Hydrogen-1 Nuclear Magnetic Resonance Investigation of Bovine Cardiac Troponin C. Comparison of Tyrosyl Assignments and Calcium-Induced Structural Changes to Those of Two Homologous Proteins, Rabbit Skeletal Troponin C and Bovine Brain Calmodulin†

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ABSTRACT: The effect of Ca^{2+} binding on the 270-MHz proton nuclear magnetic resonance spectrum of bovine cardiac troponin C (cTnC) has been examined. Assignment of resonances in the aromatic spectral region to tyrosine residues 10, 111, and 150 has been made for apo-cTnC and calcium-bound cTnC on the basis of decoupling experiments, pH titrations, temperature-induced changes, and gadolinium broadening experiments. The sequence homology which these tyrosine residues display with residues in two previously studied proteins, rabbit skeletal troponin C (sTnC) [Seamon, K. B., Hartshorne, D. J., & Bothner-By, A. A. (1977) *Biochemistry 16*, 4039] and bovine brain calmodulin [Seamon, K. B. (1980) *Biochemistry 19*, 207], was also used in the assignments. High-affinity calcium binding (up to 2 mol/cTnC) causes large alterations in the environments of tyrosines-10 and -150, indicating that the N terminus is probably buried in the protein interior. The evidence suggests that the environment of tyrosine-150 in calcium-saturated cTnC must closely resemble that of tyrosine-138 in calmodulin in that it experiences the hydrophobic core of the protein. However, there is no similarity between these environments in the apoproteins. Dramatic alterations in phenylalanine resonances are seen during the binding of the third mole of calcium, corresponding to filling the sole low affinity site. Comparison of the spectral features of cTnC with those previously reported for sTnC and calmodulin reveals many structural similarities which stem from their high degree of primary sequence homology.

Cardiac troponin C (cTnC) is the calcium-binding subunit of the troponin complex in bovine cardiac muscle (Burtnick et al., 1975). Calcium binding initiates a chain of molecular events which leads to activation of the Mg-ATPase of actomyosin and contraction in vertebrate striated muscle (Ebashi et al., 1969; Potter & Gergely, 1974).

The amino acid sequence of cTnC has been determined (van Eerd & Takahashi, 1975) and found to be quite homologous to the primary sequence of troponin C from rabbit skeletal muscle (sTnC) (Collins, 1974). Both these proteins are believed to have evolved from MCBP,[1] a high-affinity Ca^{2+}-binding protein found in abundance in many lower vertebrates (Pêchère et al., 1971). The crystal structure of the Ca^{2+}-bound MCBP has been determined (Kretsinger & Nockolds, 1973). Comparison of the amino acid sequences of these homologous calcium-binding proteins allows four Ca^{2+}-binding sites to be located in the sTnC sequence (Collins et al., 1973). In cTnC, it appears that one of the sites, the one located closest to the N terminus (site I), does not bind calcium because of extensive amino acid substitution (Kretsinger & Nockolds, 1973). This view has been confirmed by calcium-binding studies which indicate that sTnC binds 4 mol of calcium, two of high affinity (2×10^7 M^{-1}) and two of low affinity (3×10^5 M^{-1}) (2×10^5 M^{-1}) (Holroyde et al., 1980), while cTnC only binds 3 mol of calcium, two of high affinity (1×10^7 M^{-1}) and one of lower affinity (Leavis & Kraft, 1978). In each protein, the high-affinity sites also bind Mg^{2+} with low affinity (3×10^3 M^{-1}). Comparison of these data implies that the defunct cTnC site corresponds to a low-affinity site in sTnC. A chemical modification study (Sin et al., 1978) indicates that the two sites found closest to the N terminal in sTnC are the low-affinity sites (I and II), and the high-affinity sites are located in the C-terminal half of the molecule (sites III and IV).

Another protein which demonstrates primary sequence homology to troponin C and has been extensively studied is the ubiquitous calcium-binding protein calmodulin [for a recent review, see Cheung (1980)]. The sequence of this protein suggests that it should contain four calcium-binding sites (Vanaman et al., 1977), which has been verified experimentally. However, the affinities of these sites were not well established. The most recent data for calcium binding to calmodulin suggest that there are four high affinity sites with affinity constants between 3×10^5 M^{-1} and 5×10^4 M^{-1} (Crouch & Klee, 1980). Positive cooperativity is exhibited in the binding of the first 2 mol of calcium, which is associated with the major calcium-induced conformational change. Calmodulin activates a number of metabolically important enzymes, such as 3′:5′-cyclic-nucleotide phosphodiesterase (Teshima & Kakiuchi, 1974) and adenylate cyclase (Lynch et al., 1976), by forming a calmodulin–enzyme complex only when calcium is present. Apocalmodulin will not interact with these proteins. In fact, Crouch & Klee (1980) have demonstrated that 3–4 mol of calcium must be bound by calmodulin before it can activate 3′:5′-cyclic-nucleotide phosphodiesterase. In this respect, it differs from skeletal and cardiac muscle troponin C's, which are found, in either the presence or absence of calcium, as members of the troponin complex.

Calcium binding to both troponin C molecules causes large conformational changes which have been studied by a variety of spectroscopic techniques. For cTnC these have included

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[1] Abbreviations used: NMR, nuclear magnetic resonance; cTnC, bovine cardiac troponin C; sTnC, rabbit skeletal troponin C; DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Mops, 2-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; IAANS, 6-[4-(iodoacetamido)anilino]naphthalene-2-sulfonic acid; MCBP, muscle calcium binding protein (parvalbumin); photo-CIDNP, photochemically induced dynamic nuclear polarization; FMN, flavin mononucleotide.

0006-2960/81/0420-3286$01.25/0

increases in secondary structure by CD (Hincke et al., 1978), changes in intrinsic tyrosine fluorescence (Leavis & Kraft, 1978), and changes in fluorescence of the sulfhydryl-label IAANS (Johnson et al., 1978).

sTnC (Seamon et al., 1977; Levine et al., 1977) and calmodulin (Seamon, 1979, 1980) have been studied extensively by NMR techniques. This has allowed comparison of the proposed homologies based on sequence with the solution conformations of these proteins. The NMR results from this study on cTnC have allowed us to characterize this protein further and to make more general comparisons between these three homologous proteins.

Experimental Procedures

Cardiac troponin C was prepared as previously described (Hincke et al., 1978). An extinction coefficient ($E^{1\%}_{276nm}$) of 2.3 was determined by quantitative amino acid analysis with norleucine as an internal standard. This value was consistent with fringe counts obtained from sedimentation equilibrium experiments in which 4.1 fringes were assumed equivalent to 1 mg/mL (Babul & Stellwagen, 1969).

Calcium-free protein was generated by exhaustive dialysis vs. doubly distilled water in plastic ware following protein dissolution in 0.1 M EDTA, pH 8.0. The cTnC was then dialyzed against 0.15 M KCl and 25 mM Mops, pH 6.8, which had been passed through a column of Chelex-100 (Bio-Rad), and lyophilized. The protein was redissolved in D_2O to the original volume for NMR measurements. The calcium-free status of this protein was confirmed by atomic absorption measurements.

Spectra were collected on a Bruker HXS-270 spectrometer operating in the Fourier-transform mode with quadrature detection. Typical instrumental settings for the 5-mm probe were sweep width = ±2000 Hz, 4096 points, 9.8-μs pulse (~90°), and line broadening = 1.0 Hz. All spectra are reported relative to the methyl resonance of DSS and, except where noted in the text, were taken at 301 K.

Laser photo-CIDNP experiments (Kaptein, 1978) were performed by utilizing a Spectra Physics Model 164 argon ion laser operating at 3.5 W in the multiline mode with FMN as the photoprobe. Flat-bottom NMR tubes (10 mM) were used for these experiments, at an ambient temperature of 301 K. A 14.0-μs pulse (~90°) was used for the 10-mm probe. In our experimental routine, alternating light and dark spectra were taken, in which the sample was irradiated for 1.5 s prior to recording the light spectrum, with a 20-s delay between scans. Subtraction of the dark from the light spectrum allowed the specific effects of irradiation to be seen.

Two other pulse sequences were used in specific instances. (1) For complete suppression of the nuclear Overhauser enhancement effect (NOE) during decoupling of aromatic resonances, the decoupler was only turned on during the FID acquisition, and a delay of 1.5 s was incorporated between scans. (2) When decoupling experiments retaining the NOE were desired, the decoupler was left on throughout the pulse sequence. There was no delay period between scans.

Gadolinium broadening experiments were performed by adding equal amounts of $GdCl_3$ and $LaCl_3$ solutions (in 10 mM Pipes, pH 6.5) to two identical protein samples. The specific paramagnetic effects of the Gd^{3+} were seen by subtracting the Gd^{3+} spectrum from the La^{3+} spectrum.

Metal ion solutions ($LaCl_3$, $GdCl_3$, and $CaCl_2$) were prepared gravimetrically and standardized by titration with an EDTA standard solution. A syringe microburet, calibrated with H_2O gravimetrically, was utilized. The indicator eriochrome black T was used for calcium standardization and xylenol orange for lanthanide solutions.

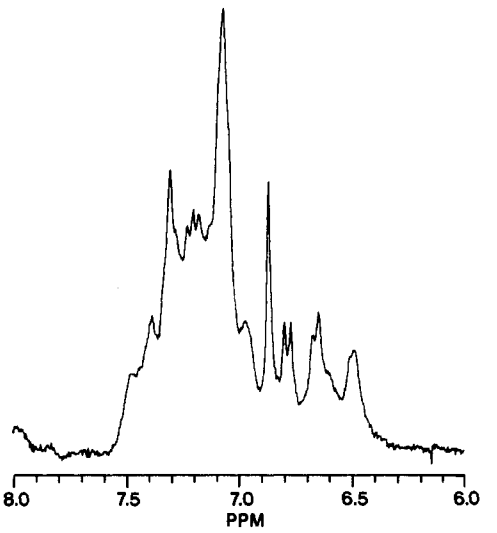

FIGURE 1: Aromatic region of the 270-MHz ^{1}H NMR spectrum of apo-cTnC in 0.15 M KCl, 25 mM Mops, and 1 mM EDTA, pH 6.8.

Protein concentrations were determined on each sample by quantitative dilution of an aliquot into a similar solvent (but H_2O not D_2O) for UV absorption measurement. pH measurements were made on a Radiometer Model PHM62 meter with a 6030-04 Ingold electrode. pH's were adjusted with solutions of NaOD or DCl and are reported as the direct meter readings, uncorrected for the deuterium isotope effect.

Results

Apo-cTnC. The aromatic region of the ^{1}H NMR spectrum of apo-cTnC is presented in Figure 1. This spectrum represents the ring protons from nine phenylalanines and three tyrosines. The protein contains no histidine or tryptophan. There are a number of upfield shifted resonances, suggesting the presence of a large amount of tertiary structure in the apoprotein.

The singlet resonance at 6.86 ppm represents the ortho and meta protons of one tyrosine (tyrosine A). This assignment, which accounts for the sharp, singlet nature of this peak (Snyder et al., 1975), is bgased on a number of observations. Slight increases in temperature (up to 43 °C) cause this singlet to break up into two coupled doublets which shift to higher field (3,5 ortho protons) and lower field (2,6 meta protons) with increasing temperature. The assignment to ortho and meta protons was based upon the relative magnitudes of the nuclear Overhauser effect (NOE) when each was decoupled. Irradiation at the meta resonance produces a larger NOE at the ortho resonance than the reverse experiment (Birnbaum & Sykes, 1978). This singlet is not coupled to any other peak in the aromatic region of the spectrum, as revealed by difference spectroscopy.

The doublet resonance at 6.77 ppm was also assigned to a tyrosine residue (tyrosine B) on the basis of its decoupling pattern (see Figure 2). It is coupled to a doublet peak at 7.06 ppm, revealed by difference spectroscopy. The resonance at 7.06 ppm appears negative in the difference spectrum, which represents the difference obtained by subtracting the spectrum with the irradiation off-resonance from that with the irradiation at 6.77 ppm, because of the negative NOE effect concomitant with decoupling. Decoupling experiments which yielded a pattern of two coupled doublet resonances were unequivocally assigned to tyrosine resonances. The relative magnitudes of the NOE in these decoupling experiments suggested that the 6.78 ppm resonance represents the ortho

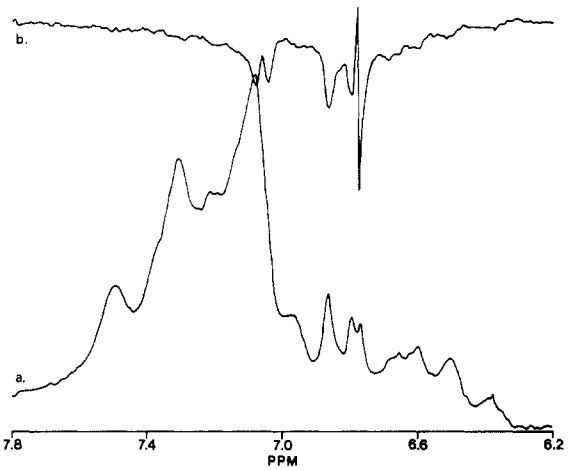

FIGURE 2: (a) Aromatic region of the 270-MHz ^{1}H NMR spectrum of cTnC in 0.15 M KCl, 25 mM Mops, pH 6.81, and ~0.6 Ca/TnC. (b) Difference NMR spectrum with decoupling applied at 6.77 ppm to locate the meta protons of tyrosine B. The control spectrum was obtained with the decoupler pulse applied at 5.92 ppm.

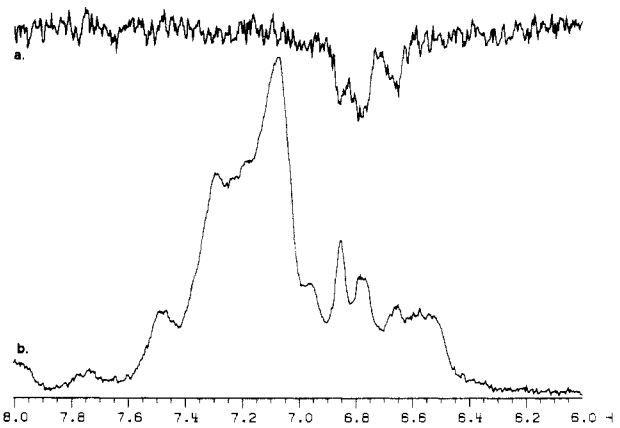

FIGURE 3: (a) Laser photo-CIDNP difference spectrum of apo-cTnC (1.1 mM). The difference spectrum represents the subtraction of the dark spectrum from the light spectrum (12 scans each). (b) The dark spectrum. The solvent is 0.15 M KCl, 25 mM Mops, pH 6.04, and 0.2 mM FMN.

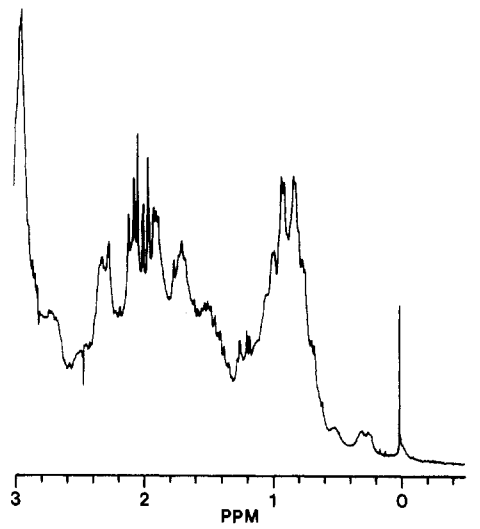

FIGURE 4: Aliphatic region of the 270-MHz ^{1}H NMR spectrum of apo-cTnC. The solvent is 0.15 M KCl, 25 mM Mops, and 1 mM EDTA, pH 6.8.

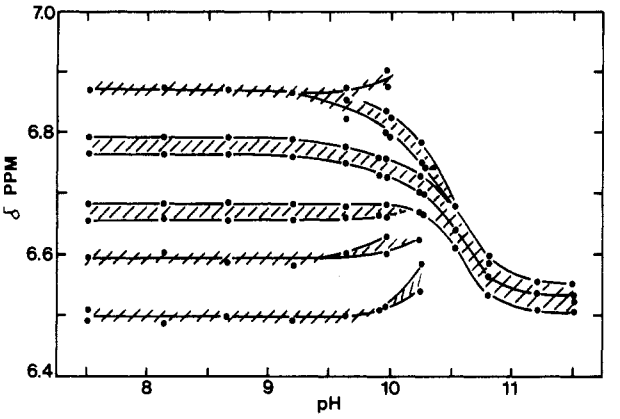

FIGURE 5: pH titration of apo-cTnC. The chemical shifts of some resonances in the aromatic region of the 270-MHz ^{1}H NMR spectrum are plotted as a function of pH. The solvent was 0.15 M KCl, 25 mM Mops, and 1 mM EDTA.

protons, while the more downfield doublet represents the meta protons.

The resonance at 6.64 ppm is believed to contain the ortho protons of tyrosine C. Laser photo-CIDNP experiments on apo-cTnC at pH 6.00 (Figure 3) indicate the presence of tyrosine ortho protons at this position and also confirm the assignment of the ortho protons of tyrosine residues A and B. The CIDNP experiment demonstrates that although tyrosine B is most exposed, tyrosines A and C are also exposed to a certain extent. It should be noted, however, that comparison with results obtained with the free tyrosine amino acid indicates that these tyrosines are all relatively unexposed.

During the course of pH titration of apo-cTnC the tyrosine C resonance at 6.64 ppm titrates upfield with the ortho resonances of the other tyrosines to a final position typical of the phenolate species in the denatured protein (see below). Decoupling experiments with irradiation at 6.64 ppm do not reveal any corresponding meta protons, even in the difference spectrum. This may indicate that they overlap the ortho protons somewhat, although not to the same extent as tyrosine A, where a sharp singlet is observed.

The aliphatic region of the apoprotein spectrum is presented in Figure 4. There are a number of upfield shifted peaks, between −0.05 and 0.3 ppm. These represent methyl groups whose environment is influenced by aromatic residues and indicate there is a well-defined tertiary structure present in the apoprotein.

pH Titration of Apo-cTnC (*Figure 5*). Increasing the pH of apo-cTnC does not affect the spectrum over the pH range 7.5–9.5. Between pH 10 and 10.2, large alterations in the phenylalanine resonances were observed as the protein began to denature. The pK_a's of the tyrosine residues, determined by the upfield titration of the ortho protons, were all about 10.5. These pK_a's are similar to the value of 10.8 obtained from the spectrophotometric titration of apo-cTnC (McCubbin et al., 1979). This would be a better indication of protein unfolding over this pH range than tyrosine pK_a.

During the titration, the ortho/meta resonances of tyrosine A at 6.87 ppm split first into an apparent triplet and then into two doublets as the meta resonance titrated downfield and the ortho upfield. The tyrosine B resonance at 6.78 ppm titrated upfield as a typical ortho resonance, as did the less well-resolved ortho protons of tyrosine C at 6.67 ppm. The upfield peak at 6.5 ppm and the shoulder at 6.6 ppm also began to move as the protein was denatured, shifting downfield to chemical shifts typical of random coil phenylalanine residues.

Ca^{2+} Saturated cTnC. The aromatic spectrum of the calcium saturated protein is shown in Figure 6a. Dramatic alterations have occurred in the phenylalanine envelope between 6.9 and 7.4 ppm with Ca^{2+} binding. In addition, a number of overlapping doublets are observed around 6.8 ppm,

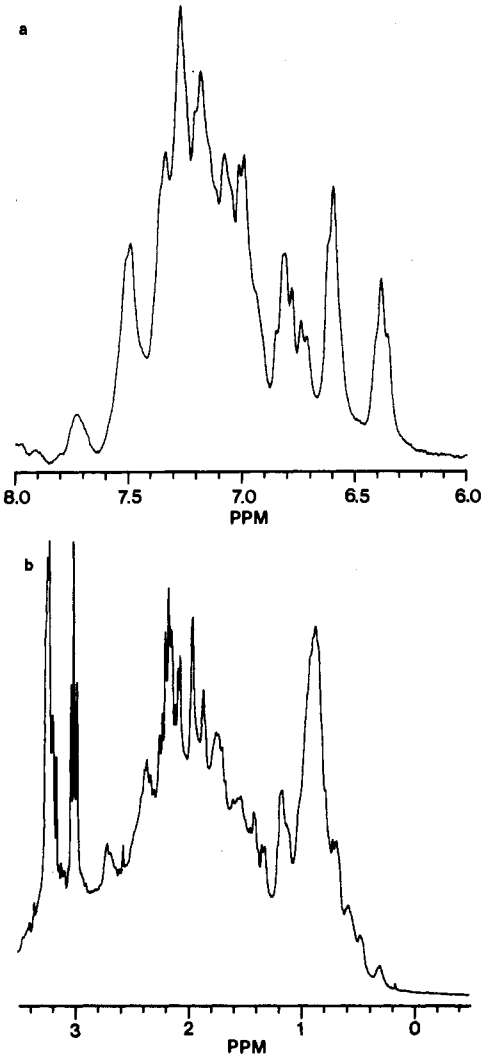

FIGURE 6: 270-MHz ^{1}H NMR spectrum of calcium-saturated cTnC in 0.15 M KCl, 25 mM Mops, pH 6.8, and 1 mM $CaCl_2$. (a) Aromatic region; (b) aliphatic region (no DSS in this spectrum).

and there are two well-defined upfield resonances at 6.60 and 6.39 ppm. Decoupling experiments (without the NOE) demonstrated that the central doublet at 6.79 ppm is coupled to a doublet at 7.06 ppm. For assignment purposes, this was called tyrosine D. A connectivity was also revealed between the doublet at 6.72 ppm and that at 6.83 ppm (tyrosine E). Two other connected doublets were found under the peaks at 6.39 and 6.60 ppm (tyrosine F).

These decoupling experiments were carried out at temperatures ranging from 27 to 80 °C (Figure 7). At higher temperatures, the phenylalanine peaks obscuring the tyrosine doublets at 6.60 and 6.39 ppm begin to shift to lower field as the structural elements making up their environment are lost. At 65 °C, each of these peaks has split into two doublets of which the upper field ones represent the coupled tyrosine doublets. At 80 °C, only a tyrosine doublet remains at 6.60 ppm, while a phenylalanine doublet at 6.43 ppm has clearly shifted downfield away from the tyrosine doublet at 6.39 ppm. It is obvious that a high degree of structure still remains at 80 °C. As well, the chemical shifts of the two coupled tyrosine doublets at 6.60 and 6.39 ppm are invariant throughout this temperature range.

Only small changes are seen at the other tyrosine resonances. Decoupling experiments at the elevated temperatures reveal that the meta resonance of tyrosine D remains at 7.06 ppm, at least to 65 °C. At 80 °C, it has shifted downfield to 7.10

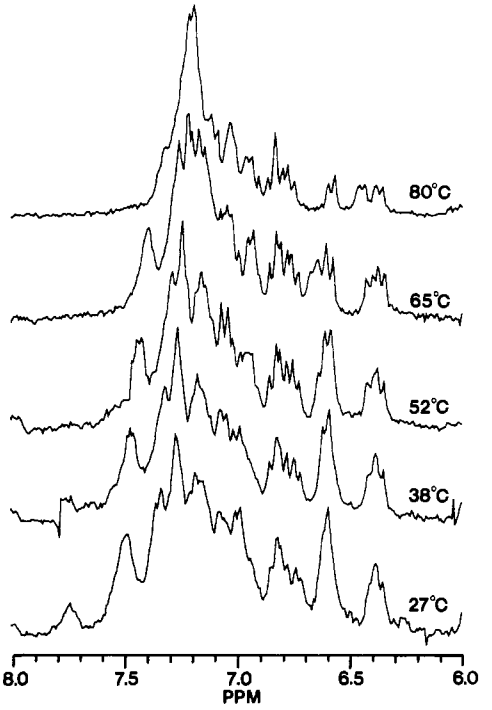

FIGURE 7: Thermal melting experiment of calcium-saturated cTnC. cTnC (1.1 mM) was dialyzed vs. 0.15 M KCl, 25 mM Mops, pH 6.78, and 1 mM $CaCl_2$ prior to lyophilization. The sample was then dissolved in D_2O to the original volume. Each spectrum represents 256 scans.

ppm, perhaps beginning to assume a random coil chemical shift (meta 7.15, ortho 6.86; Bundi & Wüthrich, 1979).

The aliphatic spectrum of the Ca^{2+}-saturated protein is shown in Figure 6b. Many changes have occurred with calcium binding. In particular, some upfield methyl resonances around 0 ppm in the apoprotein have shifted downfield.

pH Titration of Ca^{2+}-Saturated cTnC. The pH titration of the calcium-saturated protein is presented in Figure 8a. The chemical shifts of some of the peaks in the aromatic spectrum are plotted as a function of pH in Figure 8b.

Increasing the pH to 10.02 causes the overlapping tyrosine doublets at 6.8 ppm to change, forming a large peak at 6.75 ppm. This represents the ortho protons of tyrosines D and E and the meta resonance of tyrosine E. The singlet nature of this peak at pH 10.7 (Figure 8a) indicates that the ortho and meta resonances of tyrosine E are overlapping and suggests that they were originally reversed. The ortho resonance is at 6.83 ppm and titrates further upfield with increasing pH than the meta resonance at 6.72 ppm. The doublet nature of this resonance at higher pH's indicates that the ortho and meta resonances of tyrosine E are no longer overlapping. Since the meta resonance does not appear as the ortho resonance titrates upfield, it might be broadened under these conditions. At 45 °C, the meta peak does appear as a shoulder on the downfield side of the other proton resonance during the singlet to doublet transition. The ortho proton peaks titrate upfield with increasing pH, yielding an apparent pK_a of 10.6–10.7 for each tyrosine, a value not very different from the pK_a of all three tyrosines in the apoprotein. It appears that these two tyrosine residues are not particularly buried during the Ca^{2+}-binding process or that the structural elements surrounding them are not particularly stabilized by bound calcium.

However, the resonances at 6.39 and 6.60 ppm, containing the ortho and meta protons of tyrosine F, are largely unaffected by increasing pH until about pH 10.7. At this point, the tyrosine doublets begin to titrate upfield while the overlapping

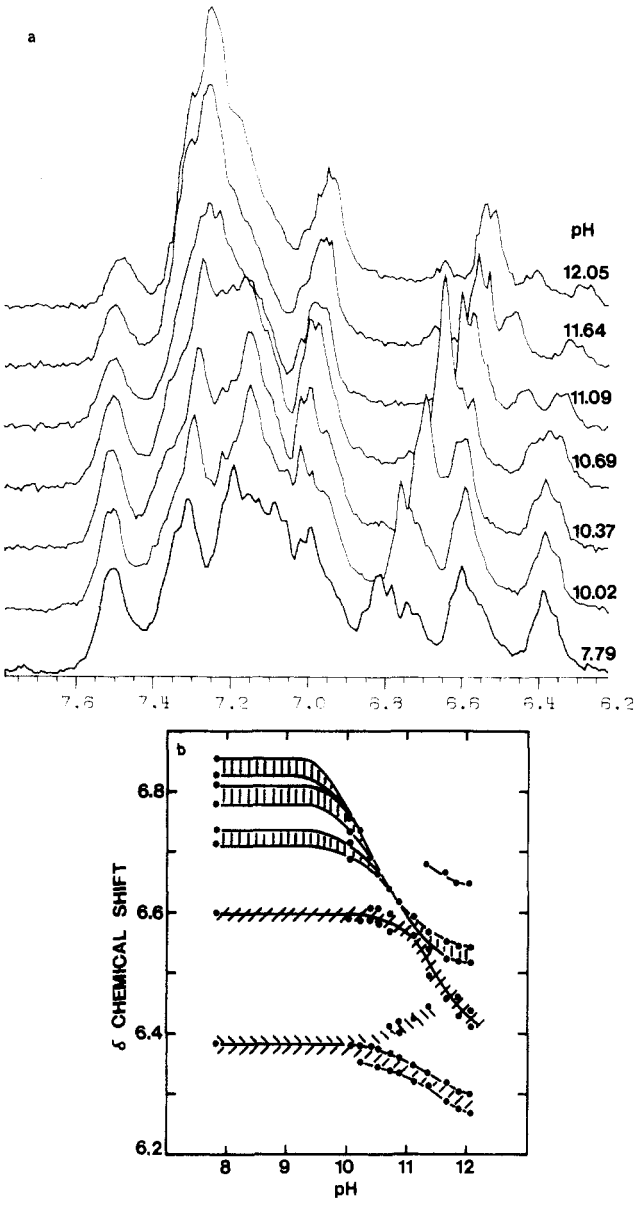

FIGURE 8: (a) pH titration of calcium-saturated cTnC. cTnC (1.1 mM) in 0.15 M KCl, 25 mM Mops, and 2 mM $CaCl_2$. The spectra are presented at increasing pH. (b) pH titration of calcium-saturated cTnC. In (b), the chemical shifts of some aromatic peaks are plotted as a function of pH. The crosshatching gives an indication of the peak width; i.e., each point represents a peak in a doublet.

phenylalanine doublets shift downfield toward chemical shifts more typical for phenylalanine ring protons (7.0–7.4 ppm). A pK_a of 11.4–11.5 can be derived from this titration curve. The magnitude of the pH-induced shift for these resonances can be used to assign the ortho and meta resonances since the meta resonance will undergo a much smaller upfield shift than the ortho resonance when the tyrosine is ionized. On this basis, the doublet at 6.39 ppm is assigned to the meta resonance and that at 6.60 to the ortho resonance. The small doublet observed at 6.65 ppm in the pH 12.04 spectrum titrates in parallel with the meta doublet of tyrosine F, suggesting that there is a slow exchange between the native structure and some intermediate denatured form (i.e., $N \rightleftharpoons N'$).

Assignment of Tyrosine Resonances. There are two aspects to assigning the tyrosine resonances elucidated in the previous section. First, the resonances in the apoprotein must be aligned with those in the calcium-bound form, and second, these must be assigned to the specific residues within the protein sequence. In this task, some use will be made of the large degree of

Table I: Chemical Shifts of Tyrosine Resonances in Homologous Calcium-Binding Proteins

	apo			plus Ca^{2+}		
	ortho 3,5	meta 2,6	pK_a	ortho 3,5	meta 2,6	pK_a
calmodulin[a,f]						
site III (99)	6.82	7.31	10.4	6.76	7.29	10.1
site IV (138)	6.70	6.72	11.9	6.55	6.36	12
sTnC[b]						
N terminal (10)	6.82	7.05[c]	10.4	6.85	7.12	NR[e]
site III (109)	6.84	7.12	10.4	6.85	7.27	NR
CB-9[d]						
site III (109)	6.83	7.06	10.6	6.64	6.51	NR
cTnC						
N terminal (5)	6.64	? (C)[g]	10.5	6.83	6.72 (E)[g]	10.8
site III (111)	6.77	7.06 (B)	10.5	6.79	7.06 (D)	10.8
site IV (150)	6.86	(o/m) (A)	10.5	6.60	6.39 (F)	11.4

[a] Seamon (1980). [b] Seamon et al. (1977); Levine et al. (1977). [c] The assignment of these coupled resonances to tyrosine-10 or -109 has not been made. [d] Birnbaum & Sykes (1978). [e] NR, not reported. [f] Calmodulin pK_a's are from Klee (1977). [g] Tyrosine labeling used in text.

sequence homology which exists between cardiac TnC and two other proteins, rabbit skeletal TnC and calmodulin.

Calmodulin possesses two tyrosines which are found within calcium-binding sites III and IV, the high affinity Ca/Mg sites. Two of the tyrosines of cardiac TnC are homologous to those of calmodulin; the third is found near the amino terminus of the protein at residue 5. In skeletal TnC, one of the tyrosine residues is at the homologous position in site III, and the other is found at amino acid position 10.

Skeletal TnC (Seamon et al., 1977) and calmodulin (Seamon, 1980) have been extensively studied by NMR techniques. Table I summarizes the tyrosine assignments for these proteins and also our proposed assignments for cardiac TnC.

We have assigned tyrosine B in the apoprotein to tyrosine D in the calcium-bound form because of the virtual equivalence of their chemical shifts. During the calcium titration (Figure 10) of cTnC, it may be seen that the ortho resonance only changes slightly as calcium is bound. This in turn allows us to assign this residue to tyrosine-111 in site III, one of the high affinity Ca/Mg binding sites. Examination of Table I demonstrates that the homologous tyrosines in site III of calmodulin and sTnC possess chemical shifts resembling free tyrosine in solution and also that these chemical shifts are relatively insensitive to calcium binding (Seamon, 1979, 1980). These properties are shared by tyrosine (B, D). The invariance of its chemical shift with calcium binding and insensitivity to temperature increases in the apoproteins and calcium-bound proteins suggest that it is a fairly exposed residue. This is also implied by its pK_a value which does not change markedly when calcium is bound.

It is of interest to note that the tyrosine residue in CB-9, a 52-residue CNBr fragment of sTnC containing only site III, possesses chemical shifts in the absence of calcium which are almost identical with those of our assigned site III tyrosine in cTnC. Apo-CB-9 has a very extended structure (Birnbaum & Sykes, 1978), lacking tertiary features. Its tyrosine residue is probably very exposed, suggesting, in turn, that tyrosine-111 in apo-cTnC is similarly exposed (pK_a's are similar as well). However, calcium-saturated CB-9 must have a different structure from that which it would assume when it is part of the much larger sTnC molecule. The ortho and meta resonances of tyrosine-109 in CB-9 are reversed and have very

different chemical shifts compared to those of calcium-saturated sTnC. The analogies can therefore only be drawn between apo-CB-9 and apo-cTnC.

Tyrosine F in calcium-saturated cTnC has been assigned to residue 150 in siie IV, the other high affinity Ca/Mg binding site, because of the similarity between its ortho and meta chemical shifts and those of tyrosine-138 in chemical-saturated calmodulin. sTnC has no such analogous tyrosine for comparison. This assignment is strengthened because of the reversed assignment of the ortho and meta resonances which both cTnC and calmodulin demonstrate (the ortho peak is downfield of the meta peak). The assignment is also supported by the elevated pK_a which tyrosine F possesses in calcium-saturated cTnC. This can be correlated with the elevated pK_a of tyrosine-138 in the apo and calcium-saturated forms of calmodulin. This assignment leads, by elimination, to the conclusion that tyrosine E corresponds to tyrosine residue 5 in calcium saturated cTnC.

Since none of the cTnC tyrosine residues have an elevated pK_a in the apoprotein, one can conclude that the homology between site IV in cTnC and calmodulin is not absolute. There must be considerable differences in the disposition of the tyrosine residue in site IV of apo-cTnC and apocalmodulin. This makes the assignment of tyrosine-150 in apo-cTnC more difficult. Because of the dissimilarities in tyrosyl pK_a's in apo-cTnC and apocalmodulin, one would not expect the chemical shifts of tyrosine-138 of calmodulin to be comparable to those of tyrosines A or C in cTnC for an assignment by homology. Calcium titration experiments (see next section, Figure 10) reveal the increase in intensity at 6.60 and 6.39 ppm corresponding to calcium binding at high affinity site IV. This can be correlated with a decrease of intensity at 6.86 ppm (tyrosine A) and also at 6.64 ppm (tyrosine C). We tentatively favor the assignment of tyrosine A to tyrosine-150, on the basis of an observation made when decoupling experiments were performed at the ortho and meta resonances of tyrosine-111 (tyrosine B) in apo-cTnC. When the decoupling is applied at these resonances, a large NOE can be seen at the tyrosine A resonance. This phenomenon can be seen quite clearly in Figure 2 and is particularly obvious when the decoupling is applied at the meta position, suggesting that tyrosine-111 and tyrosine A are close in space (i.e., occupy sites III and IV).

This argument then leads, by the process of elimination, to the assignment of the resonances of tyrosine C in the apoprotein of the N-terminal tyrosine residue at position 5. It is apparent that there is a large difference between the chemical shifts of the N-terminal tyrosine residues of sTnC and cTnC. Those of sTnC are representative of an exposed tyrosine (compare to CB-9) while the tyrosine near the N terminal of cTnC is obviously in a special, more buried, environment which is sensitive to calcium binding. Perhaps the deletion of the first calcium-binding site in the cardiac protein allows the N-terminal region to assume a different conformation than that of sTnC, possibly one more buried in the protein interior.

Gd^{3+} Broadening Experiments. So that these tyrosyl assignments could be tested, the paramagnetic relaxation reagent Gd^{3+} was utilized. Because of their similar ionic radius to calcium, but greater charge, lanthanides have been used as selective probes for calcium-binding sites (Lee & Sykes, 1980a,b; Leavis et al., 1980). Gd^{3+} binding causes large perturbations in the relaxation rates of nearby protons. This effect has an r^{-6} dependence, inducing broadening of the resonances of adjacent residues, and is conveniently detected by subtracting the broadening spectrum from one to which

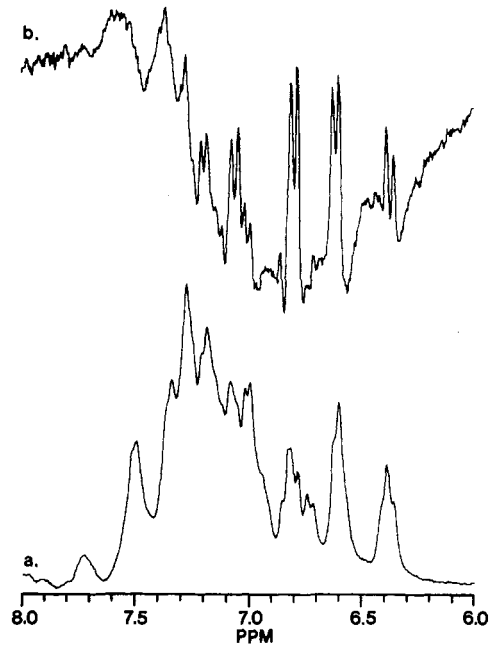

FIGURE 9: Gadolinium broadening experiment with calcium-saturated cTnC. cTnC (1.46 mM) in 0.15 M KCl, 25 mM Mops, pH 6.8, and 1 mM $CaCl_2$ was divided into two equal volumes; 0.5 equiv of $CaCl_2$ was added to each to assure calcium saturation. One sample was titrated with $GdCl_3$ (51 mM) and the other with $LaCl_3$ (49 mM). The specific broadening induced by Gd^{3+} binding was detected by subtracting the Gd^{3+} spectrum from the La spectrum. Four blocks of 256 scans were added together for each spectrum. This spectrum represents 0.13 Gd^{3+}/cTnC. (a) La spectrum; (b) difference spectrum.

gadolinium has *not* been added. The selectively broadened resonances are then observed as sharp lines in the difference spectrum.

When small aliquots of Gd^{3+} were added to calcium-saturated cTnC, the resonances ascribed to tyrosyl residues 150 and 111 are selectively broadened. In the difference spectrum, they appear as sharp doublets (Figure 9). A recent fluorescence study of terbium binding to skeletal TnC concluded that the lanthanides (Ho^{3+}, Tb^{3+}, and Gd^{3+}) were specific probes for the high affinity Ca/Mg sites (Leavis et al., 1980). This has been verified for the cardiac protein as well (M. T. Hincke, unpublished data). Therefore we consider the specific broadening of these resonances as good evidence that these tyrosyl residues are in close proximity to sites III and IV and correspond to tyrosine-111 and -150.

At higher levels of lanthanide, relaxation by Gd^{3+} broadens almost every feature of the spectrum. However, the resonances of tyrosine E (position 5) are still relatively unperturbed. This suggests that the N-terminal tyrosine residue is buried within the protein interior and also is not close to the Ca/Mg high affinity sites.

Calcium Binding. The calcium-binding process in cTnC can be divided into two stages, those which occur as the first 2 mol of calcium are bound and those which are seen when the third mole is added. Figure 10 presents the aromatic region of the spectrum as up to 3 equiv of calcium is added to the apoprotein. In Figure 11, the peak heights and chemical shifts of some selected resonances are plotted as a function of calcium added.

A general observation is that almost all spectral changes involving tyrosine resonances are complete when 2 mol of calcium is bound. Because of overlapping peaks, it is only possible to completely follow the increasing peak height of the meta resonance of tyrosine-150 as calcium is added. This titration curve correlates well with the decrease in peak height

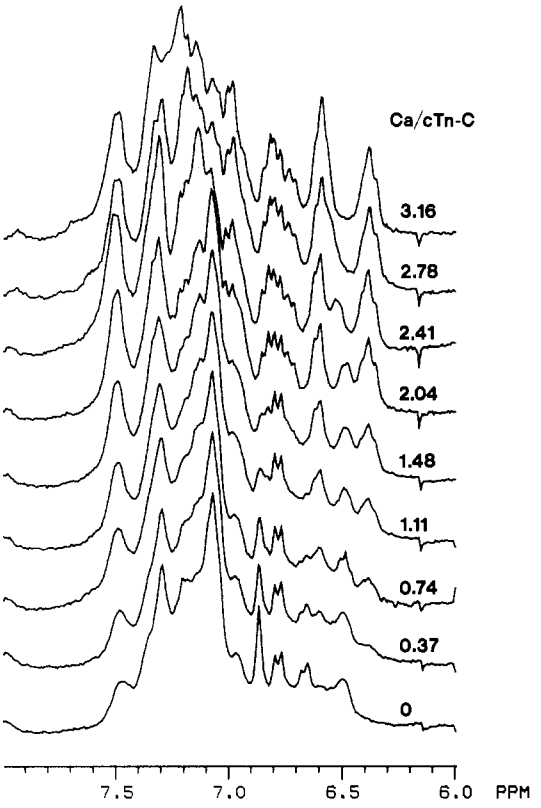

FIGURE 10: Calcium binding to apo-cTnC. cTnC (1.07 mM) in 0.15 M KCl and 25 mM Mops, pH 6.71, was titrated with calcium. The aromatic region of the spectrum is presented as a function of increasing calcium/cTnC.

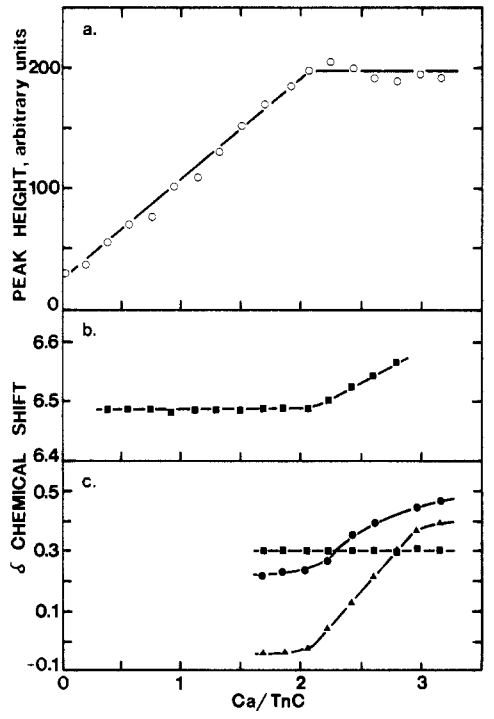

FIGURE 11: (a) Plot of the height of the plus calcium tyrosine-150 meta resonance as a function of calcium added. The heights were corrected for sample dilution by using the integrated area from 6 to 8 ppm as a normalization constant for each spectrum. In (b) and (c), several chemical shifts are also plotted as a function of added calcium. (a) Phenylalanine doublet, apo $\delta = 6.49$. (b) Upfield-shifted methyl resonances.

of the singlet at 6.86 ppm which represents the ortho and meta resonances of tyrosine-150 in the apoprotein. The increase in intensity of the 6.60-ppm peak between 2.5 and 3 Ca^{2+}/TnC is due to a phenylalanine doublet which titrates upfield from 2 to 3 Ca^{2+}/TnC to merge with the 6.60-ppm peak.

Table II: Exchange Rates Calculated for Different Ca^{2+} Conformations of cTnC Based upon the Ca^{2+}-Titration Behavior of Some Resonances

residues	resonance	Δ	K (s^{-1})
		slow exchange	
tyrosine-150	ortho protons	70 Hz	$\ll 440$
tyrosine-150	meta protons	127 Hz	$\ll 798$
		fast exchange	
phenylalanine doublet	$\delta = 6.49$	31 Hz	$\gg 195$
methyl resonance	$\delta = 0.03$	116 Hz	$\gg 700$

The behavior of these tyrosine resonances as calcium is bound to the high-affinity sites, where the resonances representing the apo form decrease in intensity as the calcium-bound resonances increase, indicates that the equilibrium between these forms of the protein are in the slow-exchange limit. That is, on the NMR time scale, the rate of interconversion is slow enough to allow each form to be seen as an individual species. This rate is $\ll 2\pi\Delta$ where Δ is the frequency separation between the resonances representing the two forms of the protein. Therefore an upper limit for the exchange rate can be calculated from these data (see Table II).

During addition of a third mole of calcium to the sample, large alterations are seen in the major phenylalanine region of the spectrum (7.0–7.4 ppm). As this represents the signals from nine phenylalanine residues (except the upfield doublets), it signifies large alterations of the protein interior accompanying the binding of a single mole of calcium to site II. A phenylalanine doublet at 6.49 ppm is observed to titrate downfield as a discrete peak (Figure 11), which suggests that the rate constant for the equilibrium between the plus and minus calcium forms is in the fast-exchange limit. A similar phenomenon is also observed for some upfield resonances at −0.03 and 0.22 ppm which also titrate downfield during binding of a third mole of calcium. In the fast-exchange limit, $K \gg 2\pi\Delta$, allowing a lower limit to be calculated for the exchange rate (see Table II).

Discussion

From the NMR data, calcium binding to cTnC can be differentiated into two steps. The binding of 2 mol of calcium to the high affinity sites causes large perturbations of tyrosine residues 150 and 5. A phenylalanine doublet is shifted upfield to the same chemical shift as the meta protons of tyrosine-150 at 6.39 ppm. There is also a phenylalanine doublet at the same position as the ortho protons of tyrosine-150 at 6.60, although it is not clear whether it occurs during Ca^{2+} binding or if the resonance is invariant with calcium binding. Spectral changes associated with tyrosine-5, located near the N terminus, are complete when the high-affinity calcium binding sites, found in the carboxy-terminal half of the molecule, have been filled. This suggests that the N-terminal region of the molecule is folded back into the protein structure and is therefore sensitive to calcium-induced conformational changes associated with the high-affinity sites. This observation does not imply the N terminus (or at least tyrosine-5) is physically close to either calcium-binding site. Such a possibility is ruled out by the Gd^{3+}-broadening experiments which demonstrate that only tyrosine-111 and -150 are close to metal ions bound at sites III and IV (within about 15 Å).

The NMR data indicate that in calcium-saturated cTnC and calmodulin, tyrosine-150 and tyrosine-138 possess very similar environments and are buried in the hydrophobic core

of the protein. Such a suggestion is based upon similar ortho and meta chemical shifts and elevated pK_a's. Seamon (1980) has predicted that tyrosine-150 in cTnC would exhibit the same spectral behavior as tyrosine-138, reflecting the hydrophobic interactions of this residue within the protein interior. However, it is important to note that while the ortho and meta resonances of tyrosine-150 in cTnC are sensitive solely to high affinity calcium binding, the chemical shift of the ortho protons of tyrosine-138 undergoes an upfield shift as the first 2 mol of calcium are added and also as the fourth calcium is bound. The meta resonance of tyrosine-138 is only sensitive to the first 2 mol of calcium. This subtle restructuring of the environment of this tyrosine in calmodulin, when the low-affinity site is occupied, is not displayed by tyrosine-150 in cTnC. This phenomenon may represent a conformational change which is unique to calmodulin.

In apocalmodulin, tyrosine-138 possesses an elevated pK_a and is resistant to chemical modification by tetranitromethane (Klee, 1977), criteria implying that it is buried and inaccessible to solvent. However, no such behavior is found for tyrosine-150 in apo-cTnC. It can be nitrated to the same extent as the other tyrosine residues and possesses the pK_a of a relatively exposed residue (McCubbin et al., 1979). Comparison of the two apoproteins by the CIDNP technique suggests that tyrosine-138 in calmodulin is buried while tyrosine-150 in cTnC is almost as exposed as tyrosine-111 (Hincke et al., 1980). The binding of calcium buries this residue. These differences are reflected in the chemical shifts of these tyrosine residues in the apoproteins and suggest that there may be large structural differences in this region of the molecule between the two apoproteins.

Analysis of the Ca^{2+} titration data suggests that the rate of exchange between the apoprotein and the protein with 2 mol of calcium bound at the high affinity sites is in the slow-exchange limit, while the rate of exchange of calcium at the low-affinity site is in the fast-exchange limit. The rates calculated in Table II suggest that the slow-exchange rate is less than 440 s^{-1}, while the rate of fast exchange is greater than 700 s^{-1}. These rates represent either the actual calcium off-rate, or the rate constant for the relaxation of the protein structure when the metal ion comes off, whichever is slower. NMR studies on sTnC, where similar phenomena are observed, have indicated that the conformational change is the rate-limiting step (Levine et al., 1977). Unfortunately, absolute values for the exchange rates cannot be determined from these data. Stopped-flow fluorescence studies utilizing cTnC labeled with the fluorescent probe IAANS have yielded similar results in that the Ca^{2+} off-rate from the low-affinity site is 230–350 s^{-1} and calcium release from the high-affinity sites is much slower (Johnson et al., 1978). Similar experiments with sTnC have suggested that the Ca^{2+} off-rate from the high-affinity sites is too slow to allow these sites to regulate contraction (Johnson et al., 1979). The low-affinity sites, however, have an exchange rate, which is fast enough to regulate striated muscle contraction (350–750 s^{-1}). These two proteins are similar, then, in that the high-affinity sites appear to play mainly a structural role while the low-affinity site(s) is(are) the regulatory ones. The reorganization of the protein interior, as evidenced by the dramatic phenylalanine changes, during the binding of calcium to the low affinity site in cTnC must represent the most important structural changes in this molecule.

Acknowledgments

We are grateful to Dr. J. H. Baldo and D. S. Hagen for their preliminary studies with our laser photo-CIDNP apparatus, to Dr. L. Lee and J. Shelling for assistance and discussion regarding the gadolinium broadening experiments, and to G. McQuaid for technical assistance with the 270-MHz spectrometer. We also thank Drs. W. D. McCubbin and R. E. Reid for fruitful discussions and T. Keri, K. Oikawa, and V. Ledsham for technical assistance. We thank Dr. P. Krahn of Dr. S. Hansen & Associates for performing calcium measurements. We are also grateful to Dr. K. B. Seamon for a copy of his manuscript prior to publication.

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Comparison of Solid State and Solution Conformations of *R* and *S* Epimers of 8,5′-Cycloadenosine and Their Relevance to Some Enzymatic Reactions†

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ABSTRACT: The C(5′)-*R* epimer of 8,5′-cycloadenosine crystallizes in the monoclinic space group $P2_1$ ($Z = 2$) with unit cell dimensions $a = 5.755$ (1), $b = 16.895$ (1), and $c = 5.511$ (1) Å and $\beta = 104.16$ (1)°. X-ray intensity data were measured on a diffractometer, and the crystal structure was determined by direct methods. Least-squares refinement converged at $R = 0.037$ for 1108 reflections. The conformation about the glycosyl bond is anti, as imposed by the 8,5′ cyclization, with $\chi_{CN} = 29.8°$. The ribose ring adopts the unusual C(1′)endo–O(4′)exo (^1_OT) conformation with pseudorotation parameters $P = 289.0°$ and $\tau_m = 49.0°$. The six-membered ring formed by the 8,5′ linkage is approximately a half-chair with C(4′) and O(4′), respectively, above and below a plane defined by the other four atoms. These results are compared with those previously reported for the corresponding *S* epimer [Haromy, T. P., Raleigh, J., & Sundaralingam, M. (1980) *Biochemistry 19*, 1718–1722]. The conformations of the sugar rings and the exocyclic groups of both epimers in the solid state are compared to the conformations in solution, as determined by analysis of the systems of proton–proton vicinal coupling constants from the ^{1}H NMR spectra. The foregoing findings are employed to examine the role of the conformational parameters of adenosine and 5′-AMP in reactions catalyzed by the appropriate enzymes.

The *R* and *S* diastereoisomers of 8,5′-cycloadenosine and its 5′-phosphate, which are carbon-bridged analogues of adenosine and 5′-AMP, have been employed as model compounds to furnish useful information about the conformation of purine nucleosides in solution (Stolarski et al., 1980) and about the conformation of adenosine and its 5′-phosphate in enzyme–substrate complexes (Raleigh & Blackburn, 1978; Dudycz & Shugar, 1979). These structurally rigid analogues may be regarded as simulating adenosine and 5′-AMP with the aglycon in the anti conformation and with the exocyclic carbinol group in the conformation trans (the *R* epimer) or gauche⁻ (the *S* epimer). Notwithstanding that the conformation of the pentofuranose ring differs from that typically found for pentofuranosyl nucleosides in solution, the anticipated invariability of this conformation on formation of enzyme–substrate complexes points to their potential utility for determination of the conformational requirements dictated by enzymes with which they may interact.

In contrast to the reported findings of Hampton et al. (1972a,b) regarding the substrate properties of these analogues, we have found that neither the *R* or *S* epimers of 8,5′-cycloadenosine nor its 5′-phosphates are substrates of the aminohydrolases of adenosine and 5′-AMP, respectively. With snake venom 5′-nucleotidase, the *S* epimer of 8,5′-cycloadenosine 5′-phosphate was also found not to be a substrate (Dudycz & Shugar, 1979), in agreement with the findings of Raleigh & Blackburn (1978). On the other hand, the *R* epimer, although a substrate for this enzyme, was hydrolyzed at a much lower rate than that reported by Hampton et al. (1972b). Since the compounds synthesized by Hampton et al. (1972a,b) exhibited physicochemical properties at variance with those synthesized by us (Stolarski et al., 1980) and Matsuda et al. (1978) according to a procedure different from that employed by Harper & Hampton (1972) and Raleigh & Blackburn (1978), it was of obvious interest to establish unequivocally the structures of the foregoing model analogues. We have therefore extended previous results obtained by ^{1}H NMR spectroscopy (Stolarski et al., 1980) with X-ray diffraction data on single crystals.

We describe here the solid-state structure of the *R* epimer of 8,5′-cycloadenosine, which fully confirms and extends the assignment previously made in solution on the basis of ^{1}H NMR spectroscopy. Since this was prepared from the *S* diastereoisomer by a change in the configuration at C(5′) of the latter, the authenticity of the *R* epimer establishes that of the *S* epimer, as well as of the corresponding 5′-phosphates (Dudycz & Shugar, 1979). After the completion of this investigation a report appeared describing the single-crystal structure of the *S* epimer of 8,5′-cycloadenosine (Haromy et al., 1980), prepared according to the procedure of Raleigh & Blackburn (1978). Thus we can make a direct comparison between the two epimers, both in the solid state and in solution, and discuss their relevance to the substrate properties of both the nucleosides and nucleotides.

Experimental Procedures

The *R* and *S* epimers of 8,5′-cycloadenosine were synthesized essentially as described by Matsuda et al. (1978). The structures of the two compounds were established, apart from X-ray diffraction for the *R* epimer as described below, by means of ^{1}H NMR spectroscopy (Stolarski et al., 1980) with the aid of a Bruker-90 spectrometer operating in the Fourier

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0006-2960/81/0420-3294$01.25/0