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Laser Photochemically Induced Dynamic Nuclear Polarization Proton Nuclear Magnetic Resonance Studies on Three Homologous Calcium Binding Proteins: Cardiac Troponin-C, Skeletal Troponin-C, and Calmodulin†

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ABSTRACT: Laser photo-CIDNP ^{1}H NMR experiments were performed with rabbit skeletal troponin-C (sTn-C), bovine cardiac troponin-C (cTn-C), and bovine brain calmodulin to study the exposure of histidine and tyrosine residues. In cTn-C, tyrosine residues, 5, 111, and 150 were exposed in the apoprotein, becoming buried as Ca^{2+} was bound. A similar phenomenon was observed for tyrosine residues 10 and 109 of sTn-C. In calmodulin, only tyrosine-99 was accessible in the apoprotein. The lack of exposure of tyrosine-138 observed with this technique correlates with the buried nature of this residue implied by other criteria. In 6 M urea each of the apoproteins was observed to be unfolded from the standpoint of the tyrosine environments. A large tyrosyl CIDNP effect was obtained for each protein which decreased as Ca^{2+} was bound, with a stoichiometry of one metal ion per protein. This was correlated for cTn-C with the appearance of "native" resonances representing tyrosine residues 111 and 150 in Ca^{2+}-saturated cTn-C, also with a stoichiometry of one. Analysis of our NMR findings, in the light of other spectroscopic and model building studies on these systems, suggests that the sole high-affinity Ca^{2+} binding site of cTn-C and sTn-C remaining in 6 M urea is site IV.

Troponin-C, from beef cardiac (cTn-C)[1] and rabbit skeletal muscle (sTn-C), and bovine brain calmodulin are calcium binding proteins which have been extensively characterized by ^{1}H NMR spectroscopy [Hincke et al. (1981), Seamon et al. (1977), and Seamon (1980), respectively]. These studies have assigned various features of the spectra, especially the tyrosine and histidine resonances, to specific residues within the primary sequence and have used the assigned resonances to monitor the large conformational changes which occur in these proteins upon Ca^{2+} binding.

These proteins are of particular interest because of their biological importance. Tn-C is the Ca^{2+} binding subunit of the troponin complex in vertebrate striated muscle. Ca^{2+} binding initiates a chain of molecular events which leads to activation of the Mg–ATPase of actomyosin and contraction (McCubbin & Kay, 1980; Perry, 1979). Calmodulin is a protein widely found in nonmuscle tissue [see Cheung (1980) for a review] which activates numerous enzymes by complexing with them only when Ca^{2+} is present. Some well-studied examples are 3′,5′-cyclic nucleotide phosphodiesterase (Techima & Kakiuchi, 1974) and adenylate cyclase (Lynch et al., 1976).

A technique has recently been introduced which allows NMR signals to be selectively detected from histidine, tyrosine, and tryptophan residues which are on the protein surface. This method is based on the laser photochemically induced dynamic nuclear polarization (photo-CIDNP) of the aromatic protons of these residues (Kaptein, 1978). A flavin dye, excited to its triplet state by brief laser irradiation, reacts reversibly with any accessible histidine, tyrosine, or tryptophan residues that

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[1] Abbreviations used: photo-CIDNP, photochemically induced dynamic nuclear polarization; NMR, nuclear magnetic resonance; FMN, flavin mononucleotide; cTN-C, bovine cardiac troponin-C; sTN-C, rabbit skeletal troponin-C; MCBP, muscle calcium binding protein (parvalbumin); Mops, morpholinopropanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

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are accessible to the dye. This leads to transient radical formation, causing nuclear polarization in the side chains of these residues. The resulting spectral changes, positive or negative enhancements of aromatic resonances, can most easily be detected by subtracting a normal spectrum ("dark") from one obtained immediately after illumination with the laser ("light"). In the aromatic part of the NMR spectrum (6–8 ppm) resonances of tryptophan, histidine, and the 2,6 protons of tyrosine are positively enhanced while the 3,5 protons of tyrosine give distinct emission lines (Kaptein et al., 1978; Jansen et al., 1978). For example, with tyrosine (TyrH), the photoreaction with the flavin dye (F) can be written as

$$F \xrightarrow{h\nu} {}^1F \rightarrow {}^3F \quad (1)$$

$${}^3F + TyrH \rightarrow FH\cdot + Tyr\cdot \quad (2)$$

$$FH\cdot + Tyr\cdot \rightarrow F + TyrH^* \quad (3)$$

$$2FH\cdot \rightarrow FH_2 + F \quad (4)$$

Here, the triplet dye (^3F) abstracts the phenolic hydrogen atom of tyrosine, and nuclear polarization (indicated by an asterisk) arises from the radical pair recombination step (eq 3). Its sign (emission or absorption) can be easily predicted from the spin-density distribution and g factors of the intermediate radicals (Kaptein, 1971). Reactions 1–3 constitute a cyclic process. Almost no net reaction occurs, although reaction 4 causes some bleaching of the dye. This technique has been used successfully to probe the surface exposure of these aromatic residues in a number of different protein systems, including dihydrofolate reductase (Feeney et al., 1980), bovine pancreatic phospholipase (Egmond et al., 1980), and bovine lactalbumin (Berliner & Kaptein, 1980).

cTn-C, sTn-C, and calmodulin are particularly suitable for NMR studies because of their low molecular weights (18 500, 18 000, and 16 500, respectively) and high solubility. Their amino acid sequences are quite homologous, suggesting their evolution from a common ancestral Ca^{2+} binding protein by gene duplication (Collins et al., 1973). The regions of their primary sequences which exhibit the greatest degree of homology are those which have been assigned as the Ca^{2+} binding sites. These domains have been identified from the X-ray structure derived for carp MCBP (parvalbumin), another homologous Ca^{2+} binding protein which has two high-affinity Ca^{2+} binding sites: the so-called CD and EF loop regions (Kretsinger & Nockolds, 1973). These homologous Ca^{2+} binding sites all have the common structural feature of a helix–loop–helix arrangement, with the six coordinating residues of the metal ion found within the loop [see Reid & Hodges (1980) for a review]. sTn-C and calmodulin bind 4 mol of calcium (Potter & Gergely, 1975; Vanaman et al., 1977), in agreement with predictions based on their sequence. cTn-C has suffered extensive amino acid substitution at one calcium-binding site and, as expected, only binds 3 mol of calcium (Leavis & Kraft, 1976).

In cTn-C and sTn-C, sites III and IV are high-affinity Ca^{2+} binding sites (Sin et al., 1978) and display the greatest degree of sequence homology. Ca^{2+} binding to these sites elicits most of the spectroscopic alterations associated with Ca^{2+} binding. In calmodulin, although some controversy exists as to which are the highest affinity sites (Kilhoffer et al., 1980), the first 2 mol of Ca^{2+} bound also elicit the majority of the conformational changes (Crouch & Klee, 1980).

These proteins are interesting candidates for photo-CIDNP experiments because of the appropriate location of the residues which are sensitive to this technique. cTn-C, sTn-C, and calmodulin each possess a homologous tyrosine residue within high-affinity Ca^{2+} binding site III, while only cTn-C and calmodulin possess a homologous tyrosine residue inside the domain of the other high affinity site, IV. In addition, calmodulin and sTn-C have a single histidine residue located in a homologous region between sites III and IV. A preliminary report of this work has already been presented (Hincke et al., 1980).

Materials and Methods

sTn-C and cTn-C were prepared as previously described (Hincke et al., 1978). sTn-C was also subjected to ion-exchange chromatography on DEAE-Sephadex A25 to remove contaminating troponin-T. A solvent system of 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, and 0.1 M NaCl was used, and the protein was eluted with a linear gradient to 0.5 M NaCl. Calmodulin was purified from bovine brain tissue (Walsh, 1978).

Protein samples were stripped of contaminating Ca^{2+} by extensive dialysis (Hincke et al., 1981) or by utilizing a column of Chelex 100 (Bio-Rad) through which the protein solutions were slowly passed (Blinks et al., 1978).

Proton NMR spectra were obtained at 270 MHz with a Brüker HX-270 spectrometer operating in the Fourier transform mode with quadrature detection and equipped with a Nicolet 1180 computer and 293B pulse unit. Typical instrumental settings were sweep width ±2000 Hz, 4096 points, 14-μs pulse ($\simeq$90 °C), and line broadening 1 Hz. The HDO resonance was suppressed with homonuclear decoupling. Chemical shifts were measured relative to the major resonance of sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) as an internal standard.

Laser photo-CIDNP experiments were performed by utilizing a Spectra Physics Model 164 argon ion laser operating at 3.5 W in the multiline mode; 10-mm o.d. flat-bottomed NMR tubes were used. The laser beam was directed into the bottom of the sample via a computer-controlled shutter and mirror. These experiments were performed at ambient temperatures (301 K).

FMN (Sigma) was dissolved in D_2O (50 mM) and small aliquots (2 or 5 μL) were added to the protein solution (1.20 mL) prior to irradiation. Urea-d_4 (98% d) was obtained from Stohler Isotope Chemicals. The model compounds Gly-His-Gly and *N*-acetyl-L-tyrosinamide were obtained from Sigma. The pH of protein solutions was measured with a 6030-04 Ingold combination electrode and a Radiometer Model PHM62 pH meter, calibrated with standard buffers (Fisher Scientific Co.). All pH values are the direct meter readings, uncorrected for the deuterium isotope effect.

Results

The CIDNP effect in model compounds is demonstrated in Figure 1. The peptide Gly-His-Gly exhibits positive enhancement of the C-2 and C-4 histidine protons, while with tyrosine there is positive enhancement of the meta (2,6) protons and much larger negative enhancement of the ortho (3,5) protons.

The accessibility of histidine and tyrosine residues in the native proteins was investigated initially. Data for sTn-C, seen in Figure 2, reveal the positive enhancement of the C-2 and C-4 histidine protons and also the negative enhancement of tyrosine ortho protons. No effect is seen for the tyrosine meta resonances as this is typically much smaller and can be canceled out by cross-relaxation. As Ca^{2+} is added to the protein, the CIDNP effect diminishes, implying that at least one of the tyrosines is being buried.[2] Similar results are obtained

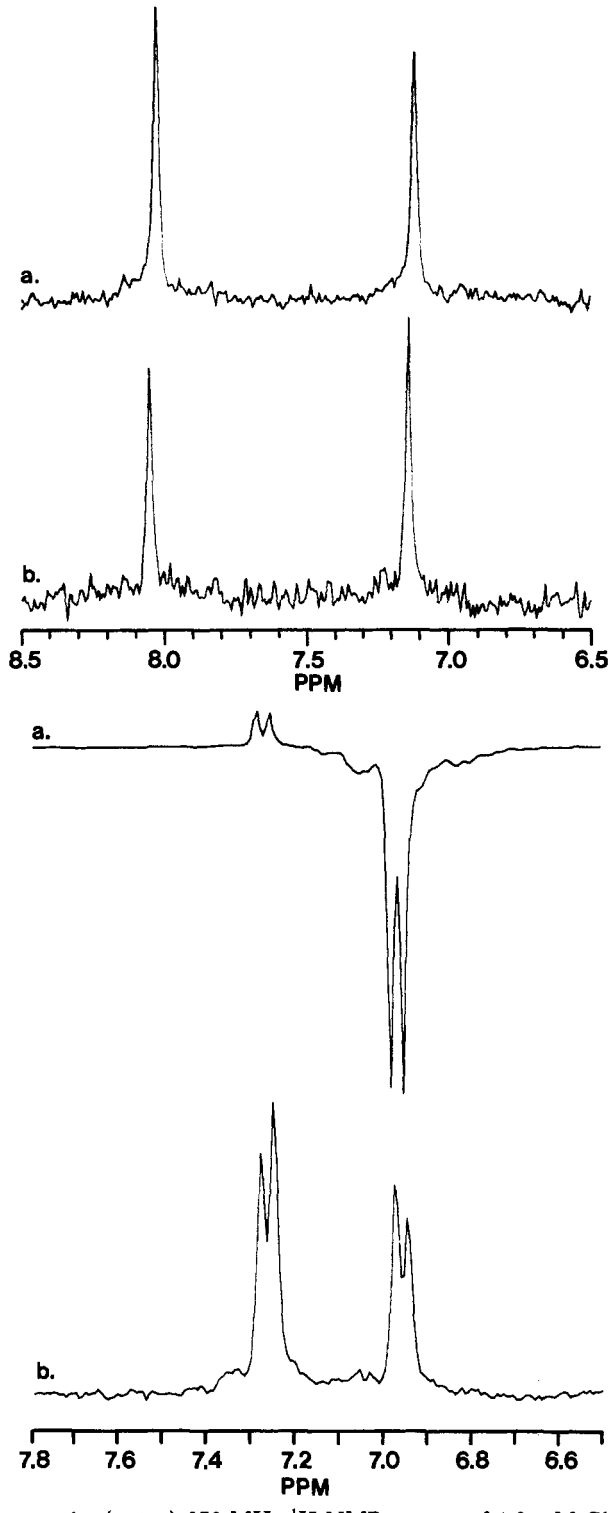

FIGURE 1: (a, top) 270-MHz ^1H NMR spectra of 1.0 mM Gly-His-Gly in 5 mM Mops, 20 mM KCl, pH 7.00, and 0.08 mM FMN. (a) CIDNP difference spectrum (light − dark) after eight scans (1.0-s irradiation) with a 20-s delay between spectra; (b) dark spectrum, 128 scans. Here the CIDNP effect represents a 16-fold enhancement. (b, bottom) *N*-Acetyl-L-tyrosinamide (1.1 mM) in 5 mM Mops, 20 mM KCl, pH 7.0, and 0.2 mM FMN. (a) CIDNP difference spectrum as before; the magnitude of the enhancement is 1.7-fold for the 2,6 meta protons and 28-fold for the 3,5 ortho protons; (b) dark spectrum, 128 scans.

with cardiac Tn-C (Figure 3), where the location of the three sets of ortho protons in the apo spectrum is indicated very

[2] The histidine results are less straightforward to interpret because of the greater variability, in our hands, of the histidine enhancement for proteins (see below).

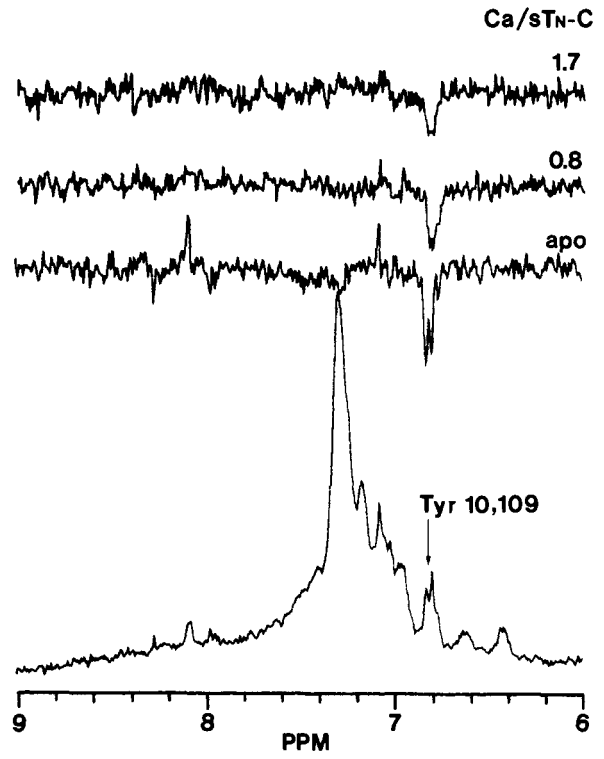

FIGURE 2: CIDNP difference spectra of sTn-C (0.4 mM) in 0.1 M KCl and 20 mM Mops, pH 7.4. Each spectrum represents eight light and dark scans (as in Figure 1a); 5 μL of FMN (50 mM) was added to the sample at the start and with each addition of calcium. The dark spectrum of apo-sTn-C is presented. The ortho resonances of tyrosine residues 10 and 109 are indicated.

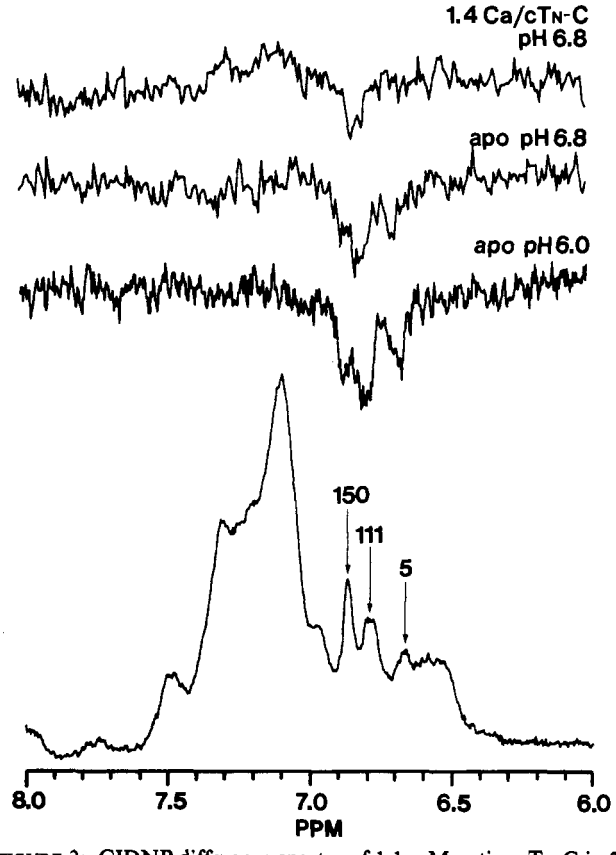

FIGURE 3: CIDNP difference spectra of 1.1 mM native cTn-C in 20 mM Mops and 0.1 M KCl. Each difference spectrum represents 12 light and dark scans (as in Figure 1a); 5 μL of FMN (50 mM) was added to each sample and with the addition of calcium. The dark spectrum of apo-cTn-c, pH 6.0, is presented as a reference spectrum with assigned tyrosine ortho resonances indicated.

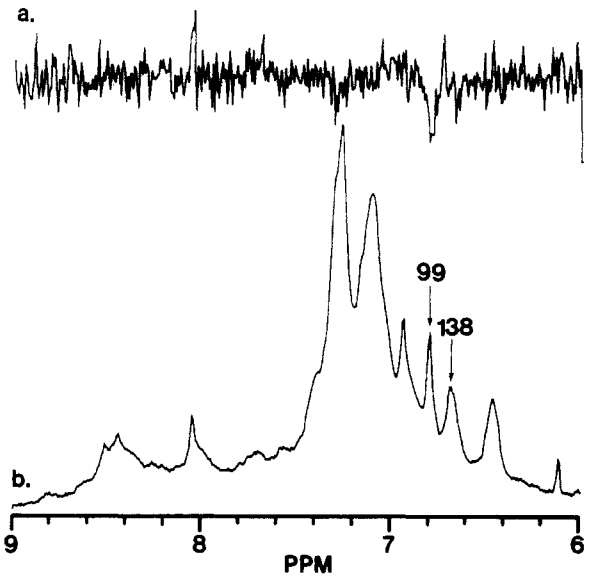

FIGURE 4: Apocalmodulin (1.3 mM) in 25 mM Mops, 0.15 M KCl, pH 7.0, and 0.18 mM FMN. (a) CIDNP difference spectrum (four scans); (b) dark spectrum, 256 scans. The ortho resonances of tyrosine residues 99 and 138 are indicated.

convincingly. The tyrosine residues to which these have been assigned are indicated (Hincke et al., 1981). Calcium binding alters the chemical shifts of residues 5 and 150; the CIDNP data suggest that these tyrosine residues are not accessible in their Ca^{2+}-bound conformation. Tyrosine-111 also becomes less exposed as Ca^{2+} is bound. Cardiac Tn-C does not possess any histidine residues.

Apo-calmodulin displays a different behavior (Figure 4). A slight enhancement is observed at the C-2 histidine resonance, and there is no effect at the C-4 histidine. Only a small effect occurs for the ortho protons of tyrosine-99. Apparently in the apoprotein this residue is less exposed than are the homologous tyrosine residues in cTn-C or sTn-C. Tyrosine-138 is not accessible to the dye under these conditions. This agrees with the buried nature of this residue deduced by other techniques. For example, it possesses a pK_a of 12.0 and 11.9 in the absence and presence of Ca^{2+}, respectively (Klee, 1977).

In concentrated urea solutions a high degree of tyrosyl exposure is observed for each of the three proteins. In Figure 5 the tyrosyl CIDNP effect obtained with cTn-C in 4.2 M urea is demonstrated. The magnitude of this phenomenon is calcium dependent, decreasing as the protein binds Ca^{2+}. The protein does indeed bind Ca^{2+} under these conditions, as evidenced by the appearance of aromatic resonances which represent features of the Ca^{2+}-bound spectrum [Figure 6; compare with Figure 10 of Hincke et al. (1981)], and the same phenomenon also occurs in 6 M urea. These results suggest that Ca^{2+} is inducing the cTn-C molecule to assume a native-type structure even in the urea solvent systems and is a further illustration of the tremendous stabilization of the protein structure by Ca^{2+} ions. Similar phenomena are observed with sTn-C and calmodulin. In each case, in 6 M urea solutions, Ca^{2+} binding decreases the accessibility of the tyrosine residues. With cTn-C it is evident that the Ca^{2+}-bound residues do not contribute to the CIDNP effect. That is, the chemical shifts of the residues in the plus Ca^{2+} protein are different from those of the same residues in the apo-unfolded structure; no CIDNP signal is observed from these "plus Ca^{2+}" resonances. It is difficult to understand the small C-2 histidine CIDNP effect observed for calmodulin unfolded in 6 M urea (approximately the same magnitude as for the native protein), which disappears as Ca^{2+} is bound. No histidine

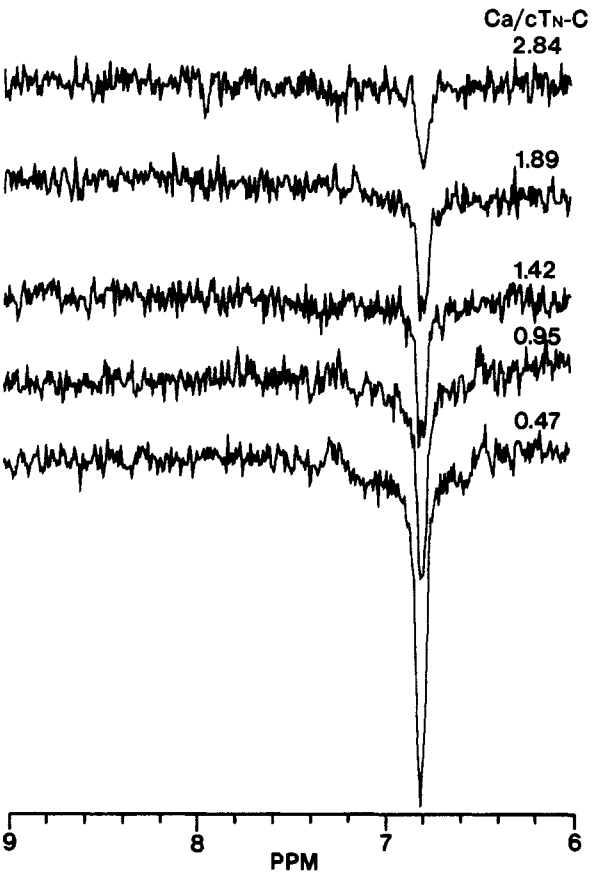

FIGURE 5: CIDNP difference spectra of cTn-C in 4.2 M urea as a function of added Ca^{2+}. Apoprotein was dissolved in 25 mM Pipes, pH 7.0, and 0.15 M KCl; 5 μL of FMN (50 mM) was added before each spectrum (eight light and dark scans) was collected.

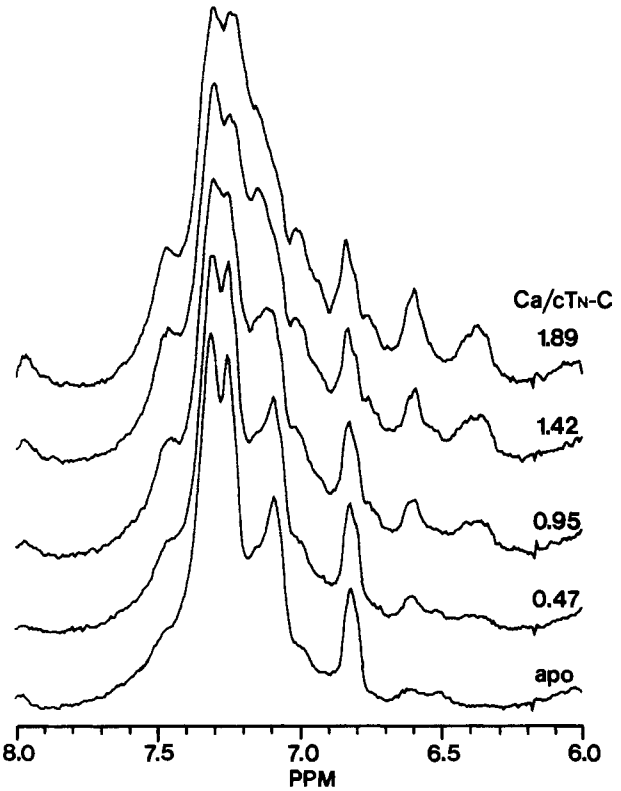

FIGURE 6: Aromatic region of the dark spectrum of cTn-C in 4.2 M urea as a function of added Ca^{2+}. Apoprotein was dissolved in 25 mM Pipes, pH 7.0, and 0.15 M KCl. Each spectrum was collected after the eight light and dark scans of the CIDNP spectrum (Figure 5) and represents 256 scans.

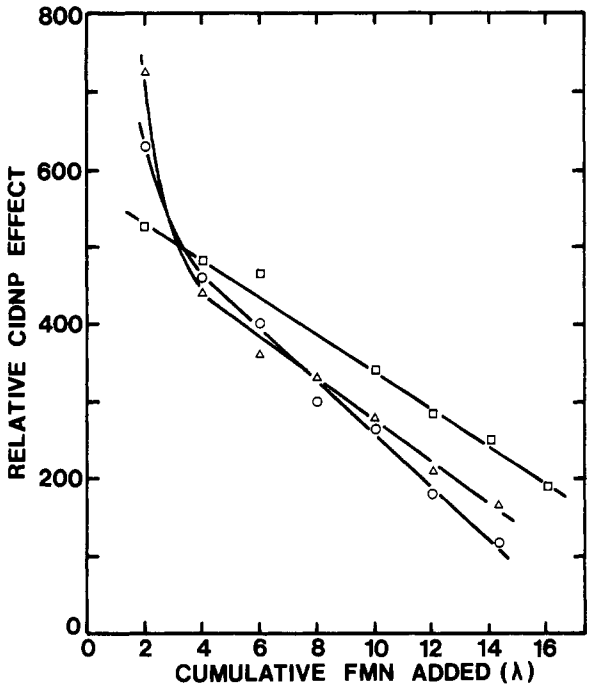

FIGURE 7: Magnitude of the CIDNP effect (arbitrary units) of ortho tyrosine protons as a function of irradiation and FMN added. *N*-Acetyl-L-tyrosinamide (1.1 mM) in 6 M urea, 5 mM Mops, and 20 mM KCl, pH 7.0 (1.20 mL). (a, O) 8 scans (1.0 s × 3.5 W, delay = 20 s); (b, △) as in (a) but 2 μL of Ca^{2+} (0.131 M) added with each FMN addition; (c, □) as in (b) but only two scans for each point (normalized by multiplying by 4).

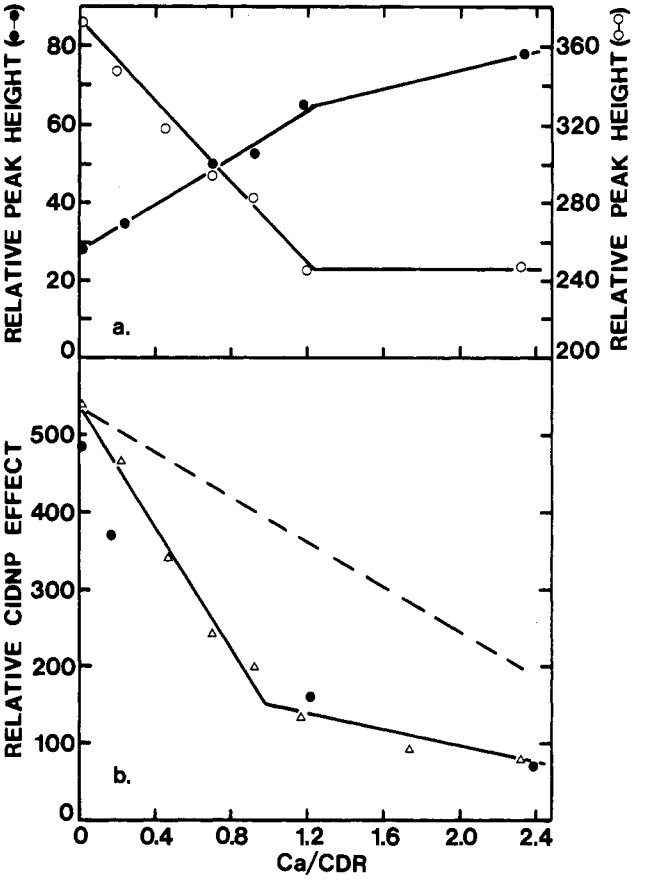

FIGURE 8: Calmodulin (0.94 mM) in 6.0 M urea, 25 mM Mops, pH 7.0, and 0.15 M KCl. (a) Plot of the peak height (arbitrary units) of some aromatic resonances in the dark spectrum (256 scans) as a function of added Ca^{2+}. (●) 6.31 ppm; (O) 6.82 ppm. (b) Tyrosyl CIDNP effect (arbitrary units) as a function of added Ca^{2+}; 2 μL of FMN (50 mM) was added for each point which is two scans (1.0 s × 3.5 W). △, ● represent different titration curves which have *not* been normalized. The dashed line is curve c from Figure 7.

CIDNP signal was observed from sTn-C in 6 M urea. The CIDNP effect at the C-2 and C-4 protons could be readily demonstrated for 1 mM Gly-His-Gly in 6 M urea.

In a study of this nature it is important to ensure that the decrease in intensity of the tyrosyl CIDNP effect is not due to destruction of the exposed tyrosyl residues or of the dye. The most convincing experiments would be with fresh protein samples for each point in a Ca^{2+} titration. This would be extremely demanding in terms of protein. We have attempted to "reuse" the samples in that each point in a titration represents the cumulative addition of FMN as Ca^{2+} is sequentially added to the same sample. At the conclusion of an experiment a sample has been irradiated many times. It is important that control experiments allow the effects of Ca^{2+} binding to be discerned from those of tyrosine or dye destruction, which would also decrease the CIDNP intensity due to progressive loss of chromophore.

Control experiments with *N*-acetyl-L-tyrosinamide in 6 M urea are shown in Figure 7. It is apparent that some process reduces the magnitude of the CIDNP effect with each subsequent series of irradiations. The presence of urea has little effect on these controls (not shown), and there is also no effect from adding Ca^{2+} with each FMN addition. Histidine controls (Gly-His-Gly) yielded similar results under these conditions (not shown). Under gentler irradiation conditions (two irradiations per point) less tyrosine destruction is noted, as a larger CIDNP effect can be obtained at the higher cumulative amounts of FMN (Figure 8, curve c). This process was less dramatic for protein samples; perhaps some protein functional groups scavenge or quench the free radicals which are responsible for the diminished effect.

Figure 8 demonstrates the Ca^{2+} titration of calmodulin in 6 M urea. In Figure 8b data from two different titration curves have been plotted together. Although one set of data represents many more irradiations and much more FMN added than the other (because more points were taken), the titration curves are virtually superimposable. As an additional feature, the control data for *N*-acetyl-L-tyrosinamide in 6.0 M urea, obtained under similar conditions (2 scans per point; see Figure 7c), has been normalized and plotted with the calmodulin data. These results suggest Ca^{2+}-dependent burying of tyrosine residues is occurring in the protein, supported by the decreasing intensity of the resonance representing the unfolded tyrosine residues as Ca^{2+} is added (Figure 8a). Other spectral features which represent the plus Ca^{2+} protein appear at the same time. The increasing peak height of a resonance at 6.31 ppm is plotted in Figure 8a. Other resonances simultaneously appear at 6.56, 6.62, and 6.76 ppm.

Controls were also performed with protein samples in which only FMN but no conformation-altering Ca^{2+} ions were added. These substantiated our conclusions that Ca^{2+} is primarily responsible for the decreasing tyrosine CIDNP effect. A control experiment in which a similar sample of cTn-C was titrated with FMN (four scans per point) is plotted with the Ca^{2+} titration data in Figure 9a. It is apparent that there is a Ca^{2+}-dependent burying of the tyrosyl residues which is complete when about 1 mol of Ca^{2+} has been added. The addition of solid urea to 8 M unfolds the protein somewhat, increasing the CIDNP signal, even when the protein is saturated with Ca^{2+}. The protein spectrum at this point suggests that there are still features representing the "native" plus Ca^{2+} protein. This would explain why 8 M urea does not return the CIDNP signal to control levels and attests to the large degree of stabilization afforded by Ca^{2+} binding. In Figure

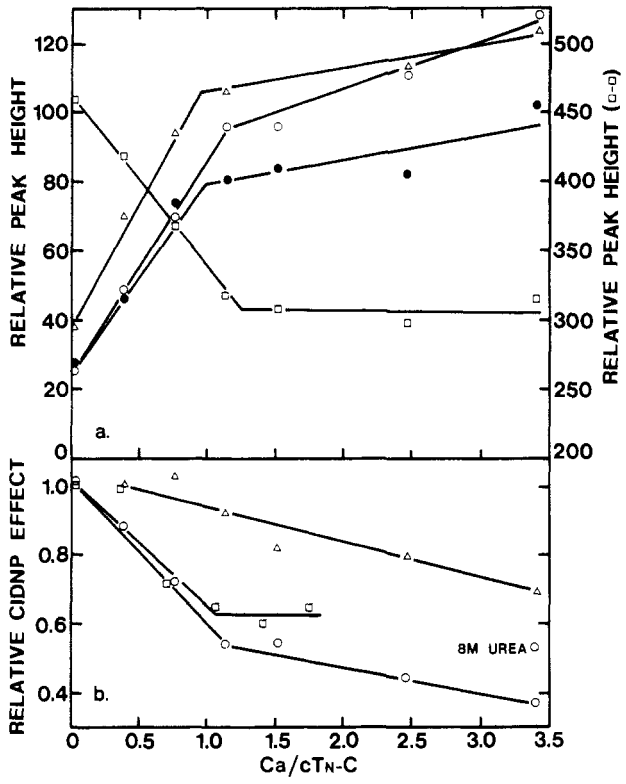

FIGURE 9: cTn-C (0.53 mM) or sTn-C (0.62 mM) in 6.0 M urea, 25 mM Mops, and 0.15 M KCl, pH 7.0. (a) Plot of the peak height of some aromatic resonances of cTn-C as a function of added Ca^{2+} in the dark spectrum (256 scans). (□) 6.84 ppm; (●) 6.35 ppm; (○) 6.60 ppm; (△) 6.76 ppm. (b) Tyrosyl CIDNP effect (arbitrary units) of cTn-C as a function of added Ca^{2+}; 2 μL of FMN (50 mM) was added for each point which is four scans (1.0 s × 3.5 W). (▲) cTn-C control, no Ca^{2+} added for each point. (○) cTn-C titration, Ca^{2+} added for each point. (□) sTn-C in 6.0 M urea, titrated as for cTn-C with Ca^{2+}, except that each point is eight scans (1.0 s × 3.5 W).

9b the appearance of native "plus Ca^{2+}" resonances in the 6 M urea spectrum of cTn-C as Ca^{2+} is added is presented. While some of the decreasing peak height at 6.84 ppm is probably due to tyrosyl destruction, as noted in the control experiment (Figure 9a), a large degree of it must correspond to the increasing peak intensity at 6.60 and 6.76 ppm, which have been previously assigned to the ortho protons of tyrosine-150 and -111 in the native plus Ca^{2+} spectrum. The resonances at 6.35 and 6.41 ppm represent the meta protons of tyrosine-150 and a phenylalanine doublet, respectively (Hincke et al., 1981). The appearance of this phenylalanine resonance is associated with Ca^{2+} binding to the high-affinity sites, as are the tyrosine-150 ortho and meta resonances at their "plus Ca^{2+}" chemical shift positions. In the native protein, Ca^{2+} titration of these resonances indicates they are responsive solely to Ca^{2+} binding to the two high-affinity sites (Hincke et al., 1981). The titration curves in 6 M urea indicate there is only one high-affinity site. Since Ca^{2+} binding to this site restores tyrosine-150 and tyrosine-111 to their native conformations, it is not clear if the metal ion is binding to site III or IV.

The tyrosine CIDNP results for sTn-C appear to be similar to those of cTn-C, i.e., Ca^{2+} binding to the protein in 6 M urea buries the tyrosine residues (Figure 9b). The effect levels off at about 1 mol of Ca^{2+} bound per molecule. In native sTn-C, the tyrosine resonances are not markers for Ca^{2+} binding, e.g., tyrosine residues 10 and 109 exhibit very similar ortho proton chemical shifts, and these do not change upon Ca^{2+} binding (Seamon et al., 1977). These chemical shifts are almost identical with that of the tyrosyl residues in urea-unfolded sTn-C (6.82 ppm), and it is not possible to make inferences about the return of "native" plus Ca^{2+} tyrosine conformations in these experiments, as was done for cTn-C.

Table I: Comparison of Aromatic Chemical Shifts of Some Resonance in Native and Urea-Unfolded Calmodulin

residue	resonances assigned by Seamon (1980) for native protein (2 mol of Ca^{2+}) (ppm)	resonances seen in 6 M urea (2 mol of Ca^{2+}) (ppm)
tyrosine-138 (meta)	6.36	6.31
tyrosine-138 (ortho)	6.65	6.62
upfield phenylalanine	6.55	6.56
tyrosine-99 (ortho)	6.76	6.76

The results for calmodulin are interesting as they indicate that although tyrosine residue(s) are buried as 1 mol of Ca^{2+} is bound, resonances strictly corresponding to the plus-Ca^{2+} resonances of tyrosine-138 are not evident in the aromatic dark spectrum. However, it is possible that the peak at 6.31 ppm represents the meta protons of this residue, previously assigned by Seamon (1980). When Ca^{2+} is bound to calmodulin (2 or 4 mol/protein), these protons possess a chemical shift of 6.36 ppm. Other resonances which appear simultaneously as Ca^{2+} is bound by the urea-unfolded protein can similarly be assigned (Table I).

These data suggest that a phenomenon similar to that observed in the cTn-C case occurs with calmodulin as Ca^{2+} is bound. Ca^{2+} binding to the urea unfolded calmodulin molecule elicits a more native conformation at the level of the tyrosyl residues in sites III and IV.

Discussion

The laser photo-CIDNP experiments with cTn-C, sTn-C, and calmodulin presented herein demonstrate that the tyrosine residues in the native apoproteins are not equally accessible to the hydrophobic FMN dye. Tyrosine-138 in calmodulin appears to be inaccessible to the FMN probe, in agreement with other techniques which have demonstrated the buried nature of this residue (Klee, 1977), and tyrosine-99 shows a smaller enhancement than tyrosine-111, for example, in cTn-C. Ca^{2+} binding by cTn-C selectively buries residues 5 and 150 while tyrosine-111 remains exposed to the solvent. In sTn-C the ortho resonances of the two tyrosines overlap and therefore cannot be distinguished.

Even in cardiac and skeletal Tn-C, where the largest exposure of the tyrosyl residues is indicated in the apoprotein, comparison with model compounds such as *N*-acetyl-L-tyrosinamide (Figure 1b) suggests the accessibility is low when compared to that of the free amino acid. It is tempting to ask if the degree of enhancement can be correlated with the slightly elevated pK_a's that the tyrosine residues possess in each protein. In apo-cTn-C, each of the three tyrosines has a pK_a of ca. 10.8, as do those in apo-sTn-C (McCubbin et al., 1979). Also tyrosine-138 of calmodulin has a pK_a of 12 (Klee, 1977). Comparison with values for exposed tyrosine residues in proteins (9.5–10; Nozaki & Tanford, 1967) indicates these residues are probably not freely exposed on the protein surface. However, the pK_a of tyrosine-99 in calmodulin (10.4 and 10.1 in the absence and presence of Ca^{2+}) indicates it is an exposed residue (Klee, 1977). While the spectrophotometric titration result is seemingly at odds with the CIDNP data on tyrosine-99, it should be borne in mind that the two techniques may not be directly comparable. In order to generate a

Table II: Chemical Shifts of Tyrosine-109 in Apo and Plus Ca^{2+} CB-9 and sTn-C

	apo (ortho, meta) (ppm)	plus Ca^{2+} (ortho, meta) (ppm)
sTn-C[a]	6.84, 7.12	6.85, 7.27
CB-9[b]	6.83, 7.06	6.64, 6.51[c]

[a] Seamon et al. (1977); Levine et al. (1977). [b] Birnbaum & Sykes (1978). [c] Note the reversed nature of these resonances.

CIDNP effect, there appear to be two potential mechanisms for the transient radical formation: electron transfer and hydrogen atom abstraction (Feeney et al., 1980). Electron transfer presumably requires the aromatic ring of the dye to make contact with that of the protein residue, so that the tyrosine ring would have to be free from obstruction by other residues, at least on one side. On the other hand, for hydrogen atom abstraction from an OH group (a situation analogous to spectrophotometric titration), only this group need be accessible. If the former mechanism were the operative one for the CIDNP effect, one could envision a situation where a tyrosine, because of obstruction by other residues, would be rendered inaccessible insofar as the photo-CIDNP experiment is concerned, but would be free to titrate its hydroxyl group because of its exposure to solvent.

The three apoproteins are largely unfolded by 6 M urea, but native-like features in the aromatic spectrum reappear as Ca^{2+} is bound. Titration of these resonances, representing the plus Ca^{2+} protein, indicates that in 6 M urea only one high-affinity site remains in each protein. Ca^{2+} binding to a single high-affinity site in cTn-C induces a native protein structure at the level of tyrosine residues 111 and 150. This observation emphasizes the importance of long-range interactions in Ca^{2+} binding to the individual sites, irrespective of whether Ca^{2+} binding to site III or site IV is responsible for the phenomenon. These interactions have been postulated to play a key role in the mechanism of Ca^{2+} binding to the homologous MCBP proteins (Reid & Hodges, 1980). Clearly, the structural changes observed upon Ca^{2+} binding to urea-unfolded cTn-C also occur when the native protein binds Ca^{2+}, and by implication, when sTn-C binds Ca^{2+}.

Other spectroscopic studies on Ca^{2+} binding to sTn-C in 6 M urea have also demonstrated that only one of the high-affinity sites binds Ca^{2+} under these denaturing conditions. Further, such binding evokes almost all of the circular dichroism and fluorescence changes which are observed when Ca^{2+} binds to the native molecule (Leavis et al., 1980; Nagy & Gergely, 1979). These workers concluded that the sole high-affinity site in 6 M urea corresponded to site III, primarily because the increase in tyrosine fluorescence observed when sTn-C binds Ca^{2+} was assumed to originate from tyrosine-109, located within the Ca^{2+} binding loop of this site. The large increase in tyrosyl fluorescence observed when the sTn-C fragment CB-9, containing only site III, binds Ca^{2+} (Nagy et al., 1978) was considered to support this assignment (Nagy & Gergely, 1979). However, it has been demonstrated by ^1H NMR (Birnbaum & Sykes, 1978) that the environment of tyrosine-109 in Ca^{2+}-bound CB-9 is completely different from that in the Ca^{2+}-saturated sTn-C molecule. This can be illustrated by comparing the chemical shifts of tyrosine-109 under these different conditions (Table II). Examination of Table II reveals that although the chemical shifts of tyrosine-109 are similar in apo-CB-9 and apo-sTn-C, reflecting an exposed environment (Hincke et al., 1981), Ca^{2+} binding induces a large alteration in the environment of tyrosine-109 in CB-9 but very little change in the case of sTn-C. This difference suggests the fluorescent changes induced by Ca^{2+} in sTn-C and CB-9 might not be attributed to the same source, although a similar fluorescence enhancement is observed in each case.

There are also other reasons for reconsidering the assignment of site III as the high-affinity site in 6 M urea. X-ray structural studies of MCBP, containing two high-affinity Ca^{2+} binding sites which exhibit sequence homology with sites III and IV in troponin-C's, have indicated the carbonyl group of phenylalanine residue 57 serves as a donor to Ca^{2+} bound to the CD loop. However, the side chain of this aromatic residue is within 5 Å of the Ca^{2+} atom bound to the EF loop (Donato & Martin, 1974). The EF loop is homologous to site IV, while the CD loop has sequence homology to site III in Tn-C, in which phenylalanine-57 has been replaced by tyrosine-109 in sTn-C and tyrosine-111 in cTn-C. Models of sTn-C based on the X-ray structure derived for Ca^{2+}-bound MCBP have indicated tyrosine-109 would be a close proximity to the metal ion bound at site IV (Kretsinger & Barry, 1975).

Spectroscopic studies have indicated that there is energy transfer from the Phe-57 side chain to Tb^{3+} bound at the Ca^{2+} binding site in the EF loop (Moews & Kretsinger, 1975). Terbium luminescence studies with sTn-C and MCBP (Miller et al., 1975) and cTn-C (Brittain et al., 1976) demonstrate near-identity in circularly polarized emmission (CPE) line shape and dissymmetry factors, indicating closely similar binding sites for Tb^{3+} in these proteins. Therefore it was concluded that in each of these proteins a homologous aromatic residue was involved in the energy transfer, this being the Y residue of the CD Ca^{2+}-binding loop which was in close proximity to the metal ion bound in the EF loop.

Additional evidence to support the proposed orientation of tyrosine-111 relative to site IV stems from Gd^{3+} binding experiments with cTn-C (Hincke et al., 1981). When Gd^{3+} was added to the Ca^{2+}-saturated native protein, the resonances representing the ortho and meta protons of tyrosine residues 111 and 150 were selectively broadened, indicating their proximity to the paramagnetic ions bound at the high-affinity sites. The ortho resonances of tyrosine-111 were broadened to a greater extent than the meta resonances, indicating that the ortho protons are closer to the bound metal ion than are the meta protons. Tyrosines-109 and -111 in sTn-C and cTn-C, respectively, are proposed to occupy the Y ligand position of site III, coordinating the metal ion to the peptide carbonyl group. The NMR results specify a geometry with the aromatic side chain directed away from the bound ion. It is difficult to conceive of an orientation in which the ortho protons of this residue could be closer to the site III metal ion than the meta protons. However, the model of sites III and IV constructed from coordinates based on the parvalbumin X-ray structure (Kretsinger & Barry, 1975) predicts that the tyrosine residue will be suitably oriented relative to the metal ion bound at site IV.

Energy transfer from a tyrosine residue in sTn-C to bound Tb^{3+} has been demonstrated for the native protein and the protein unfolded in 6 M urea (Leavis et al., 1980). The stoichiometry for Tb^{3+} binding is 2 mol/Tn-C in the native protein, but only one binding site remains in 6 M urea, as is observed for Ca^{2+}. Because a similar degree of Tb^{3+} fluorescence is seen in each case, these workers suggested site III and not site IV was the remaining high-affinity site in 6 M urea, since in a largely unfolded protein the other binding sites "were considered" to be too far away from tyrosine for energy transfer to occur. This assumption neglects the fact

that energy transfer in the native protein is presumed to occur between the aromatic tyrosyl side chain and the nearby site IV metal ion, as discussed above. Moreover, Nagy & Gergely (1979) have demonstrated that under both native and denaturing (6 M urea) conditions three α-helical segments, each containing 9–10 residues, are formed when Ca^{2+} is bound to sTn-C. Evidently large conformational changes occur in regions of the denatured molecule which are distant from the single Ca^{2+} binding site. It is equally likely, therefore, that this extensive conformational change in urea is brought about by Ca^{2+} binding to site IV, bringing tyrosine-109 into close proximity with a metal ion bound at site IV even when binding at site III itself was abolished.

An initial premise in our interpretation of the NMR evidence considered to support this proposal is that the chemical shifts of tyrosyl residues 111 and 150 are dependent upon their environment, which is primarily determined by metal binding to the nearby site IV in the native plus Ca^{2+} protein, where 2 mol of Ca^{2+} is bound. The results presented here indicated that the same environment is created for these residues when only 1 mol of Ca^{2+} is bound to the protein in 6 M urea. It seems likely that a similar phenomenon occurs in sTn-C, although the ortho chemical shifts of tyrosine-109 are not suitable probes to allow us to monitor it.

This conclusion stresses the fundamental nature and importance of long-range interactions between residues of sites III and IV in Ca^{2+} binding to each site. The presence of these interactions is also indicated by studies with fragments of sTn-C, specifically those containing site III (CB-9), site IV (TH2) and both sites III and IV (TR2) (Leavis et al., 1978). The Ca^{2+} affinity of TR2 was similar to that of the high-affinity sites of native sTn-C, with an affinity of 5×10^7 M^{-1} for 2 mol of Ca^{2+}. However, the affinities of the peptides containing the single sites were much reduced: 2.2×10^5 M^{-1} for CB9 and 2.5×10^4 M^{-1} for TR2. Clearly, important interactions exist between these two regions of the molecule which affect Ca^{2+} binding at each site. Such a phenomenon has also been dramatically shown for carp parvalbumin (Coffee & Solano, 1976; Derancourt et al., 1978). When the CD and EF Ca^{2+} binding loops are separated, the Ca^{2+} affinity of each site is reduced by 4–5 orders of magnitude.

The similar phenomena observed for cTn-C and calmodulin in 6 M urea at the level of the tyrosine residues in sites III and IV suggest that, in 6 M urea at least, site IV is the highest affinity site in calmodulin.

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Analysis of Cooperativity Observed in pH Titrations of Proton Nuclear Magnetic Resonances of Histidine Residues of Rabbit Cardiac Tropomyosin†

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ABSTRACT: We have investigated in detail the cooperativity which we had previously observed in the pH titration profiles of the histidine residues of rabbit tropomyosin [Edwards, B. F. P., & Sykes, B. D. (1978) *Biochemistry 17*, 684]. Nonpolymerizing tropomyosin was prepared by carboxypeptidase digestion, and the titration profiles of its histidine residues were compared with those of undigested tropomyosin which was fully polymerized (in 0.1 M KCl) throughout the titration. We have concluded that both histidine-153 and histidine-273 have significant cooperativity in their pH titrations only in polymerized tropomyosin, that the cooperativity arises from an intrinsic pH-dependent conformational transition which links the two residues together and not from the known pH dependence of the polymerization, and that the best model for the cooperativity is a biallosteric adaption of the Monod–Wyman–Changeux formalism involving two classes of binding sites for the same ligand (protons). Three other models which postulated either a Hill transition, an interaction with a neighboring residue that also titrates, or a pH-dependent polymerization were also considered.

Tropomyosin molecules are found in the thin filaments of muscle sarcomeres where they function in the regulation of muscle contraction by calcium ions. The rodlike tropomyosin molecules (~420 by 20 Å; Caspar et al., 1969) form a continuous ribbon on both sides of the double helix of globular actin molecules by an end-to-end overlap of ~20 Å. Each tropomyosin rod covers seven actin molecules and has one troponin molecule bound to it (Hanson & Lowy, 1963; Ebashi et al., 1969). In resting muscle the tropomyosin molecules block the interaction between myosin and actin that generates tension. When calcium ions are temporarily released from the sarcoplasmic reticulum in response to a nerve impulse, troponin becomes saturated with calcium ions and undergoes a conformational change that moves the tropomyosin molecules to a nonblocking position. Harrington (1979) has recently reviewed the extensive literature on the mechanisms of muscle contraction and its regulation.

Tropomyosin is a coiled coil of two α-helical polypeptide chains, based upon theoretical considerations (Crick, 1953), measurements of optical rotary dispersion (Cohen & Szent-Gyorgyi, 1957), electron micrographs (Caspar et al., 1969), and the amino acid sequence (Stone & Smillie, 1978). The X-ray crystallographic structure at 20-Å resolution (Phillips et al., 1979) has confirmed the earlier work.

We have used ^{1}H nuclear magnetic resonance to investigate the relationship between the physical properties of purified tropomyosin molecules and their function on the thin filaments (Edwards et al., 1977; Edwards & Sykes, 1978, 1980). In our study of the resonances from the histidine residues of tropomyosin (Edwards & Sykes, 1978), we observed two anomalies which required further examination: (1) the resonance from the C-2 proton of histidine-153 broadened into a square multiplet of resonances under certain conditions, and (2) the pH titration profiles of the most up- and downfield resonances of this multiplet were cooperative. In that paper we suggested that the multiple resonances derived from interconverting conformations of differing pK_a. However, we postponed an analysis of the cooperativity because we did not know if the different resonances of the multiplet came from individual species.

We have since determined that the different conformational forms are associated with the thermal unfolding of the coiled coil (Edwards & Sykes, 1980). The C-2 resonances from histidine-153 and histidine-276 of α,α'-tropomyosin are single peaks at 34 °C and below so long as the protein is fully reduced. Formation of a cross-link between cysteine-190 and cysteine-190′ destabilizes the middle region of tropomyosin (Lehrer, 1978) and lowers the temperature at which the multiple histidine resonances appear. We have analyzed the pH titration profiles of the histidine C-2 resonances in the ^{1}H NMR spectra of this "low-temperature" conformation and found them still to be cooperative. In this paper we present a physical explanation for the cooperativity, and we discuss four models that could explain the data.

Experimental Procedures

We have recently presented our biochemical and NMR procedures in detail (Edwards & Sykes, 1980). All the experiments discussed in this paper used rabbit cardiac tropomyosin which has two identical α chains (Mak et al., 1979). The nonpolymerizing tropomyosin (αTM-NP) was prepared

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