Ca^{2+} and Mg^{2+} Dependent Conformations of Troponin C as Determined by ^1H and ^{19}F Nuclear Magnetic Resonance†

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ABSTRACT: The effects of pH and pCa variation on the 250-MHz proton magnetic resonance spectrum of troponin C have been examined. Assignments of resonances in the aromatic region to histidine-125, and to tyrosines-10 and -109, have been made. The tyrosine peak assignments are confirmed by the spectrum of a derivative of troponin C in which the tyrosine rings have been iodinated. Two upfield shifted phenylalanine resonances in the spectrum of the Ca^{2+}-free protein have been identified. Three distinct conformational states of the protein are revealed by Ca^{2+} titration. These correspond to states of the protein with: (1) no Ca^{2+} bound; (2) Ca^{2+} bound only at one or both of the high affinity binding sites; and (3) Ca^{2+} bound at both high and one or both of the low affinity binding sites. The upfield phenylalanine resonances and an upfield aliphatic resonance are shifted and broadened as the third binding site (first low affinity site) is occupied by Ca^{2+}. This change is not effected by Mg^{2+}, which is shown to produce spectral changes characteristic of the binding of metal only at the high affinity sites. A fluorinated derivative was prepared by reacting the protein with bromotrifluoroacetone, a sulfhydryl alkylating reagent. The fluorine resonance of this derivative shifts downfield upon adding Ca^{2+}. The same shift is effected by the addition of Mg^{2+} indicating that the fluorine probe is sensitive to conformational changes induced by binding of metal at the high affinity sites only.

The protein complex, troponin, is involved in the regulation of striated muscle activity (Ebashi and Endo, 1968). It is composed of three distinct subunits, TN-C,[1] TN-T, and TN-I, of which only the first can bind Ca^{2+} (Greaser and Gergely, 1971). It is generally accepted that the contraction of striated muscle is initiated by changes of TN-C induced by its interaction with Ca^{2+}. The molecular weight of TN-C is approximately 18 000 (Greaser and Gergeley, 1971; Hartshorne and Dreizen, 1972). The primary structure of TN-C has been determined (Collins et al., 1973) and shows strong homology to MCBP, a high affinity Ca^{2+} binding protein found in abundance in many lower vertebrates (Pechère et al., 1973). The latter has been studied extensively and the crystal structure of MCBP from carp has been determined (Kretsinger and Nockolds, 1973). By comparison of the amino acid sequence of carp MCBP with the sequence of TN-C, four Ca^{2+} binding sites were located for TN-C (Collins et al., 1973; Weeds and McLachlan, 1974). An overall structure for TN-C has also been suggested based on the crystal structure of carp MCBP (Kretsinger and Barry, 1975).

Several studies on the binding of Ca^{2+} to troponin and TN-C have been reported, of which the most detailed is that of Potter and Gergely (1975). Their results indicate that there are six cation binding sites in TN-C, two Ca^{2+} specific binding sites, two Mg^{2+} specific binding sites, and two sites that can bind Ca^{2+} and Mg^{2+}. In the absence of Mg^{2+}, the four sites that can bind Ca^{2+} can be separated into two classes, each of two sites, with binding constants of 2×10^7 and 3×10^5 M^{-1}. The higher affinity sites have the ability to bind Mg^{2+}.

It is known that the binding of Ca^{2+} to TN-C can effect a conformational change and this has been detected using a variety of techniques (Murray and Kay, 1972; Van Eerd and Kawasaki, 1972; Potter et al., 1974; Head and Perry, 1974; Ohnishi et al., 1975; McCubbin and Kay, 1975; Levine et al., 1976). It was our intention to examine the proton NMR spectra of TN-C over a range of Ca^{2+} concentrations in order to detect changes in the protein's tertiary structure accompanying the binding of Ca^{2+} at the various sites. As intrinsic probes of a protein's structure tyrosine ring protons have been shown to be sensitive both to local motions and local environments (Snyder et al., 1975). These resonances can be identified by the pH dependence of their chemical shifts due to the ionization of the hydroxyl proton. It seemed particularly attractive to monitor these resonances as a function of metal binding to TN-C since it was indicated by several studies that one of the two tyrosines of the protein participates in the binding of Ca^{2+}. Sequence homology (Weeds and McLachlan, 1974; Kretsinger and Barry, 1975; Tufty and Kretsinger, 1975) with carp MCBP places tyrosine-109 of TN-C at one of the binding sites and the evidence from tyrosine fluorescence data (Van Eerd and Kawasaki, 1972) and enhanced Tb^{3+} fluorescence (Miller et al., 1975) are consistent with this prediction.

The sole cysteine of TN-C, cysteine-98, has been shown to be sensitive to metal binding to TN-C (Potter et al., 1974, 1976) which is predicted by the sequence homology placing it in an α-helical region connected to one of the binding sites. A sulfhydryl specific reagent, bromotrifluoroacetone, has been used in incorporating a trifluoroacetonyl group into proteins and has been shown to be a sensitive monitor of changes in tertiary structure near the labeled residue (Huestis and Raftery, 1972; Bode et al., 1975; Bendall and Lowe, 1977). We have prepared the trifluoroacetonyl derivative of TN-C and

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[1] Abbreviations used: TN-C, troponin C; TN-T, troponin T; TN-I, troponin I; MCBP, muscle calcium binding protein; $NaDodSO_4$, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; TSP, trimethylsilylpropionic acid; HFB, hexafluorobenzene; BrTFA, bromotrifluoroacetone; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; TFA-TN-C, trifluoroacetonylated troponin C.

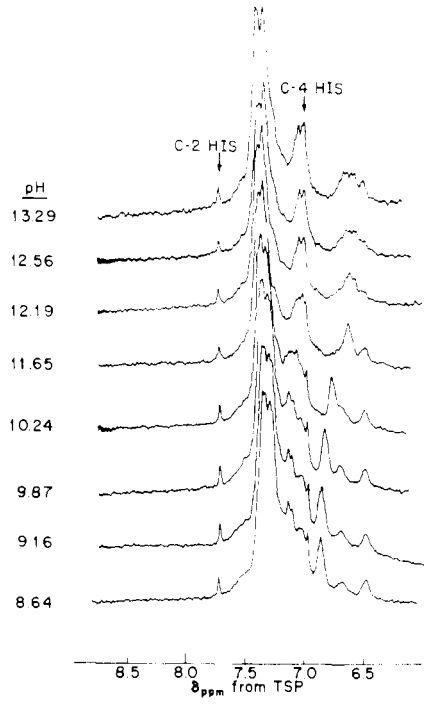

FIGURE 1: Aromatic region of TN-C in the Ca^{2+}-free state as a function of pH; [TN-C] $\simeq 1 \times 10^{-3}$ M, 0.1 M KCl, 50×10^{-3} M EGTA. Samples were prepared as described in Materials and Methods. The histidine C-2 and C-4 proton resonances are labeled.

have monitored the fluorine resonance as a function of Ca^{2+} and Mg^{2+}. The fluorine results are consistent with the conclusions derived from the proton NMR spectra of TN-C at various stages of Ca^{2+} and Mg^{2+} binding.

Materials and Methods

Protein Purification. Troponin was prepared from rabbit skeletal muscle by the method of Ebashi et al. (1971). TN-C was purified by chromatography of whole troponin on SP-Sephadex, in 6.0 M urea-33.0 mM citrate-1.0 mM dithiothreitol (pH 5.2). TN-I and TN-T bind to the column, and the TN-C is collected in the void volume. TN-C used in the experiments was judged pure by $NaDodSO_4$ gel electrophoresis. Protein concentrations were determined by the method of Lowry et al. (1951).

Preparation of Iodinated TN-C. TN-C was iodinated using lactoperoxidase (Morrison and Bayse, 1970). The 10-mL reaction mixture consisted of 0.1 mM TN-C, 0.2 mM KI, 50 mM sodium phosphate buffer at pH 7.0, 0.1 mM H_2O_2, 0.25 IU of lactoperoxidase, and either 5 mM EGTA or 1 mM $CaCl_2$. The reaction was started by addition of the lactoperoxidase and monitored by the decrease in tyrosine fluorescence at 308 nm with excitation at 280 nm. When the fluorescence intensity stopped decreasing, aliquots of KI and H_2O_2 were added such that the initial concentrations were maintained, until the final fluorescence intensity was ~5% of the original intensity.

Preparation of Trifluoroacetonyl TN-C. TN-C was dialyzed 24 h against 10 mM Tris-HCl buffer at pH 7.5, 0.1 mM DTT, 2 mM EGTA, and 2 mM EDTA. The protein was then dialyzed against the same buffer without the DTT under nitrogen. The reaction was carried out at room temperature. The 10-mL reaction mixture contained 0.2 M Tris-HCl buffer at pH 7.5, 0.16 mM TN-C, 2 mM EGTA, and 0.16 M BrTFA. The reaction was started by addition of the appropriate amount of undiluted BrTFA directly into the reaction mixture. At pH 7.5 the BrTFA hydrolyzes rapidly so it is necessary to add the reagent last and to maintain rapid stirring. The protein is allowed to react 15 min and then dialyzed immediately against 10 mM Tris-HCl buffer at pH 7.5, and 1 mM EGTA.

Preparation of Ca^{2+}-Free Samples. Two methods were used to produce Ca^{2+}-free TN-C. (1) TN-C (~1 mg/mL) was exhaustively dialyzed vs. 0.01 M KCl-0.2 mM EGTA-0.1 mM dithiothreitol (pH 7.0), then lyophilized, and dissolved in D_2O at a final concentration of ~10 mg/mL TN-C-2.0 mM EGTA-0.1 M KCl. (2) TN-C (~3 mg/mL) was dialyzed vs. 2.0 mM Tris-HCl-0.1 mM dithiothreitol (pH 7.0). Chelex-100 previously equilibrated to the potassium form was then added (~1 g wet Chelex/3 mL solution) to the sample and stirred for 0.5 h at 50 °C. The resin was then removed by filtration, the protein solution lyophilyzed, and the residue redissolved in 0.1 M KCl-D_2O solution. pH titrations in the Ca^{2+}-free state were carried out in the presence of 50 mM EGTA.

Ca^{2+} titrations were effected by adding aliquots of 0.1 M or 0.05 M $CaCl_2$ solution to Ca^{2+}-free samples prepared by either of the two methods. Aliquots of 1.0 M $MgCl_2$ were used for the Mg^{2+} titration.

Ca^{2+} Analysis. Two methods were used for determination of total Ca^{2+}. (1) Protein was precipitated with 5% Cl_3CCOOH and Ca^{2+} in the supernatant was determined by atomic absorption spectrophotometry (Baker et al., 1969). A Perkin-Elmer Model 303 instrument was used for this purpose. (2) Protein was precipitated using 5% Cl_3CCOOH and Ca^{2+} in the supernatant was determined by calcein fluorescence (Wallach and Steck, 1963) using a Farrand Mark I spectrophotometer equipped with corrected excitation and emission.

pH Titrations. pH titrations were carried out with a Radiometer Model 62 pH meter equipped with a GKS73041 electrode. pHs were adjusted with 0.2 M KOD or 0.2 M DCl. pHs reported are not corrected for the deuterium isotope effect.

NMR Spectra. Proton spectra were run at 250 MHz and fluorine at 235.2 MHz in the correlation mode (Dadok and Sprecher, 1974). Typical spectra were the average of 1000 scans with 1500-Hz sweep width, 1-s sweep time, and an exponential filtering of 0.5 Hz to reduce noise. Optimal digital filtering as described by Ernst (1966) was employed to enhance spectral resolution for the study of the tyrosine meta proton resonances. Proton chemical shifts were measured from the HDO lock and adjusted to chemical shifts from internal TSP by adding 4.72 ppm. Chemical shifts were measured from the external HFB lock for the fluorine experiments. All spectra were taken at 23 °C.

Results

pH Titration and Peak Assignments. The low-field portion of the 250-MHz proton magnetic resonance spectrum of Ca^{2+}-free TN-C (<0.2 Ca^{2+}/mol of TN-C) is shown in Figure 1, which shows the positions of the aromatic ring proton signals and their dependence on pH in the range pH 9 to pH 14. The known sequence of TN-C requires that these peaks be assigned to the aromatic ring protons of one histidine (His-125), two tyrosines (Tyr-10 and Tyr-109), and ten phenylalanines. In addition, slowly exchanging peptide NH or unexposed side-chain amide hydrogens can produce signals in this spectral region.

The signals arising from histidine and tyrosine may be identified by their pH titration behavior. Thus, examination of these spectra shows that the peak located at δ 6.88 ppm at pH 8.64 migrates to higher field as the pH is raised, eventually appearing at pH 13.29 as two doublets centered at δ 6.66 ppm and δ 6.62 ppm each with an 8-Hz spin-spin splitting. Because of interference by the peak at δ 6.67 ppm, the titration curve is difficult to obtain precisely. Figure 2 shows the approximate

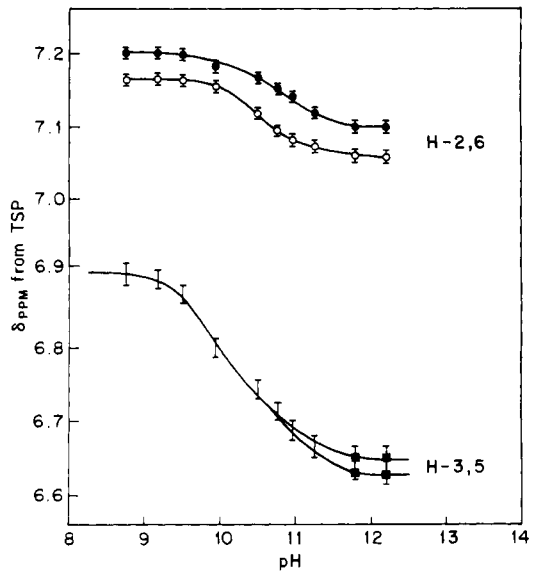

FIGURE 2: Chemical shifts of the ortho (H-3,5) and meta (H-2,6) protons of tyrosine-10 and tyrosine-109 in Ca^{2+}-free TN-C as a function of pH. The ortho proton (H-3,5) resonances for each tyrosine could not be followed separately until the high pH values where their positions are indicated (■). The meta (H-2,6) proton resonances could be followed separately at all pHs in the resolution enhanced spectra (Figure 3) and are labeled separately (○ and ●).

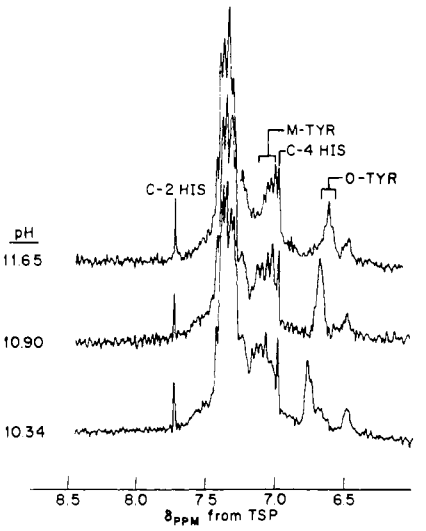

FIGURE 3: Resolution enhanced spectra of the aromatic region of Ca^{2+}-free TN-C at different pHs. The histidine C-2 and C-4 proton resonances are indicated as are the ortho (O-TYR) and meta (M-TYR) ring proton resonances of the tyrosine residues.

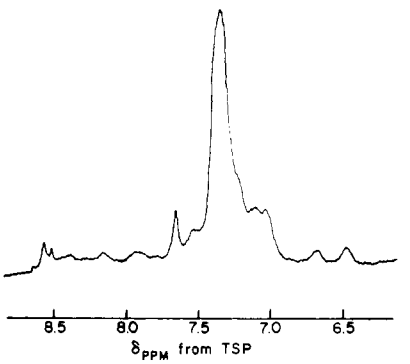

FIGURE 4: Aromatic region of the Ca^{2+}-free iodinated derivative of TN-C; [TN-C] ~ 1×10^{-3} M, 0.1 M KCl, pH 6.6. TN-C was made Ca^{2+}-free using the Chelex-100 technique (Materials and Methods).

titration curve. In this figure, the error bars are set equal to the width of the observed resonance at half-height, and the final positions of the centers of the two doublets are shown as circles. Titration curves of the theoretical form may be fitted to this diagram, with pK_as of 10.4 ± 0.2. The position, splitting, and pH dependence of these peaks are appropriate for the signals from the ortho protons of tyrosine. The original intensity of the peak corresponds to four protons, and its resolution into two doublets clearly establishes that the original peak represents the ortho protons of both tyrosine-10 and tyrosine-109.

The signal from the protons in the meta positions of the tyrosine rings is expected at lower applied field. Changes in the spectrum over the pH range 9–14 in the region δ 7.0–7.2 ppm indicate that there are peaks in this region that are titrating in a parallel way with the ortho protons. The changes may be visualized much more clearly in the resolution-enhanced spectra shown in Figure 3, where the doublets from the meta protons may be distinguished and followed separately.

In the same way, the signals from the histidine ring protons were assigned by titration in the region pH 6.0 to pH 8.0, confirming the assignment of Levine et al. (1976). The pK_a deduced from the titration curve for the histidine in the Ca^{2+}-free state of TN-C was 7.2 ± 0.2.

The original preparation up to pH 8 also yields low-intensity absorptions in the region δ 8.0–8.7 ppm, which are assigned to slowly exchanging peptide NH signals, on the basis of their chemical shift, disappearance at high pH, and nonreappearance on returning to low pH. The pH dependent spectral changes are otherwise reversible.

The remainder of the resonance signals must be assigned to the ring protons of the phenylalanines including the signals at δ 6.44 and at δ 6.67. These last two are at unusually high field for phenylalanine resonances. Their assignments as phenylalanine resonances are strengthened by the insensitivity of their chemical shifts to pH.

Spectra of Iodinated TN-C. TN-C was iodinated using lactoperoxidase. The reaction was monitored by the decrease in tyrosine fluorescence. The fluorescence intensity decreased to ~5% of the fluorescence of the native protein regardless of whether EGTA or Ca^{2+} was present in the reaction mixture. The iodinated TN-C was made Ca^{2+}-free and the spectrum recorded (Figure 4). It can be seen that the peak at 6.88 ppm in the pH 8.64 spectrum of native TN-C (Figure 1), assigned to the ortho protons of both tyrosines, is absent in the spectrum of the iodinated derivative. The upfield shifted resonances at 6.44 ppm and 6.67 ppm are in the same position as those exhibited by the native protein and do not seem affected by the iodination. This confirms the assignment of the peak at 6.88 ppm in the spectrum of the native Ca^{2+}-free TN-C to the ortho protons of the tyrosines and further confirms the assignment of the upfield resonances at 6.44 ppm and 6.67 ppm as not being due to tyrosine ring protons but rather to phenylalanine ring protons. The resonances which appear between 7.5 ppm and 8.0 ppm in the spectrum of the iodinated derivative which are not evident in the spectrum of the native protein are attributed to the meta protons of the iodinated tyrosine rings and the ortho proton of the monoiodo derivative of tyrosine.

Ca^{2+} Titration and Conformational Change. Titration with Ca^{2+} ion was performed in two ways: by direct addition of calcium chloride to Ca^{2+}-free TN-C and by the use of Ca^{2+}-EGTA buffer system to establish the free Ca^{2+} concentration. The fractions of the several sites of TN-C filled with Ca^{2+} could be calculated using the binding constants reported by Potter and Gergely (1975) and, in the case of EGTA buffering,

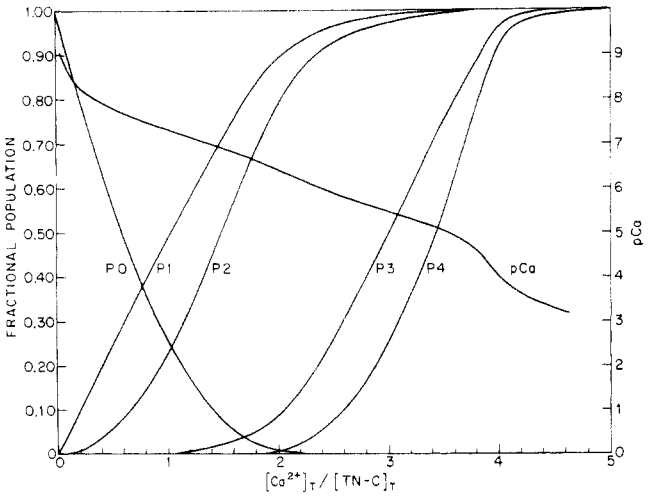

FIGURE 5: Fractional populations, P_i's, as a function of the ratio of total Ca^{2+} to total TN-C. The free Ca^{2+} concentration is also plotted. The P_i's were calculated using binding parameters: $n = 2$, $K_a = 2.1 \times 10^7$ M^{-1}, and $n = 2$, $K_a = 3.2 \times 10^5$ M^{-1}.

the EGTA binding constants compiled by Sillén and Martell (1964). Spectra of TN-C with a given degree of Ca^{2+} loading obtained by the two methods were indistinguishable from each other.

The fraction of the ith site occupied by Ca^{2+} (f_i) was calculated for arbitrary free Ca^{2+} ion concentrations, $[Ca^{2+}]$, from the relation

$$f_i = \frac{[Ca^{2+}]}{[Ca^{2+}] + K_i} \tag{1}$$

where K_i is the dissociation constant for the ith site. The total calcium concentration was then obtained in each case from the relation

$$[Ca^{2+}]_{total} = [Ca^{2+}] + [P]\left(\sum_{i=1}^{4} f_i\right) \tag{2}$$

where [P] = total protein concentration. The f_i's as a function of total Ca^{2+} were then tabulated. In discussing the various species of TN-C, differing only in their Ca^{2+} content, we define certain site populations, as follows:

$$P_0 = \prod_{k=1}^{4} (1 - f_k)$$
$$P_i = \prod_{k=1}^{i} f_k \tag{3}$$

In this notation, P_0 is the fraction of protein in which none of the four sites is occupied, P_1 the fraction in which a specific high affinity site is occupied, P_2 the fraction in which both high affinity sites are occupied, P_3 the fraction in which both high affinity and a specific low affinity site are occupied, and P_4 the fraction in which all four sites are occupied. These P_i's represent the predominant species of TN-C that will exist in solutions of different free Ca^{2+} concentrations. These can be calculated over a range of free Ca^{2+} from the f_i's determined with eq 1. This tabulation can in turn be related to total Ca^{2+} with eq 2. In practice the P_i's were tabulated as a function of the ratio of total Ca^{2+} to total protein, $[Ca^{2+}]_{total}/[P]$. The P_i's and free Ca^{2+} are plotted as a function of $[Ca^{2+}]_{total}/[P]$ in Figure 5.

To determine the P_i's for a particular NMR sample, the total Ca^{2+} and total protein were measured. In the samples where EGTA was not present the ratio, $[Ca^{2+}]_{total}/[P]$, was calculated and the P_i's could be determined from Figure 4. When EGTA was used as a Ca^{2+} ion buffer knowledge of total Ca^{2+}, total protein, and total EGTA allowed a determination of free Ca^{2+} using the iterative computer program of Perrin and Sayce (1967). All possible combinations of filled and unfilled sites on TN-C were accounted for in the computation. With knowledge of the free Ca^{2+}, the P_i's could be determined from Figure 5.

Spectra of TN-C are shown in Figure 6 as a function of the site populations, P_i. The spectrum of the Ca^{2+}-free TN-C in Figure 6a is virtually identical with the spectrum of Ca^{2+}-free TN-C at pH 8.64 (Figure 1) with the exception that the histidine C-2 and C-4 proton signals are now observed at δ 8.46 and 7.23, respectively. The high-field phenylalanine peaks are labeled C and D. In the aliphatic region, two distinct high-field resonances are seen at δ 0.166 ppm and δ −0.162 ppm (labeled E and F, respectively). A sharp singlet observed at δ 2.16 ppm has been assigned by Levine et al. (1976) to the *N*-acetyl group of TN-C.

As the high affinity sites become occupied (Figure 6, a–d), as indicated by the increase in P_1 from 0.1 to 0.91 and P_2 from 0.01 to 0.46, a redistribution of phenylalanine signal amplitude is observed with intensity at δ 7.19 (labeled B) increasing and intensity at δ 7.34 (labeled A) decreasing. The histidine, tyrosine ortho proton, phenylalanine C and D, and E and F resonances are not visibly affected.

As the low affinity sites become occupied (Figure 6, d–f), further changes are evident. (1) Resonances C and D broaden visibly and peak D shifts to lower field. (2) Resonance F broadens and shifts downfield. (3) There is a continued increase in phenylalanine signal intensity at δ 7.34 (B) and a decrease in intensity at δ 7.19 ppm (A).

In the final stages of Ca^{2+} binding (Figure 6, g and h): (1) there is a general broadening of the aromatic and aliphatic signals. (2) There is a redistribution in intensity of the methyl signals, with more intensity occurring to higher field. (3) Phenylalanine resonances C and D merge into the main aromatic peak. (4) The tyrosine ortho proton signals remain unaffected.

To relate these changes to binding of Ca^{2+} at particular sites in the protein, we have attempted to correlate spectral changes with the changes in the populations, P_i. Figure 7a shows plots of the chemical shifts of phenylalanine peak D against the P_i. It can be seen that the chemical shift of peak D does not start changing until P_1 is ~0.75 and P_2 ~0.6. An approximately linear relationship between P_3 and the chemical shift is evident. It can be seen that there is a noticeable change in chemical shift of peak D before any appreciable increase in P_4. The same relationships between the chemical shift of peak F and the P_i's exist (Figure 7b). We conclude that the changes in chemical shift of peak D and peak F are related to P_3, or occupation of a low affinity site.

The other obvious spectral change in the aromatic region is the change in the relative intensities of the two central phenylalanine peaks (labeled A and B in Figure 6). This change occurs over the entire range of Ca^{2+} concentration and is associated with progressive binding at all sites. When 91% of the high affinity sites are occupied by Ca^{2+} (Figure 6d, $P_1 = 0.91$), the intensities of peaks A and B are approximately equal.

These results allow us to define two aromatic spectral characteristics with respect to the occupation of the high affinity sites: equal intensity of the central phenylalanine peaks A and B, and no change in chemical shift of phenylalanine peak D with respect to its chemical shift in the Ca^{2+}-free protein. The spectral characteristics of the binding of Ca^{2+} to the low affinity sites are: a continued increase in intensity in the central phenylalanine peak B, and the downfield shift of phenylalanine peak D.

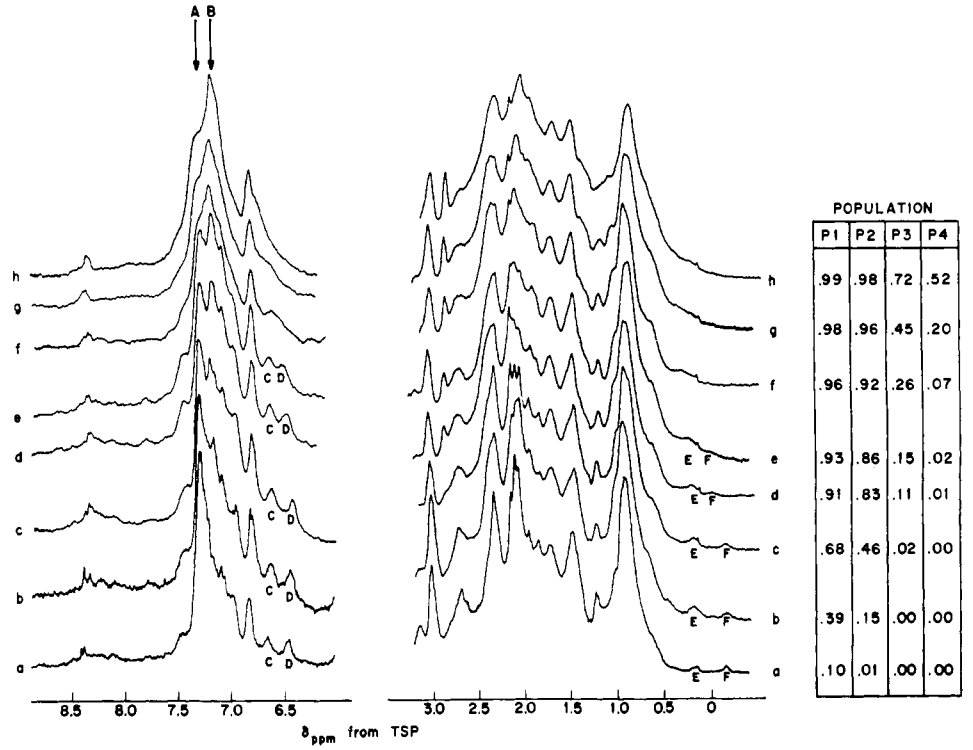

FIGURE 6: Aromatic and aliphatic regions of the spectra of TN-C at various stages of Ca^{2+} binding; [TN-C] $\simeq 1.0 \times 10^{-3}$ M, 0.1 M KCl, pH 6.6. Protein samples for spectra a–c were prepared by adding aliquots of 0.05 M $CaCl_2$ to Ca^{2+}-free samples prepared by the Chelex-100 technique (Materials and Methods). Samples for spectra d–h contained 2.0×10^{-3} M EGTA as a Ca^{2+} ion buffer. The fractional populations P_i are given for each spectrum. (See text.) Intensity at δ 7.34 and 7.19 ppm is indicated by arrows A and B, respectively. Phenylalanine peaks C and D and aliphatic peaks E and F are labeled in spectra a–e. The peak at δ 2.86 ppm appearing in spectrum d and increasing in intensity through spectrum h is due to resonances from EGTA.

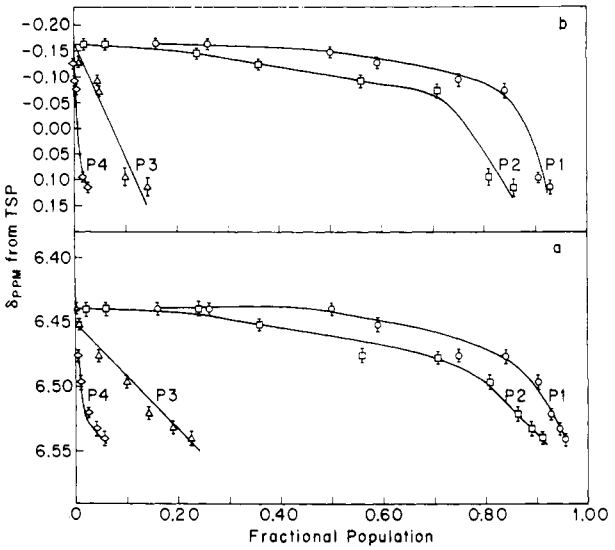

FIGURE 7: Chemical shifts of (a) phenylalanine peak D and (b) aliphatic peak F as a function of P_i: P_1 (○); P_2 (□); P_3 (△); P_4 (◇).

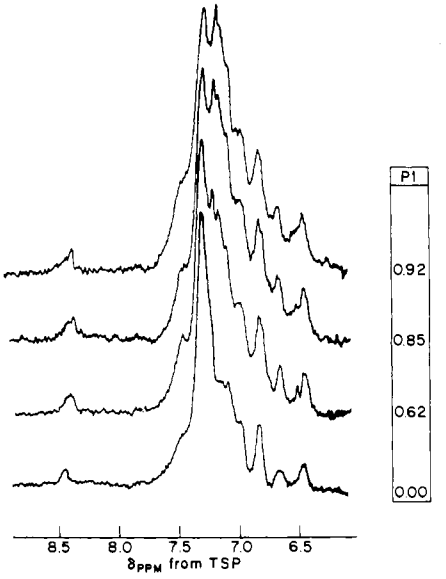

FIGURE 8: Aromatic region of TN-C at various stages of Mg^{2+} binding; [TN-C] $\simeq 1.0 \times 10^{-3}$ M, 0.1 M KCl, pH 6.6. Ca^{2+}-free protein was prepared by the Chelex-100 method (Materials and Methods). Aliquots of 1.0 M $MgCl_2$ were added to the Ca^{2+}-free protein. The fractional populations, P_1, were calculated directly from the $[Mg^{2+}]_{total}/[TN\text{-}C]$ ratio. The binding parameters used in the calculation were: $n = 4$, $K_a = 4 \times 10^3$ M^{-1}.

Effects of Mg^{2+} Ion. According to Potter and Gergely (1975), four Mg^{2+} ions bind to TN-C, two competing with Ca^{2+} for the high affinity sites and two binding at Mg^{2+} specific sites. We have accordingly examined the spectral changes produced by Mg^{2+} binding. The aromatic regions of Ca^{2+}-free TN-C at various stages of Mg^{2+} binding are shown in Figure 8. The site populations, P_1, given in the figure correspond to the fraction of each Mg^{2+} site occupied. The binding parameters were those of Potter and Gergely (1975). As the Mg^{2+} content increases there is a redistribution of intensity at positions A and B of the central phenylalanine peaks. This represents spectral changes associated with the binding of metal at the high affinity sites. Phenylalanine peaks C and D remain unaffected as do peaks E and F (not shown). The tyrosine ortho proton peak does not change position or line width upon Mg^{2+} binding. The spectrum of Ca^{2+}-free TN-C in the presence of 7 mM Mg^{2+} is the same as the Mg^{2+}-saturated spectrum, P_1

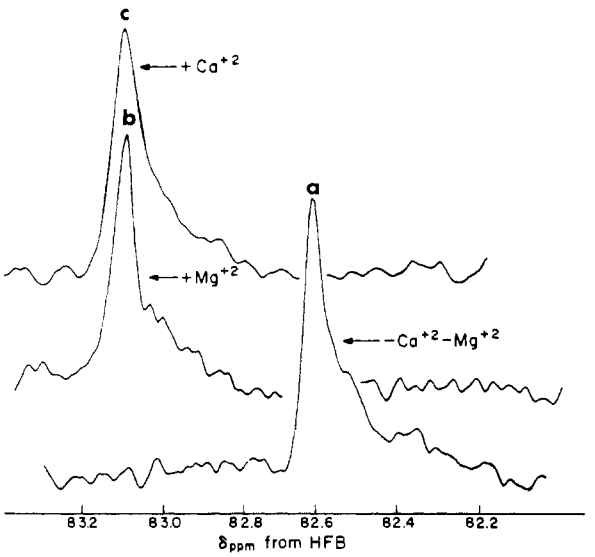

FIGURE 9: ^{19}F NMR spectra of TFA-TN-C; [TN-C] ~ 1×10^{-3} M, 0.1 M KCl, 0.01 M Tris-HCl, pH 7.0 in H_2O; (a) absence of Ca^{2+} and Mg^{2+}; (b) presence of Mg^{2+}; and (c) presence of Ca^{2+}. The sample was made metal-free using the Chelex-100 technique (Materials and Methods). The Mg^{2+} and Ca^{2+} samples were made by adding the appropriate amount of 1.0 M $MgCl_2$ or 0.1 M $CaCl_2$ to the metal-free protein such that the final free metal concentrations were 7 mM for Mg^{2+} and 1 mM for Ca^{2+}. Ca^{2+} was determined for each sample to verify that there was no preloading of the protein with Ca^{2+}.

= 0.92, in Figure 8. The spectrum corresponding to 92% of the Mg^{2+} sites being occupied (Figure 8, P_1 = 0.92) closely resembles the spectrum corresponding to 91% of the high affinity sites being occupied with Ca^{2+} (Figure 6d). This suggests that the conformations of protein with either Ca^{2+} or Mg^{2+} bound at the high affinity sites are very similar.

Effect of Ca^{2+} and Mg^{2+} on TFA-TN-C. TN-C was reacted with BrTFA and the extent of reaction determined by the method of Brown and Seamon (1977, and manuscript in preparation). This method quantitates the amount of reacted cysteine by determining cysteic acid content of the protein after any alkylated cysteine is reduced with $NaBH_4$. The reduction of the trifluoroacetonyl group is necessary in order to make the cysteine derivative stable to acid hydrolysis under oxidative conditions. Fluorine spectra were taken over a range of 40 000 Hz and only a single fluorine peak could be detected. Since it was determined that the cysteine was modified and only a single fluorine peak is observed, we assign this peak to the trifluoroacetonyl derivative of cysteine-98 of TN-C.

The alkylated TN-C (~0.7 mol of alkylated cysteine/mol of TN-C) was made Ca^{2+} free and the effect of added Mg^{2+} and Ca^{2+} on the fluorine spectrum observed. The fluorine resonance in the cation-free state appears as a single resonance at 82.6 ppm downfield of external HFB (Figure 9a). Upon adding Mg^{2+} (in an amount sufficient to saturate all four Mg^{2+} sites) to the cation free protein, the resonance shifts downfield to 82.1 ppm (Figure 9b). The effect of adding Ca^{2+} to the metal-free protein is the same; the resonance shifts to 82.1 ppm (Figure 9c). The Ca^{2+} added to the metal free protein was enough to saturate all four Ca^{2+} binding sites. The line width of the fluorine resonance is approximately 15 Hz in the absence of Mg^{2+} or Ca^{2+} and increases to 20 Hz upon addition of either of the metals. These same results were obtained in a metal-free protein system prepared with EGTA.

Discussion

The spectrum of TN-C in the Ca^{2+}-free state indicates that the protein is at least in part in a definite conformation, not a random coil. This follows from the presence in the spectrum of the metal free protein of the two high-field phenylalanine ring resonances C and D and the high-field resonances E and F. The anomalous chemical shifts of these resonances require that the groups be in a structured environment, with the most probable explanation being that they are subjected to ring current shifts from neighboring aromatic rings. This assignment is strengthened by the report of Cave et al. (1976), who have assigned phenylalanine resonances at δ 6.25 ppm in carp MCBP using decoupling techniques. Based on the structure of the Ca^{2+} saturated form of carp MCBP, Parello et al. (1975) have calculated resonance shifts in the region δ 5.0 to 6.5 ppm for aromatic rings in the interior of the protein.

Previous studies have suggested that TN-C upon binding Ca^{2+} undergoes a conformational change resulting in a more compact structure (Murray and Kay, 1972). Our results are consistent with this interpretation. The upfield shift in phenylalanine signal intensity within the main peak, on addition of Ca^{2+} could represent further structuring of a hydrophobic core as the four binding sites become progressively occupied. The upfield shift of main group methyl signal intensity is also consistent with structuring of a hydrophobic core, composed of aliphatic amino acid side chains as well as aromatic residues. The broadened appearance of the spectrum in the Ca^{2+}-saturated state could result from either a greater distribution in chemical shifts for amino acid residues or a more efficient dipolar relaxation of protons. As the protein goes to a more compact structure, it would be expected that a greater number of amino acid residues would be in different environments. This would result in a larger dispersion of resonance positions for side chains of the same amino acid. In a more compact structure the correlation time for a particular residue could be increased from that in a less restricted structure due to steric interactions which would limit its motion: hydrogen bonding, hydrophobic associations, etc. Generally, this results in a broadening of the proton signal from that particular residue.

As spectral monitors of metal binding, the chemical shifts of phenylalanine peak D and high-field peak F are related to binding of Ca^{2+} at a low affinity site. Whether the changes are caused by binding at a single low affinity site or partly by binding at either low affinity site is not demonstrated. However, occupation of both sites does not seem to be required. The [Ca^{2+}]-dependence of the chemical shift of the phenylalanine peak D and peak F is consistent with the value for the low affinity binding constant measured by Potter and Gergely (1975). These results are all consistent with a two-stage conformational change. The change induced by the high affinity binding is indicated by the initial upfield shift of phenylalanine intensity. The downfield shifts of phenylalanine peak D and peak F are indicative of the second conformational change due to binding at the low affinity sites. Accompanying the specific low affinity site spectral changes is a continued upfield shift in phenylalanine intensity. This indicates that binding of Ca^{2+} at the low affinity sites does produce a conformational change which affects a number of phenylalanine rings and establishes that TN-C with Ca^{2+} bound at the low affinity sites is in a different conformation from that when just the high affinity sites are occupied. Recently, a fluorescence probe attached to the single cysteine of TN-C has been shown to be sensitive to both high affinity and low affinity binding (Potter et al., 1976). Other conformational probes have been sensitive only to the binding of Ca^{2+} at the high affinity sites.

Tyrosine rings do not appear to be directly involved or affected by Ca^{2+} binding in any of the sites. This is especially

noteworthy since studies using fluorescence (Van Eerd and Kawasaki, 1972) and sensitized emission from Tb^{3+} (Miller et al., 1975) have all placed a tyrosine close to or at a binding site. In addition, the sequence homology between carp MCBP and TN-C indicates that Tyr-109 occurs at a binding site (Kretsinger and Barry, 1975; Weeds and McLachlan, 1974). In the crystal structure of carp MCBP (Kretsinger and Nockolds, 1973), it is, however, the carbonyl group of the homologous phenylalanine which coordinates Ca^{2+}, the phenylalanine ring protruding outwardly. If tyrosine-109 participated in the binding of Ca^{2+} through its peptide carbonyl, then it is possible that binding of metal ion at that site would not perturb the ring protons. Further, the pK_a value (10.4) for the tyrosine rings is close to the pK_a typical of a fully exposed tyrosine ring (10.1). The observed pK_a value agrees well with that of Lehrer and Leavis (1974), obtained using fluorescence techniques. The insensitivity of the chemical shift of the ortho protons suggests that the rings are not further buried as Ca^{2+} is added. This is supported by the spectrum of the iodinated derivative of TN-C which indicates that both tyrosines are iodinated by the lactoperoxidase reaction. The absence of the peak assigned to the ortho protons of the tyrosines in the spectrum of the iodinated derivative is accompanied by an almost complete loss of tyrosine fluorescence (~5% that of native TN-C). TN-C contains no tryptophans and any intrinsic protein fluorescence must therefore be attributed to the fluorescence from the tyrosine rings since the phenylalanine fluorescence is extremely weak (Undenfriend, 1969). Iodinated tyrosine rings are nonfluorescent (Cowgill, 1967) and therefore the extent of iodination of the tyrosines in TN-C can be monitored by the decrease in fluorescence. Since both tyrosines have been reported to contribute to the fluorescence of TN-C (Lehrer and Leavis, 1974), a complete loss of fluorescence upon iodination confirms that both tyrosines are being iodinated. The fluorescence intensity is decreased to the same extent whether the reaction is carried out in the presence or absence of Ca^{2+}. Since the iodination by lactoperoxidase depends on the accessibility of the tyrosine ring to the enzyme (Morrison and Bayse, 1970), both tyrosine rings must be on the surface of the protein in the Ca^{2+}-free and Ca^{2+}-saturated conformations of TN-C. It has also been reported that both tyrosines of TN-C are nitrated by tetranitromethane in the presence and absence of Ca^{2+} (McCubbin and Kay, 1975). In contrast to these results Van Eerd and Kawasaki (1972) report an increase in the rotational correlation time exhibited by the tyrosine rings, from 14.5 to 29.5 ns, upon addition of Ca^{2+} to TN-C, suggesting that one or both of the rings is in a more rigid environment in the Ca^{2+}-saturated state. Assuming that the ortho protons of the tyrosines are relaxed predominantly by the meta protons of the same ring, we can calculate the expected increase in line width of the ortho protons upon an increase in correlation time, as described above, to be ~3.5 Hz at a resonance frequency of 260 MHz. The line width of the ortho proton resonance in the Ca^{2+}-free state is 20 Hz. Because of the overlapping of other resonances upon the addition of Ca^{2+}, an increase in 3 Hz in the line width of the ortho proton resonance would be difficult to detect.

The Mg^{2+} results indicate that Mg^{2+} binds to the high affinity sites of TN-C and effects a similar conformational state as that produced by Ca^{2+} binding to the same sites. This confirms Potter and Gergely's (1975) assignment of the low affinity sites of TN-C as Ca^{2+} specific. Other probes have reflected the binding of Mg^{2+} to the high affinity sites. In particular probes attached to cysteine-98 have shown to be sensitive indicators of Ca^{2+} and Mg^{2+} binding (Potter et al., 1976). The fluorine resonance of the trifluoroacetonyl cysteine-98 derivative of TN-C shows the same sensitivity to Mg^{2+} as it does to Ca^{2+}, indicating that the probe is only sensitive to binding of metal at the high affinity site. This supports the prediction that the cysteine is in a region near one of the high affinity sites (Kretsinger and Barry, 1975). An interpretation of the nature of the conformational change causing the shift of the fluorine resonance is difficult; however, it has been suggested that fluorine resonances show a downfield shift in going from a position exposed to solvent on the surface of a protein to a more buried position (Hull and Sykes, 1976). The downfield shift of the fluorine resonance upon binding of metal by TN-C is consistent with the proton NMR results, indicating a more compact conformation for the TN-C with bound metal.

If it is assumed that the conformational changes of TN-C are equivalent in the isolated subunit and in the whole troponin complex, then the conformation of TN-C with metal ions in all four sites would be that existing in the active or contracting muscle. In the relaxed state the Ca^{2+}-specific sites would not be occupied; if, however, the free Mg^{2+} concentration was high enough ($\sim 2.5 \times 10^{-4}$ M),[2] then the high affinity sites would be occupied by Mg^{2+}. It has recently been reported that the free Mg^{2+} concentration in frog muscle (Cohen and Burt, 1977) and barnacle muscle (Tiffert et al., 1977) is above 4 mM. Since our results have shown that Mg^{2+} and Ca^{2+} effect similar changes by binding at the high affinity sites, it follows that in relaxed muscle the conformation of TN-C approaches that found when the high affinity sites are occupied by Ca^{2+}, i.e., the cation free conformation is not found. The relevant physiological change of troponin, that occurring in the transition of relaxed to contracting muscle, would be produced by the binding of Ca^{2+} to the Ca^{2+} specific sites (the low affinity binding sites of the isolated TN-C subunit). It is significant that Weber and Murray (1973) and Potter and Gergely (1975) have suggested that occupation of the Ca^{2+} specific sites is necessary for full activation of myofibrillar ATPase, although there is not complete agreement on the role of the high affinity sites.

We feel that the results presented here on the isolated TN-C can form the basis for future studies on the behavior of the intact troponin.

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[2] Potter and Gergely (1976) report that Mg^{2+} binds to the intact troponin complex in four sites with association constants of 4×10^4 M^{-1}. Each Mg^{2+} site would then be approximately 91% occupied at a free Mg^{2+} concentration of 2.5×10^{-4} M.

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