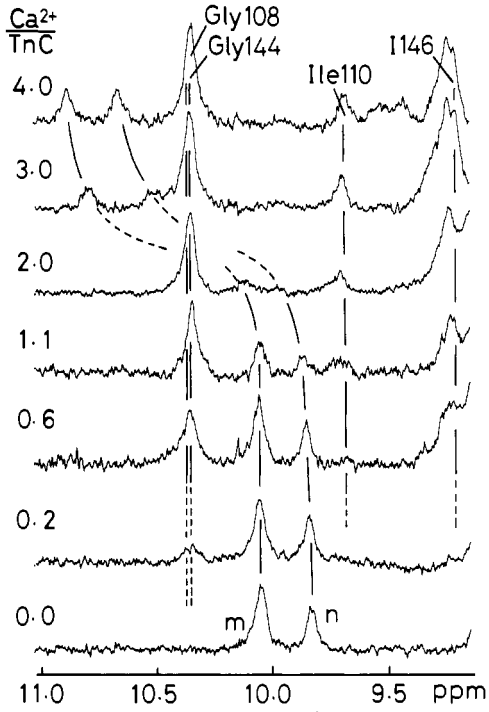

FIGURE 5: Low-field region of 500-MHz ^{1}H NMR spectra of rabbit skeletal muscle TnC as a function of molar ratio of Ca^{2+}/TnC. Experimental conditions are same as described in Figure 4.

4′) appears at 7.92 ppm. These results indicate the slow exchange between apo-TnC and Ca_2TnC.

Two resolved peaks c′ (6.59 ppm) and d′ (6.47 ppm) that are assignable respectively to δ-protons of Phe-C and Phe-D of Ca_2TnC emerge during the first half of titration. This observation is also attributed to the slow exchange process. For apo-TnC these two phenylalanine δ-proton resonances are presumably in the crowded region of 7.0–7.3 ppm.

Resonances of aromatic protons of Phe-72 and Phe-B (peaks a and b) do not change at Ca^{2+} contents of 0–2 mol per TnC. With increasing Ca^{2+} content from 2 to 4 mol, these phenylalanine resonances shift continuously to lower fields. These results indicate that fast conformational exchange occurs between Ca_2TnC and Ca_4TnC. An increase of the Ca^{2+} content from 2 to 4 mol does not cause any further change in the resonances of His-125, Phe-C, and Phe-D.

Our results of His-125 are rather similar to the results of Levine et al. (1977) although they did not use DTT. Our results are, however, at variance with those reported by Seamon et al. (1977). They reported that histidine ring protons did not show spectral change during the first half of titration.

Figure 5 shows the low-field amide proton region at various Ca^{2+} contents. At Ca^{2+} contents of 0–2 mol, amide proton signals of Gly-108, Gly-144, Ile-110, and Ile-146 vary in a slow-exchange manner, while at Ca^{2+} contents of 2–4 mol other amide proton resonances denoted as m and n in the figure shift to lower fields in a fast-exchange manner.

Figure 6 shows an expanded view of two tyrosine ring proton resonances as a function of Ca^{2+}/TnC. During the first half of titration, three doublet tyrosine resonances newly appear at 6.86 (p), 6.82 (q), and 6.76 ppm (r) and grow in intensity at the expense of doublet resonances of δ-protons of Tyr-A (6.83 ppm) and Tyr-B (6.80 ppm) in the slow-exchange manner. During the last half of the titration, the resonances of p and r do not show any shift, while only peak q shifts downfield sigmoidally to 6.82 ppm. The sequential assignment indicates that two doublet resonances, p (6.86 ppm) and r (6.76

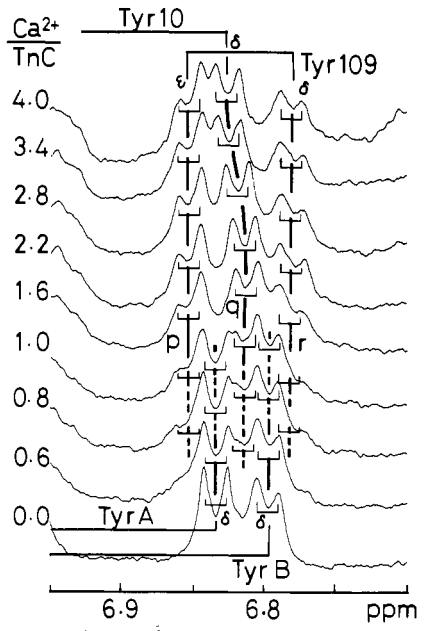

FIGURE 6: Expanded view of the Tyr-10 and Tyr-109 resonance part of the 500-MHz ^{1}H NMR spectra as a function of the molar ratio of Ca^{2+}/TnC. Experimental conditions are same as described in Figure 4.

ppm), arise from, respectively, ε- and δ-protons of Tyr-109. Thus, the doublet peak r (6.82 ppm) and another doublet peak at 7.16 ppm (not shown) can be assigned to δ- and ε-protons of Tyr-10, respectively. The present assignments of Tyr-109 and Tyr-10 of Ca_4TnC are not consistent with the results reported by Levine et al. (1977) but are in accordance with those on the tryptic fragments of TnC by Levine and Dalgarno (1983). It is of interest to note that resonances of Tyr-10 change in a slow-exchange manner during the first half of the titration and in a fast-exchange manner during the last half of the titration.

In order to clarify the characteristics of the hydrophobic cluster, we examined NOE, which represents the closeness of two residues. Figure 7 shows NOE difference spectra in which the spectrum observed during irradiation of a peak is subtracted from the normal spectrum. In spectrum b in which the doublet peak h of Phe-72 δ-protons was irradiated, the intraresidue NOE effects were observed on peaks i and m of Phe-72, and interresidue enhancement was observed on peaks g and j of Phe-B and peaks a and b of Ile-A. In spectrum c in which doublet peak g of Phe-B was irradiated, the intraresidue NOE effects were observed on peaks j and l of Phe-B, and interresidue effects appeared on peaks h and i of Phe-72 and peak k of Phe-Z. No NOE effect was observed on peaks of Ile-A. In spectrum d in which peak a of the γ-methyl proton of Ile-A was irradiated, the effects were observed on peaks h, i, and m of Phe-72, peak g of Phe-B, peak k of Phe-Z, peak f of Asp-71, unknown peak e, and peak c of Val-A.

Discussion

Hydrophobic Cluster. For apo-TnC, ring protons of Phe-72 and Phe-B resonate at higher fields than those of other Phe's as shown in Figures 4 and 7. γ-Methyl protons of Ile-A are observed at the highest field (−0.10 ppm). Such secondary high-field shifts observed for Phe-72, Phe-B, and Ile-A are probably due to the ring current effect, suggesting that these residues are closely assembled. Figure 8a is a schematic diagram of the NOE for apo-TnC, in which the relationship of residues connected by NOE as revealed by NOESY and NOE difference experiments is shown. These results indicate an

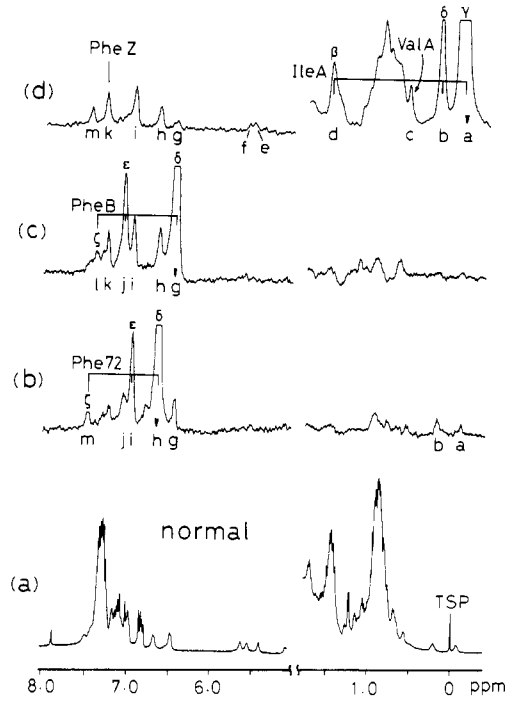

FIGURE 7: Aromatic and aliphatic regions of 400-MHz ^1H NMR NOE difference spectra of apo-TnC: [TnC] = 1 mM, [DTT] = 10 mM, 0.2 M KCl, pH 8.0, and 40 °C. Peak symbols are marked under the spectrum. Assignments are indicated. (a) Control spectrum with no irradiation. (b) Irradiation at 6.66 ppm (peak h) of high-field-shifted Phe-72 resonance. (c) Irradiation at 6.46 ppm (peak g) of high-field-shifted Phe-B resonance. (d) Irradiation at −0.10 ppm (peak a) of high-field-shifted Ile-A resonance. Negative NOEs are phased so as to give positive peaks.

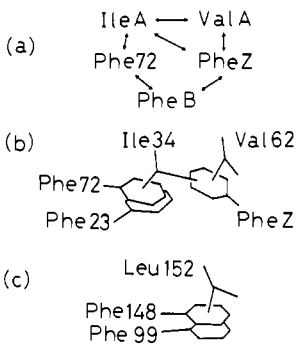

FIGURE 8: (a) NOE schematic diagram of hydrophobic residues of apo-TnC. (b) Spatial arrangements of amino acids of the N-terminal-half domain of apo-TnC and Ca_4TnC. (c) Spatial arrangements of amino acids of the C-terminal domain of Ca_4TnC.

existence of a cluster of hydrophobic residues including Phe-72, Phe-B, Phe-Z, Ile-A, and val-A. It can be suggested that the hydrophobic cluster consisting of these residues is similar to that observed for apo-CaM (Ikura et al., 1983). Taking into account the NOE diagram shown in Figure 8a as well as the sequential homology between TnC and CaM, Phe-B is presumably assigned to Phe-23. Evans et al. (1980) noted that this phenylalanine was either Phe-19, -23, -26, -72, or -75. The analogy of the appearance of interresidue NOE between TnC and CaM allows us to assign Val-A and Ile-A, respectively, to Val-62 and Ile-34. Evans et al. (1980) assigned the δ-methyl proton doublet signal (0.20 ppm) of Ile-A to one of Ile-16, -34, -58, -59, and -70.

The NOE diagram obtained for apo-TnC leads to the following conclusions: (1) side chains of Phe-72 and Phe-23 are themselves oriented so as to bring their ring planes into partial overlap; (2) γ- and δ-methyl groups of Ile-34 are directed toward the rings of Phe-72 and Phe-Z; (3) the side chain of Val-62 is situated on the ring plane of Phe-Z. The spatial arrangement of these amino acids of apo-TnC is schematically depicted in Figure 8b. Such arrangements of hydrophobic residues in the N-terminal-half domain of apo-TnC are similar to those in the crystal structure (Sundaralingam et al., 1985; Herzberg & James, 1985a).

For Ca_4TnC, interresidue NOE effects between ring protons of Phe-C and -D were observed as evidenced by the cross-peaks marked by asterisks in Figure 3. An NOE was also observed between ring protons of Phe-C and methyl protons of either of Val, Leu, or Ile. Taking into account the homology between TnC and CaM and the crystallographic results, it is probable that these hydrophobic residues are Phe-148 (C), Phe-99 (D), and Leu-152. Assignments of the two phenylalanines are same as those reported on the C-terminal tryptic fragments of TnC by Drabikowski et al. (1985). The structural implication obtained here is shown in Figure 8c.

Drabikowski et al. (1985) also reported on the basis of NOE experiments that the aromatic ring of Phe-99 points toward the β-sheet segment between two calcium-binding loops of the C-terminal fragment with two calciums. As shown in Figure 7d, noticeable NOEs were observed for the N-terminal-half domain of apo-TnC among methyl proton signals of Ile-34 and α-proton signals of Asp-71 and an extremely low-field-shifted α-proton signal of an unknown residue (peak e of 5.62 ppm in Figure 7d). Thus, it can be said that the N-terminal-half domain of apo-TnC and the C-terminal-half domain of Ca_4TnC have similar hydrophobic regions.

Table I shows the chemical shift values of some resonances assigned on the basis of the sequential assignment and the comparison of NOE diagrams between TnC and CaM.

β-Sheet Structure. In order to obtain information about the conformation of TnC, three empirical rules are utilized: (1) the extreme low-field shift of the amide proton resonance is indicative of the formation of a hydrogen bond (Pardi et al., 1983; Wagner et al., 1983; Dalgarno et al., 1983a) (2) the extreme low-field shift of the α-proton resonance is a sign of the formation of β-sheet structure (Dalgarno et al., 1983a,b); (3) NOE can be observed between two close α-protons in the β-sheet conformation as judged from the crystal structure (Moews & Kretsinger, 1975; Szebenyi et al., 1981).

For Ca_4TnC, as shown in Figure 5 and Table I, amide protons of Ile-110 and Ile-146 appear at extremely low fields, suggesting that these amide protons form hydrogen bonds. α-Protons of Tyr-109, Ile-110, Arg-145, and Ile-146 appear at extremely low fields, implying that these residues form β-sheet structure. NOE was observed between α-protons of Asp-111 and Arg-145 (data not shown), indicating the spatial proximity of these protons.

Drabikowski et al. (1985) reported that a part of the C-terminal-half tryptic fragment of TnC assumes antiparallel β-sheet structure in solution. The present results as well as Drabikowski's results indicate that the twin-strand antiparallel β-sheet structure is formed between the two calcium-binding loops of the C-terminal half of Ca_4TnC and that the loops are connected by two hydrogen bonds between Ile-110 and Ile-146, one from Ile-110 NH to Ile-146 CO and the other from Ile-146 NH to Ile-110 CO. These results are comparable to those reported previously for the C-terminal-half fragment of CaM by Ikura et al. (1985). They reported that hydrogen bonds are formed between Ile-100 and Val-136 of the C-terminal fragment of CaM with two Ca^{2+}, and these two residues correspond to Ile-110 and Ile-146 of TnC.

In the case of apo-TnC, as shown in Figure 5, amide proton resonances of Ile-110 and Ile-146 do not appear at extremely

Table I: Chemical Shifts (ppm) of Assigned Resonances of Rabbit Skeletal Muscle Troponin C[a]

residue	no.	NH	α	β	others
			(a) Apo-TnC		
Tyr-A				2.98	6.83 (δ), 7.08 (ϵ)
Phe-B	23		3.54	2.51, 2.70	6.46 (δ), 7.08 (ϵ), 7.41 (ζ)
Ile-A	34			1.46	0.74 and 0.98 (γ), 0.20 (γ), −0.10 (δ)
Val-A	62				0.56 (γ), 1.77 (β)
Thr-A	69	7.96	5.39	4.02	1.02 (γ)
Ile	70	8.70	4.84	1.85	0.98 (γ)
Asp	71	8.40	5.66	2.82, 3.26	
Phe-A	72	8.41	4.05	3.14, 3.30	6.66 (γ), 6.97 (ϵ), 7.49 (ζ)
Tyr-B			4.38	3.06	6.80 (δ), 7.12 (ϵ)
His	125				8.01 (C2), 7.06 (C4)
			(b) Ca_4TnC		
Tyr-Y	10		4.40		7.16 (δ), 6.82 (ϵ)
Phe-B	23				6.70 (δ), 7.11 (ϵ), 7.43 (ζ)
Phe-A	72				6.74 (δ), 7.10 (ϵ), 7.33 (ζ)
Phe-D	99		3.36	3.18, 2.84	6.47 (δ), 6.95 (ϵ), 7.25 (ζ)
Asn	105	8.69	4.99	2.58, 3.29	
Ala-A	106	8.78	4.94	1.86	
Asp	107	8.54	4.49	2.67, 3.08	
Gly-A	108	10.34	3.41, 4.00		
Tyr-X	109	7.98	5.29	2.90, 3.35	6.76 (δ), 6.86 (ϵ)
Ile-A	110	9.74	5.05	1.93	0.70 and 1.74 (γ), 0.92 (γ), 0.22 (δ)
Asp	111	8.51	4.47	2.75, 3.08	
His	125		4.59	3.02	7.91 (C2), 7.02 (C4)
Gly-B	144	10.34	3.53, 4.06		
Arg	145	7.82	4.72	3.29	
Ile-B	146	9.08	5.39	2.57	2.44 and 1.86 (γ), 1.31 (γ), 0.89 (δ)
Phe-C	148		3.60	2.62, 2.28	6.59 (δ), 7.18 (ϵ), 7.33 (ζ)

[a] Experimental conditions are as follows: [TnC] = 1.5 mM; [KCl] = 0.2 M; [DTT] = 10 mM; 40 °C. Data for NH protons are obtained in 85% H_2O (pH 6.2), and those for α-proton and side-chain protons are obtained in D_2O (pH 7.5). Chemical shifts are relative to internal TSP.

low field, suggesting that two hydrogen bonds between Ile-110 and Ile-146 are broken or weakened. α-Proton resonances of Tyr-109, Ile-110, Arg-145, and Ile-146 are not at extremely low fields, suggesting that the β-sheet structure is not formed or is loosely formed. In case of the C-terminal fragment of CaM without Ca^{2+}, the loops are also in the β-sheet structure, and the hydrogen bond between the amide group of Val-136 and the carboxyl group of Ile-100 still remains. The structure of the C-terminal-half domain of TnC is different from that of CaM at this point.

α-Protons of Thr-69, Ile-70, and Asp-71 appear at extremely low fields for apo-TnC, indicating formation of the β-sheet structure. Amide proton signal of Ile-70 appears at extremely low field, indicating formation of a hydrogen bond.

Another interesting observation is that amide protons of Gly-108 and Gly-144 are shifted significantly downfield for Ca_4TnC (Figure 5). This is also attributable to the hydrogen bonding. Herzberg and James (1985b) did not report hydrogen bonding of glycines in their crystal structure work of turkey skeletal TnC with two calciums. On the other hand, Moews and Kretsinger (1975) indicated that Gly-95 of parvalbumin forms a hydrogen bond between its amide group and the side-chain carboxyl group of Asp-90. The homology of the amino acid sequence between parvalbumin and TnC indicates that Gly-95 and Asp-90 of parvalbumin correspond to Gly-144 and Asp-139 of TnC, respectively. It seems most likely that the amide group of Gly-144 is hydrogen-bonded to the side-chain carboxyl group of Asp-139. In a similar manner, there may be a hydrogen bond between Gly-108 and Asp-103. Ikura et al. (1985) reported hydrogen bonds of Gly-98 and Gly-134 amide protons of the C-terminal-half fragment of CaM with two Ca^{2+}. These two glycines of CaM correspond to Gly-108 and Gly-144 of TnC.

Amide protons of both Gly-108 and Gly-144 for apo-TnC do not appear at lower fields. This fact suggests that hydrogen bonds between Gly-108 and Asp-103 and between Gly-144 and Asp-139 are not formed for apo-TnC. It was reported that Gly-98 forms a hydrogen bond for the C-terminal fragment of CaM without Ca^{2+} (Ikura et al., 1985). These results show a difference between the two molecules.

Ca^{2+}-Induced Conformational Change. In the course of Ca^{2+} titration, two types of spectral change were observed. A type 1 change occurs at $[Ca^{2+}]/[TnC] = 0–2$ and is characterized by the slow-exchange process. A type 2 change is observed at $[Ca^{2+}]/[TnC] = 2–4$ and is characterized by the fast-exchange process. A number of resolved resonances are categorized into either type of spectral changes, type 1 or type 2. Two such types of spectral change have been reported by Seamon et al. (1977) and Levine et al. (1977). However, changes observed here are different from those reported by them in some respects. The spectral change of Tyr-109 belongs to type 1. On the contrary, Seamon stated that tyrosine ring protons were not visibly affected in the first half of titration. It is of interest to note that δ- and ϵ-proton resonances of Tyr-10 exceptionally show both types of change.

Phe-148 and Phe-99 in the C-terminal-half domain form a hydrophobic cluster when 2 mol of Ca^{2+} bind to the C-terminus. However, there is no evidence that for apo-TnC these two phenylalanines form a hydrophobic cluster, although a hydrophobic cluster is formed in the N-terminal-half domain of apo-TnC.

When a further 2 mol of Ca^{2+} bind to the N-terminal-half domain, Phe-23 and Phe-72 are still in the hydrophobic cluster as evidenced by further ring current shifts to lower fields. These results are in accordance with those of Seamon et al. (1977) but not with those of Levine et al. (1977).

As shown in Figures 4–6, residues that belong to type 1 are Phe-99, Gly-108, Tyr-109, Ile-110, His-125, Gly-144, Arg-145, Ile-146, and Phe-148, and all are in the C-terminal-half domain. Resonances of these residues are governed by the slow-exchange process when the first 2 mol of Ca^{2+} bind to the C-terminal-half domain. Residues that belong to type 2 are Phe-23, Ile-34, Val-62, Ile-70, and Phe-72, and all are in the N-terminal-half domain. Resonances of these residues are

governed by the fast-exchange process when a further 2 mol of Ca^{2+} binds to the N-terminal-half domain. Thus, we confirmed the previous conclusion that type 1 reflects the conformational change the C-terminal-half domain and that type 2 reflects those of the N-terminal half (Seamon et al., 1977). This situation is similar to that of CaM (Ikura et al., 1983). All these results except for Tyr-10 as mentioned below support the independence of two domains as revealed by X-ray studies (Sundaralingam et al., 1985; Hertzberg et al., 1985a,b) as well as calorimetric studies (Tsalkova & Privalov, 1980).

As already stated, δ- and ϵ-protons of Tyr-10 show both types of change. This is the only evidence that the two domains communicate to each other. The structure of apo-TnC is presumably different from the independent dumbbell-like structure of Ca_2TnC so that Tyr-10 may interact with the C-terminal-half domain for apo-TnC. Binding of 2 mol of Ca^{2+} to the C-terminal-half domain leads to such dumbbell-like structure as reported. Thus, Tyr-10 may behave as type 1. Further addition of Ca^{2+} to the N-terminal-half domain affects again Tyr-10 and gives rise a type 2 change. Hincke et al. (1981) reported that the environment of Tyr-5 in the Ca^{2+} low-affinity site of bovine cardiac TnC is affected by calcium binding to the high-affinity sites. Recently, fluorescence dynamics study showed that apo-TnC might not have a dumbbell structure (Steiner & Norris, 1987). These results may be related to the behavior of the resonances of Tyr-10.

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