Proton Nuclear Magnetic Resonance Investigation of Synthetic Calcium-Binding Peptides†

Jean Gariépy,* Brian D. Sykes, Ronald E. Reid, and Robert S. Hodges

ABSTRACT: The interaction of Ca^{2+} with three synthetic analogues of the high-affinity site III of rabbit skeletal troponin C has been studied with proton magnetic resonance at 270 MHz. The resonance patterns observed for tyrosine-109, phenylalanine-119, and the acetyl group located on the N-terminal residue of each peptide are indicative of the unfolded nature of the fragments in the absence of calcium. Ca^{2+}-induced changes in the environment of residues located in the Ca^{2+}-binding loop (Asp-103, Tyr-109, and Glu-114) as well as in the N- and C-terminal regions of the "helix–loop–helix" calcium-binding unit (Phe-99, Phe-102, Phe-119, and Ala 98) were followed by their characteristic proton resonances. Lactoperoxidase iodination and laser photochemically induced dynamic nuclear polarization experiments demonstrated the exposure of tyrosine-109 in the presence and absence of calcium in all the analogues and also aided in the assignment of the tyrosine-109 ring protons. An upfield-shifted resonance associated with phenylalanine-99 and/or phenylalanine-102 ring protons was observed in the calcium-saturated spectra. The exchange rate for the meta (2,6) proton resonances of tyrosine-109 indicates a transition to a slower rates as the N terminal of the calcium site is elongated. This finding correlates with the observed increase in the calcium-binding constant of the synthetic analogues occurring upon peptide elongation. The Ca^{2+}-binding constants determined by circular dichroism were 10^2 M^{-1}, 10^4 M^{-1}, and 10^5 M^{-1} for the 21-, 26-, and 34-residue peptides, respectively (Reid et al., 1981). These results illustrate the importance of the N-terminal helical region in stabilizing the cation in the peptide-binding loop. Finally, the observation of changes in phenylalanine-119 resonances located in the C terminal of our smallest fragment coupled with the increased calcium-binding constants observed for the 21- and 26-residue peptides (K_{Ca} of 10^5 M^{-1}) in 50% trifluoroethanol in aqueous buffer (helix-inducing conditions) is consistent with the involvement of the C-terminal region in the Ca^{2+} stabilization process.

Cell division, muscle contraction, and other metabolic activities have been shown to be sensitive to intracellular Ca^{2+} levels (Ebashi & Endo, 1968; Greaser & Gergely, 1971; Gnegy et al., 1976; Marcum et al., 1978; Cohen et al., 1978; Wong & Cheung, 1979). Calcium can modulate these cellular events by acting as a chemical signal that alters the structure of regulatory proteins such as troponin C and calmodulin. Alignment of the primary sequence of these calcium-binding proteins revealed regions of strong homology corresponding to the calcium-binding regions (Barker et al., 1978; Vogt et al., 1979; Reid & Hodges, 1980) as deduced from the sequence homology with carp MCBP[1] whose crystal structure has been determined (Kretsinger & Nockolds, 1973). These crystallographic studies lead to the hypothesis that each calcium-binding site exists in a helix–loop–helix conformation, with the six coordinating residues of the metal ion found within a central 12-residue loop flanked on both sides by α-helical regions.

MCBP's (parvalbumins) from the white muscle of hake and carp have been shown to bind 2 mol of calcium/mol of protein with high affinity (Pechère et al., 1971; Nockolds et al., 1972) while calmodulin and the calcium-binding component of the troponin complex (TnC) from rabbit skeletal muscle have been shown to bind 4 mol of calcium/mol of protein (Potter & Gergely, 1975; Klee et al., 1980). TnC has two classes of Ca^{2+}-binding sites: two high-affinity sites, which can competitively bind Mg^{2+}, and two lower affinity, calcium-selective sites (Potter & Gergely, 1975). It is generally accepted that calmodulin has four high-affinity calcium-binding sites. The Ca^{2+}-binding sites in these three proteins show K_{Ca} values ranging from 10^5 to 10^8 M^{-1}. Such affinity differences could be partially interpreted in terms of tertiary structure considerations, i.e., prefolding occurring in some Ca^{2+}-binding regions. However, the preparation of enzymatic and synthetic fragments of rabbit skeletal troponin C (Leavis et al., 1978; Reid et al., 1980, 1981) and carp MCBP (Derancourt et al., 1978) has indicated that the primary sequence of such regions will also significantly dictate the ability of these sites to bind divalent cations.

Our group has recently synthesized a series of analogues of the high-affinity Ca^{2+}-binding site III of rabbit skeletal troponin C by a combination of solution and solid-phase peptide synthesis. The advantages of chemically synthesizing such sites have been described earlier (Reid et al., 1980). We have shown that a synthetic 34-residue fragment of this particular site possesses approximately the same affinity for calcium as does the naturally obtained 52-residue cyanogen bromide fragment (CB9) of this region (Leavis et al., 1978). Attempts to further shorten the N terminus of this analogue resulted in a significant decrease in the observed Ca^{2+}-binding constant (K_{Ca} going from 10^5 to 10^2 M^{-1}) (Reid et al., 1981). The small size of our analogues makes them attractive for

† From the Department of Biochemistry and the Medical Research Council of Canada Group in Protein Structure and Function, University of Alberta, Edmonton, Alberta, Canada T6G 2H7. *Received June 16, 1981.* This investigation was supported by research grants from the Medical Research Council of Canada, the Medical Research Council Studentship (J.G.), and a research allowance (J.G.) from the Alberta Heritage Foundation for Medical Research.

[1] Abbreviations: Ac(A^{98})STnC(98–123)amide, synthetic N-terminal acetylated rabbit skeletal troponin C fragment, residues 98–123 with a C-terminal amide and alanine substituted at position 98; CB9, cyanogen bromide fragment 9, residues 84–135 of rabbit skeletal troponin C; CIDNP, chemically induced dynamic nuclear polarization; DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; FID, free induction decay; FMN, flavin mononucleotide; NMR, nuclear magnetic resonance; Pipes, 1,4-piperazinediethanesulfonic acid; MCBP, muscle calcium-binding protein; TnC, troponin C; CD, circular dichroism; TFE, trifluoroethanol.

0006-2960/82/0421-1506$01.25/0

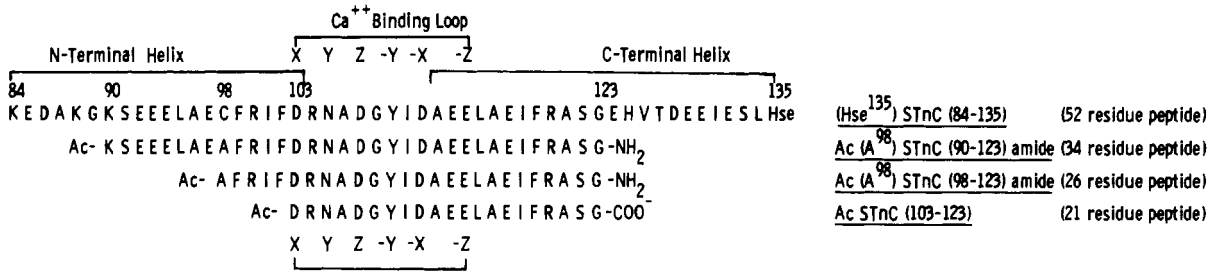

FIGURE 1: Amino acid sequences of rabbit skeletal troponin C (STnC) cyanogen bromide fragment 9 (CB9) and of the synthetic analogues as described under Materials and Methods. Symbols X, Y, Z, -X, -Y, and -Z represent the proposed Ca^{2+} coordinating ligands. Regions corresponding to the N- and C-terminal helices as well as the Ca^{2+}-binding loop are indicated. HSe, homoserine; Ac, N^{α}-acetyl; NH_2, C^{α}-amide.

NMR studies. The proton NMR spectrum is relatively simple to interpret, and fluorine or other types of nuclear probes can be synthetically integrated to permit the study of the dynamic events occurring during Ca^{2+} addition. In this study we report the proton nuclear magnetic resonance results of three synthetic peptides involving the region 90–123 of TnC.

Materials and Methods

Preparation of Peptides. The synthesis and purification approaches used to prepare AcSTnC(103–123), Ac(A^{98})-STnC(98–123)amide, and Ac(A^{98})STnC(90–123)amide were described extensively elsewhere (Reid et al., 1981). Peptide concentrations were determined by amino acid analysis.

Preparation of Iodinated Peptides. Lactoperoxidase was used to catalyze the iodination of the single tyrosine present in Ac(A^{98})STnC(98–123)amide and Ac(A^{98})STnC(90–123)amide by methodology previously described (Seamon et al., 1977; Morrison & Bayse, 1970). The 10-mL reaction mixture consisted of 0.1 mM peptide, 0.1 mM KI, 50 mM sodium phosphate buffer, pH 7.0, 0.1 mM H_2O_2, 0.30 IU of lactoperoxidase (Sigma), and 5 mM EGTA. The iodination was initiated by the addition of the enzyme and monitored by the decrease in tyrosine fluorescence at 305 nm (λ_{ex} = 280 nm). Successive additions of KI and H_2O_2 were made to ensure total iodination of the tyrosine. When the residual fluorescence was reduced to 5% of the original intensity, the reaction was stopped by the addition of 100 μL of a 50 mM DTT solution.

Preparation of NMR Samples. The peptides were dissolved in 10 mM Pipes–0.1 M KCl–0.5 mM DSS in D_2O, pH 6.8. All solutions used were treated with Chelex 100 resin (Bio-Rad). The resin was then removed by Millipore filtration. Calcium titrations were performed by adding aliquots of 0.01 and 0.1 M $CaCl_2$ solutions to the Ca^{2+}-free samples.

Metal Ion Analysis. All calcium stock solutions were prepared from reagent grade $CaCl_2 \cdot 2H_2O$ dissolved in D_2O. The Ca^{2+} content was determined by EDTA titrations with murexide as the end point indicator (Blaedel & Knight, 1954). The initial calcium content of our stock peptide solutions was estimated by flameless atomic absorption on a Varian Techtron Model AA-6 spectrometer.

^{1}H NMR Spectra. ^{1}H NMR experiments were performed with a Bruker HXS 270-MHz spectrometer operating in the Fourier transform mode and equipped for quadrature detection. A typical spectrum was obtained from 1000 acquisitions, using a 1-s acquisition time, with a 9-μs pulse width (~90°), a ±2000-Hz sweep width, and a line broadening of 1 Hz.

Laser Photo-CIDNP Spectra. The CIDNP (chemically induced dynamic nuclear polarization) experiment (Kaptein, 1978) involves the use of a photo-excited flavin dye (FMN) that interacts specifically with tyrosine, histidine, and tryptophan residues in proteins. A Spectra Physics Model 164 argon ion laser was utilized to illuminate the flavin-containing sample. The 10-mm flat-bottomed NMR tubes used contained typically 1.2 mL of a 0.5 mM peptide solution (10 mM Pipes, 0.1 M KCl, 0.5 mM DSS in D_2O, and 5 mM $CaCl_2$, pH 6.8). A 5-μL aliquot of a 50 mM FMN stock solution was added to the NMR tube. The data were acquired in an alternating pattern, i.e., one spectrum of the nonirradiated sample followed by one of the irradiated sample, with a 10-s delay between the light and dark spectra. The light spectra were irradiated with 3.5 W of power for 1 s prior to accumulation of the FID. Blocks of eight spectra were collected with the same instrument settings as described above. The laser photo-CIDNP ^{1}H NMR method has been recently described by Hincke et al. (1981a) in their study of calcium-binding proteins.

Results

Primary Sequence of Analogues. The sequences of the synthetic analogues are presented in Figure 1. The first sequence represents the cyanogen bromide fragment containing the Ca^{2+} high-affinity site III of rabbit skeletal troponin C (CB9) (Collins et al., 1977). The synthetic peptides have an identical sequence with that of the native fragment CB9 except for the replacement of cysteine-98 by alanine, in order to avoid problems associated with sulfhydryl groups. Their lengths vary from 21 [AcSTnC(103–123)] to 26 [Ac(A^{98})STnC(98–123)amide] and finally 34 amino acid residues [Ac(A^{98})-STnC(90–123)amide], through the extension of the N-terminal region of the molecule. The peptides were acetylated at the N terminus, and the C terminus was blocked as an amide except for the 21-residue analogue, which contains an unblocked α-carboxyl group. By protecting the ends of these fragments, we do not generate extra charges that are absent in the natural protein.

Assignment of Resonances in the Apopeptides. Looking at Figure 2, we note that the apopeptide spectra reveal the presence of two sharp doublets, which have central resonances around 7.08 and 6.82 ppm. These doublets can be assigned to the meta (2,6) and ortho (3,5) protons of tyrosine-109, respectively. Similar resonance patterns were observed for bovine skeletal apo cardiac TnC tyrosine-111 (Hincke et al., 1981b), rabbit apo skeletal TnC tyrosine-109 (Seamon et al., 1977), and apo CB9 tyrosine-109 (Birnbaum & Sykes, 1978). The 21-residue analogue possesses only phenylalanine-119, the resonance of which is readily assigned to the multiplet region situated around 7.3 ppm, and resembles the free amino acid spectrum. All other analogues show a more complex phenylalanine region because their sequence contains three phenylalanines (Phe-119, Phe-102, and Phe-99) instead of one. Nevertheless, all the phenylalanine resonances are located in the region extending from 7.2 to 7.4 ppm as similarly observed for the free amino acid itself.

In the aliphatic region, the CH_3 protons of the acetylated N-terminal residue can be seen as a sharp singlet in the 2.00-ppm region. Its resonance position fluctuates only slightly

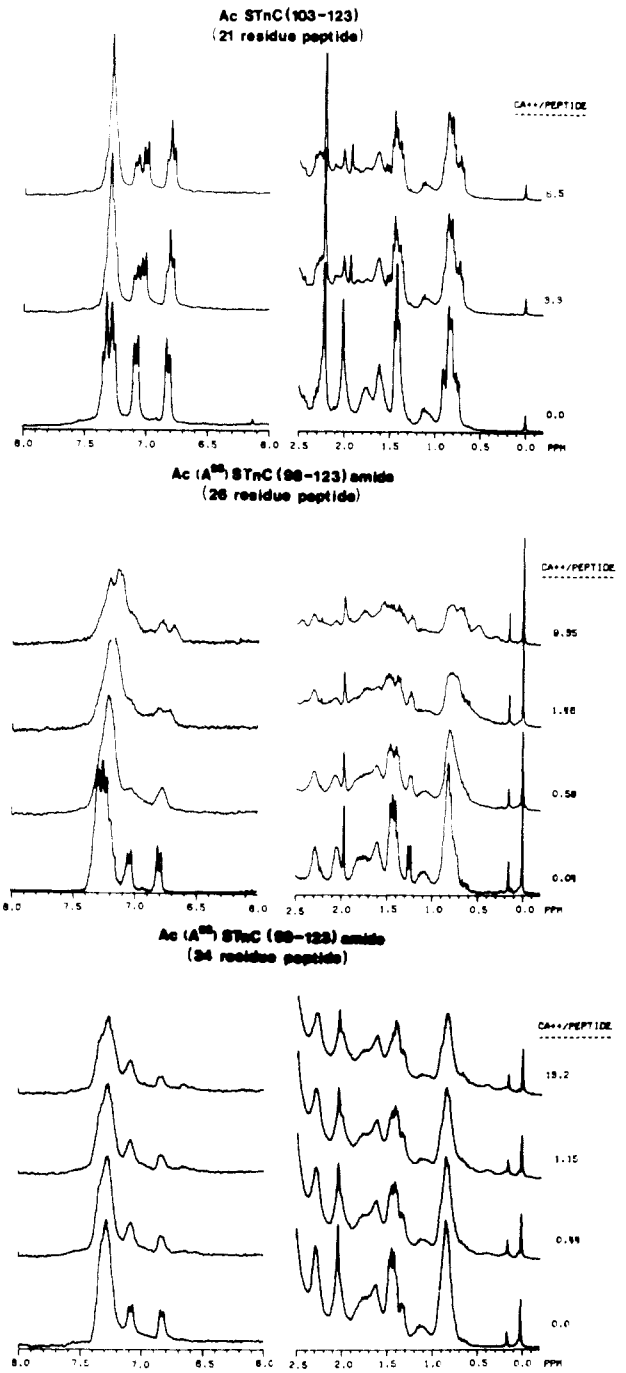

FIGURE 2: ^1H NMR spectra of the synthetic peptides at various stages of calcium addition. [peptide] $\simeq 5 \times 10^{-4}$ M in 10 mM Pipes–0.1 M KCl–0.5 mM DSS (pH 6.8). Resonances at 0.00 and 0.18 ppm arise from the presence of DSS.

going from the 21-residue apopeptide to the longer fragments. This fact points out again the unfolded nature of these peptides, since temperature denaturation of carp parvalbumin shifts the acetyl protons of the N terminal from 2.13 to 2.00 ppm (Cave et al., 1979). The sharp doublet situated at 1.24 ppm in the 26-residue peptide has been tentatively assigned to the methyl protons of alanine-98. This assignment is based on the absence of such a resonance in the other analogues and because Ala-98 represents the acetylated N terminus of the 26-residue analogue. Finally, all the glutamic acid residues present in the 26-residue peptide are located at the beginning of the C-terminal region of this fragment. The γ-CH_2 protons of these amino acids generate a clean signal at 2.30 ppm, which will be used to investigate this part of the molecule upon calcium addition.

Effect of Calcium Addition. Figure 2 shows the effect of calcium addition on each of the analogues. In the aromatic region of the 21-residue peptide, the sharp tyrosine ortho (3,5) and meta (2,6) doublets are decreased in intensity upon the addition of calcium, and a new set of doublets appears. In addition, the new set of doublets shifts continuously upfield with increasing calcium concentration (for example, the new meta (2,6) doublet shifts from 7.08 to 7.01 ppm). From the behavior of the tyrosine resonance we can suggest that two populations of the fragment exist in the presence of calcium: one which is not affected by calcium and may not bind calcium and a second which binds calcium with an off rate large enough so that the spectra are in the NMR fast-exchange limit.

This pattern is very different for the 26- and 34-residue fragments. First of all, there is no fraction of either peptide that is not affected by calcium binding. Second, especially for the 34-residue fragment, the off-exchange rate for calcium must be much smaller so that the spectra are in the NMR slow-exchange rate with the calcium-bound spectrum coexisting with and sequentially replacing the calcium-free spectrum. The off rate for calcium for the 26-residue peptide is intermediate in that there is considerable slow exchange broadening of the calcium-free and calcium-bound peptide resonances when the peptide is fractionally saturated with Ca^{2+}. The meta resonance of the 26-residue free peptide is broad at a calcium to peptide ratio of 0.58. This transition to slower exchange processes may be explained as increased stabilization of the Ca^{2+} ion in the binding site as the peptides are increased in length.

The phenylalanine region of the 21-residue analogue indicates that Ca^{2+} does affect the Phe-119 located in the C-terminal region of this peptide. It has been proposed that the C-terminal α helix of this site in rabbit skeletal troponin C and CB9 is Ca^{2+} insensitive (Nagy et al., 1978). However, circular dichroism would not detect changes in side-chain environment. It should be noted here that the resonances of His-125 of CB9 remain unaffected by Ca^{2+} addition (Birnbaum & Sykes, 1978).

The two other analogues show a broadened upfield-shifted phenylalanine region in the presence of the calcium. The 34-residue analogue has an additional phenylalanine resonance in the calcium-saturated protein positioned at 7.07 ppm. This resonance corresponds to phenylalanine protons as shown in the iodination experiment (Figure 3). Such an assignment was also made in CB9. This different resonance pattern suggests that the environment of Phe-102 and Phe-99 is affected by the lengthening of the peptide from 26 to 34 residues. The Ca^{2+} sensitivity of these aromatic residues correlates well with their location in the proposed Ca^{2+}-sensitive N-terminal region (Nagy et al., 1978; Reid et al., 1981).

In the 21-residue peptide, Asp-103 is acetylated. The acetyl group methyl proton peak centered at 2.01 ppm in the Ca^{2+}-free fragment is split into two distinct singlets (2.01 and 1.92 ppm) in the presence of calcium. On extension of the N-terminal region only peak broadening is observed with the N-terminal acetyl group.

The sharp doublet centered at 1.24 ppm in the 26-residue peptide was assigned to Ala-98 methyl protons. This doublet broadens considerably as a function of Ca^{2+} addition, which suggests that the binding of Ca^{2+} affects the environment of residues as far along as Ala-98 in the N-terminal region.

The 26- and 34-residue fragments also show characteristic shifted CH_3 resonances in the 0.15–0.50-ppm region, in the presence of calcium. These resonances are most likely due to the interactions of leucine or isoleucine side chains with aro-

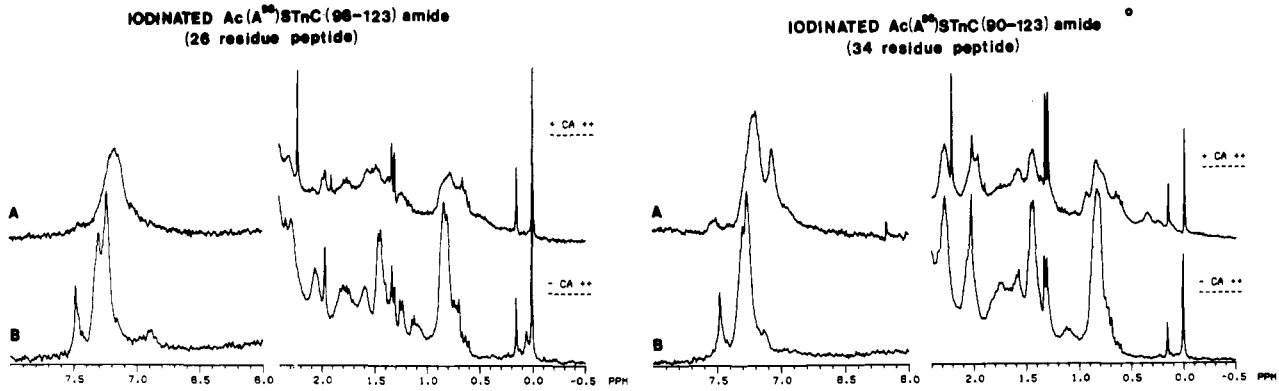

FIGURE 3: ^{1}H NMR spectra of the iodinated synthetic peptides. (A) Excess Ca^{2+}; (B) no Ca^{2+}. The new tyrosine-109 meta proton singlet is positioned at 7.47 ppm. Experimental conditions are the same as in Figure 2. The sharp resonances observed at 1.3 and 2.3 ppm are contamination artifacts from the iodination step.

matic rings. Calcium thus promotes the rearrangement of the peptide to allow the positioning of a phenylalanine or tyrosine ring near one of the leucine or isoleucine residues. The use of Kretsinger's (Kretsinger & Nockolds, 1973) molecular coordinates for parvalbumin CD and EF loops suggests that only I^{101}–F^{102}, Y^{109}–I^{110}, and I^{118}–F^{119} could come in close proximity to allow such possible ring-shifted upfield resonances in our analogues.

Glu-113, Glu-114, and Glu-117 are all located at the beginning of the C terminus of the Ca^{2+}-binding loop. These amino acids can be uniquely detected in the 26-residue analogue since their γ-CH_2 protons produce a resonance located at 2.28 ppm. This region in the 34-residue peptide is further complicated by the addition of four glutamic acid residues on extension of the N terminal. The resonance broadens and a new resonance appears at 2.44 ppm as the metal ion concentration increases. This agrees with Glu-114 being one of the ligands in the coordination sphere and helix induction of this region upon Ca^{2+} binding (Reid et al., 1981).

Ca^{2+} Titration of Iodinated Peptides. In order to study the effect of calcium addition on the meta (2,6) protons of tyrosine-109 and to aid in assigning resonances, we have selectively removed the ortho (3,5) protons from the ring by using the lactoperoxidase iodination method (Morrison & Bayse, 1970).

In Figure 3, the spectra of two iodinated peptides are presented. The ortho (3,5) proton resonances centered around 6.82 ppm have disappeared, and the meta (2,6) proton doublet (7.07 ppm) has now become a singlet shifted downfield to 7.47 ppm. This resonance in both analogues almost vanishes in the presence of calcium, suggesting they are either broadened or shifted into the main phenylalanine envelope. This observation, coupled with the appearance of a resonance at 7.07 ppm in the Ca^{2+}-saturated spectrum of the modified 34-residue fragment, suggests that this resonance arises from a phenylalanine contribution in the unmodified peptide (see Figure 2). This peak (7.07 ppm) was assigned to the combined effect of meta (2,6) tyrosine protons and phenylalanine ring protons in CB9 (Birnbaum & Sykes, 1978). Birnbaum & Sykes (1978) commented that the possible presence of a rather significant amount of unfolded CB9 in their Ca^{2+}-saturated spectrum may explain the appearance of residual meta (2,6) proton resonance at 7.07 ppm.

A comparison of the apoiodinated peptides to the unmodified peptides shows subtle modifications in the phenylalanine splitting patterns. Since this chemical modification is selective for tyrosine only and the reaction was carefully monitored, it suggests that the environment of our phenylalanines is affected by the substituents on the tyrosine ring. A general upfield shift (0.03 ppm) of the region situated between 7.2 and 7.4 ppm can be observed when comparing the unmodified and iodinated apopeptides.

Laser Photo-CIDNP of Ca^{2+}-Saturated Peptides. The laser photo-CIDNP spectrum provides an immediate assignment of the tyrosine ortho (3,5) protons and an indication of tyrosine exposure in the peptide. This arises because the tyrosine ortho (3,5) protons experience a characteristic enhanced emission in the free amino acid, and the appearance of enhanced emission in the peptide indicates exposure of the tyrosine to the dye. In Figure 4, the calcium-saturated spectrum of each of our analogues is shown. The large negative enhancements in the laser photo-CIDNP difference spectra immediately demonstrate the assignment of the tyrosine protons in the calcium-saturated spectra.

In the laser photo-CIDNP difference spectra for the free tyrosine amino acid, the meta (2,6) protons show a small enhanced absorption. However, in proteins, with slower overall rotational correlation times, this can be reversed by cross relaxation of the meta (2,6) and ortho (3,5) protons carrying the much larger negative enhancement of the ortho protons to the meta protons. This suggests that the resonance at 6.7 ppm in the calcium-saturated 26-residue peptide is the upfield-shifted meta protons. The situation is less convincing for the 34-residue fragment.

The interaction of the flavin dye with the tyrosine ring suggests that tyrosine-109 is exposed in all the analogues, in agreement with the results obtained on skeletal troponin C (McCubbin & Kay, 1975; Seamon et al., 1977) and CB9 (Birnbaum & Sykes, 1978). This is quite revealing since the Ca^{2+}-induced UV difference spectra for our analogues have a blue-shifted tyrosine absorption band around 280 nm (Reid et al., 1981), pointing out the possible movement of tyrosine to a potentially more hydrophilic environment. This was not observed in CB9 (Nagy et al., 1978) and gives support to the idea that some residues not present in our analogues are interacting with tyrosine-109 in CB9.

Discussion

Our group has currently been involved in developing a dynamic model to describe calcium-induced protein folding in troponin C, calmodulin, and related proteins. Upon Ca^{2+} addition to these Ca^{2+}-modulated proteins, large increases in the α-helical content and a general increase in compactness of these molecules have been observed by intrinsic fluorescence (Leavis & Kraft, 1978; Dedman et al., 1977; Dabrowska et al., 1978; Cox et al., 1979) and circular dichroism (Leavis & Kraft, 1978; Liu & Cheung, 1976; Wolff et al., 1977; Hincke et al., 1978; Walsh et al., 1978). ^{1}H NMR spectroscopy has been used to study the conformational changes in these proteins

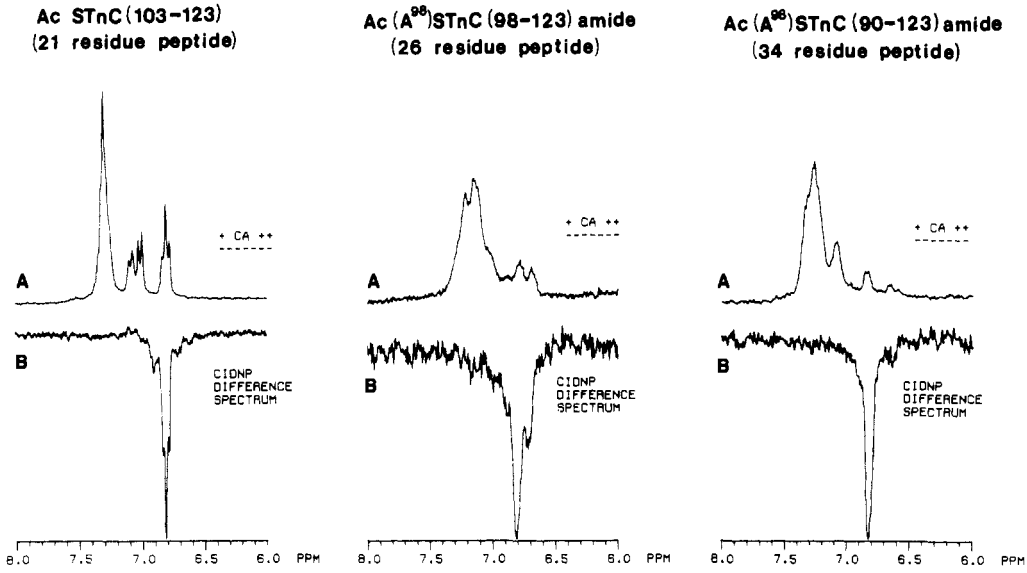

FIGURE 4: ^{1}H NMR laser photo-CIDNP difference spectra of the aromatic region of each analogue in the presence of calcium. (A) ^{1}H NMR spectra of the aromatic region; (B) CIDNP difference spectra as described under Materials and Methods. The sharp emission peak corresponds to the resonance position of the ortho protons of tyrosine-109.

(Seamon et al., 1977; Levine et al., 1977, 1978; Seamon, 1980; Hincke et al., 1981b). However, it is extremely difficult to probe local molecular events in a single calcium-binding site in these multi-calcium-binding site proteins. To overcome this problem, we have prepared synthetic analogues of a high-affinity calcium-binding site ranging from 21 to 34 amino acids in length to obtain simpler, more interpretable spectra than are obtained from the native proteins in terms of Ca^{2+} effects on particular amino acid side chains.

For the apopeptides, the phenylalanines and tyrosine resonances resemble the spectra obtained for model compounds. Also the N-terminal acetyl methyl protons in all the apo analogues show the resonance position observed in heat-denatured parvalbumin (Cave et al., 1979). Similarly the amount of ellipticity determined by CD measurement at 222 nm reveals that only small conformational changes exist between the calcium-free peptides in the presence and absence of urea (8 M) (Reid et al., 1981). Thus, it can be concluded that these analogues are largely unfolded in the absence of calcium.

Addition of calcium produces definite metal-induced broadening and shifting of protein resonances associated with the coordinating residues Asp-103 (+X) acetyl group, Tyr-109 (−Y), and Glu-114 (−Z) side chains. These events coincide with similar occurrences in the spectral regions associated with Phe-98, Phe-101, Phe-119, and Ala-98 side chains. Such results confirm that concerted changes can be observed in both the helical regions and the cation-binding loop. It also partly verifies our assumption that the circular dichroism investigation of the α-helical content of these fragments indirectly measures metal stabilization events in the binding region.

The exposure of the tyrosine-109 ring in the presence and absence of calcium was demonstrated by the lactoperoxidase iodination and the laser photo-CIDNP experiments. Results agree with similar conclusions drawn for rabbit skeletal troponin C and its cyanogen bromide fragment, CB9 (McCubbin & Kay, 1975; Seamon et al., 1977; Birnbaum & Sykes, 1978). Furthermore, the iodination of the 26- and 34-residue peptides has revealed that the resonances located in the 6.5–7.1-ppm region in the apopeptides are associated only with tyrosine-109 ring protons. The CIDNP results made the assignment of the ortho (3,5) protons of tyrosine-109 unambiguous (6.82 ppm) in all Ca^{2+}-saturated fragments. The meta (2,6) proton resonance position appears shifted upfield from the ortho protons in these Ca^{2+}-saturated analogues. This is in agreement with a similar conclusion drawn from the ^{1}H NMR Ca^{2+} titration of CB9 (Birnbaum & Sykes, 1978) and bovine cardiac TnC (Hincke et al., 1981b). Birnbaum & Sykes (1978) suggested a localized shielded environment for the tyrosine meta (2,6) protons due to the proximity of carboxyl groups from one of the ligands. This also agrees with possible charge effects concluded from small CD spectral changes observed for CB9 (Nagy et al., 1978).

The upfield shift of the meta (2,6) protons upon Ca^{2+} binding is significantly greater for CB9 (6.51 ppm) than for our 34-residue analogue (6.63 ppm) even though both fragments possess similar binding constants. The extra amino acids in CB9 may affect the shielding of these meta (2,6) protons. In addition, the ultraviolet difference analysis in the aromatic region of CB9 and rabbit skeletal TnC has pointed out red spectral shifts upon Ca^{2+} addition. Nagy et al. (1978) commented that such a result was consistent with the calcium-induced movement of Phe-99 and Phe-102 to a more hydrophobic environment. For the synthetic peptides, the presence of the perturbant generates blue shifts in the tyrosine region of the difference spectra. We interpret all these spectral differences as an indication that the environment of the aromatic side chains of CB9 is modified significantly by the presence of extra residues located in the C-terminal region (Figure 1).

It is interesting to note that the resonances of Phe-99, Phe-102, and Phe-119 ring protons are affected by calcium addition. The appearance of a Ca^{2+}-sensitive upfield resonance at 7.07 ppm in the 26 and 34 amino acid analogues suggests that this contribution comes from further shielding of the side chain of Phe-99 and/or Phe-102. This is consistent with the blue shift observed in the ultraviolet difference spectrum through a charge neutralization upon Ca^{2+} chelation.

^{1}H NMR allows us to investigate some thermodynamic aspects of Ca^{2+}-induced peptide folding. The lengthening of the N-terminal region of the calcium-binding site can dramatically increase its Ca^{2+}-binding ability (K_{Ca} from 10^2 to 10^5 M^{-1}). Since the carbonyl group of tyrosine-109 is thought to be involved in the chelation of calcium by analogy with MCBP (Kretsinger & Nockolds, 1973), it is of interest to discuss qualitatively the various exchange rates observed for the ring meta (2,6) protons in each of the synthetic fragments.

In going from the 21-residue to the 34-residue analogue, the exchange rate appears to decrease so that the NMR spectra go from the fast-exchange limit to the slow-exchange limit. For the 21-residue fragment (part of the population of which remains unfolded), only a lower limit for the off rate constant (much greater than 100 s^{-1}) can be obtained from the spectra by using the shift of the calcium sensitive meta (2,6) protons upon calcium addition. For the 26-residue peptide, we have determined by a computer line-shape analysis of the broadening of the meta (2,6) proton resonances as a function of added calcium that the off rate constant is 135 ± 50 s^{-1}. This k_{off} value agrees well with the 230–350-s^{-1} range obtained for the low-affinity sites of cardiac TnC (Johnson et al., 1978). For the 34-residue peptide only an upper limit on the off rate constant (much less than 100 s^{-1}) can be obtained, from the fact that no exchange contribution to the line broadening is observed. The value also agrees well with that calculated from the previously measured binding constant (K_{Ca} of 3.8×10^4 M^{-1}) and the assumption that the on rate is diffusion controlled (i.e., 10^7 M^{-1} s^{-1}) (Hammes & Schimmel, 1970).

The Ca^{2+} off rate (k_{off}) can be described in terms of compactness of the coordination sphere surrounding the metal. Compactness is dependent on both the type and the proper positioning of the ligands around the cation. Lanthanide-induced ^{1}H NMR shifts (Lee et al., 1979) have permitted the estimation of average distances of metal to the various ligand nuclei. Results suggested that such distances were larger in CB9 than in the intact Ca^{2+}-binding protein parvalbumin. The loss of molecular compactness was correlated to the fact that the binding constant for CB9 was 3 orders of magnitude lower than for MCBP (10^5 vs. 10^8 M^{-1}). We can consider that the extra residues of parvalbumin aided metal binding through at least partial prefolding of the sites. The positioning of the ligands is also apparently dependent on the amount of calcium-inducible α helix present near the binding loop since the lengthening of the N-terminal region raises the binding constant significantly. Circular dichroism results showed a large increase in the α-helical content for the apopeptides in 50% TFE. In such a hydrophobic environment (50% TFE), all the fragments bound calcium with a similar binding constant in the order of 10^5 M^{-1}. The constancy of the binding constant in this medium points out the need of the C-terminal helical region for Ca^{2+} binding since the 21-residue analogues only possess the C-terminal region in addition to the calcium loop. Calcium-sensitive environmental changes in the C-terminal region have been revealed from the ^{1}H NMR spectra of our fragments (Phe-119, Glu-113, Glu-114, and Glu-117). The binding constant of a 12-residue analogue of the binding loop (lacking both N- and C-terminal regions) (Reid et al., 1980) remains low (10^2 M^{-1}) in 50% TFE and confirms our view that the C terminal is probably essential to the affinity of the site for calcium. The importance of the type of ligands forming the binding loop remains to be tested by new synthetic analogues.

These peptides are excellent models to study the thermodynamics of protein folding. First, Ca^{2+} is a benign perturbant compared to strong denaturants like urea or heat. Second, the small size of these peptides will most likely provide a simple two-state model for protein folding (unfolded $\rightleftharpoons$ folded) compared to the multisite calcium-binding proteins. For example, the present model for skeletal troponin C suggests that for the Ca^{2+}-specific sites (low-affinity sites), the binding constant, K_{Ca}, depends mostly on the enthalpy term, $\Delta H°$, the entropy contribution, $\Delta S°_{reaction}$, being rather small (Potter et al., 1977; Moeschler et al., 1980). If we analyze the source of a large $\Delta H°$ term, the present hypothesis (Filimonov et al., 1978) suggests a change in heat capacity during unfolding or denaturation arising from the exposure of apolar groups to water. This explanation does not apply in the case of our analogues since the exposure of such aromatic residues as Tyr-109 has been demonstrated in the presence and absence of the metal. Similarly, it is doubtful that the formation of helical segments helps to bury apolar groups in such small peptides. In fact, the use of hydrophobic conditions has partly proven that our side chains located in the α-helical region are exposed (Reid et al., 1981).

Any significant contribution to the heat of unfolding would come from the rupture of intramolecular hydrogen bonds. Interactions of water molecules with polar groups otherwise involved in the Ca^{2+} chelation or in intramolecular H bonding would lead to a negative thermal effect (Privalov & Khechinashvili, 1974). This would better explain the alteration of the $\Delta H°$ term by addition of α-helical segments on each side of the calcium loop. This enthalpy value for Ca^{2+} binding to high- or low-affinity sites remains around −30 to −40 kJ/site (Moeschler et al., 1980).

Finally, in view of large calcium-induced conformational changes observed by CD and ^{1}H NMR, we have to consider the possibility of the increasing binding constant value arising from a significant positive entropy of reaction, $\Delta S°_{reaction}$, following the relations

$$-RT \ln K_{Ca} = H° - T\Delta S°_{reaction} \quad (1)$$

$$\Delta S°_{reaction} = \Delta S°_{conformation} - \Delta S°_{hydration} \quad (2)$$

We can probably predict that the entropy involved in peptide conformation, $\Delta S°_{conformation}$, is comparable to the large hydration entropy for calcium, $\Delta S°_{hydration}$, i.e., −183.9 J K^{-1} mol^{-1} (Filimonov et al., 1978). We base our prediction on the value of the binding constant for the analogues (10^4–10^5 M^{-1}), which corresponds to the binding ability of low-affinity binding sites of TnC (Potter et al., 1977; Moeschler et al., 1980). The difference between both entropy terms of comparable magnitude would yield, following relation 2, a small $\Delta S°_{reaction}$ contribution. This would agree with the expected entropy contribution for a low-affinity site in troponin C (Moeschler et al., 1980).

In summary, this work has demonstrated the usefulness of synthetic Ca^{2+}-binding peptides as models for studying calcium-induced folding. The selected dimensions and primary sequence of these analogues have enabled a technique like ^{1}H NMR to be more informative in terms of local Ca^{2+}-induced events.

Acknowledgments

J.G. wishes to acknowledge the indispensable help of Judy Shelling in the operation of the NMR spectrometer in the early stages of this investigation and Jaclyn Dorsey for typing and correcting the manuscript.

References

Barker, W. C., Ketcham, L. K., & Dayhoff, M. O. (1978) in *Atlas of Protein Sequence and Structure* (Dayhoff, M., Ed.) Vol. 5, Suppl. 3, p 273, The National Biomedical Research Foundation, Silver Spring, MD.

Birnbaum, E. R., & Sykes, B. D. (1978) *Biochemistry 17*, 4965–4971.

Blaedel, W. J., & Knight, H. T. (1954) *Anal. Chem. 26*, 743–746.

Cave, A., Pages, M., Morin, P., & Dobson, C. M. (1979) *Biochimie 61*, 607–613.

Cohen, P., Burchell, A., Foulkes, J. G., Cohen, P. T. W., Vanaman, T. C., & Nairn, A. C. (1978) *FEBS Lett. 92*, 287–293.

Collins, J. H., Greaser, M. L., Potter, J. D., & Horn, M. J. (1977) *J. Biol. Chem. 252*, 6356–6362.

Cox, J. A., Winge, D. R., & Stein, E. A. (1979) *Biochimie 61*, 601–605.

Dabrowska, R., Sherry, J. M. F., Aromatorio, D. K., & Hartshorne, D. J. (1978) *Biochemistry 17*, 253–258.

Dedman, J. R., Potter, J. D., Jackson, R. L., Johnson, J. D., & Means, A. R. (1977) *J. Biol. Chem. 252*, 8415–8422.

Derancourt, J., Haiech, J., & Pechère, J.-F. (1978) *Biochim. Biophys. Acta 532*, 373–375.

Ebashi, S., & Endo, M. (1968) *Prog. Biophys. Mol. Biol. 18*, 123–184.

Filimonov, V. V., Pfeil, W., Tsalkova, T. N., & Privalov, P. L. (1978) *Biophys. Chem. 8*, 117–122.

Gnegy, M. E., Uzunov, P., & Costa, E. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3887–3890.

Greaser, M. L., & Gergely, J. (1971) *J. Biol. Chem. 246*, 4226–4233.

Hammes, G. G., & Schimmel, P. R. (1970) in *The Enzymes* (Boyer, P. D., Ed.) Vol. 2, p 67, Academic Press, New York.

Hincke, M. T., McCubbin, W. D., & Kay, C. M. (1978) *Can. J. Biochem. 56*, 384–395.

Hincke, M. T., Sykes, B. D., & Kay, C. M. (1981a) *Biochemistry 20*, 4185–4193.

Hincke, M. T., Sykes, B. D., & Kay, C. M. (1981b) *Biochemistry 20*, 3286–3294.

Liu, Y. P., & Cheung, W. Y. (1976) *J. Biol. Chem. 251*, 4193–4198.

Johnson, J. D., Collins, J. H., Robertson, S. P., & Potter, J. D. (1978) *Circulation 58*, 1171.

Kaptein, R. (1978) in *Nuclear Magnetic Resonance Spectroscopy in Molecular Biology* (Pullman, B., Ed.) p 211, D. Reidel Publishing Co., Dordrecht, The Netherlands.

Klee, C. B., Crouch, T. H., & Richman, P. G. (1980) *Annu. Rev. Biochem. 49*, 489–515.

Kretsinger, R. H., & Nockolds, C. E. (1973) *J. Biol. Chem. 248*, 3313–3326.

Leavis, P. C., & Kraft, E. L. (1978) *Arch. Biochem. Biophys. 186*, 411–415.

Leavis, P. C., Rosenfeld, S. S., Gergely, J., Grabarek, Z., & Drabikowski, W. (1978) *J. Biol. Chem. 253*, 5452–5459.

Lee, L., Sykes, B. D., & Birnbaum, E. R. (1979) *FEBS Lett. 98*, 169–172.

Levine, B. A., Mercola, D., Coffman, D., & Thornton, J. M. (1977) *J. Mol. Biol. 115*, 743–760.

Levine, B. A., Thornton, J. M., Fernandes, R., Kelly, C. M., & Mercola, D. (1978) *Biochim. Biophys. Acta 535*, 11–24.

Marcum, M., Dedman, J. R., Brinkley, B. R., & Means, A. R. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3771–3775.

McCubbin, W. D., & Kay, C. M. (1975) *FEBS Lett. 55*, 183–187.

Moeschler, H. J., Schaer, J. J., & Cox, J. A. (1980) *Eur. J. Biochem. 111*, 73–78.

Morrison, M., & Bayse, G. S. (1970) *Biochemistry 9*, 2995–3000.

Nagy, B., & Gergely, J. (1979) *J. Biol. Chem. 254*, 12732–12737.

Nagy, B., Potter, J. D., & Gergely, J. (1978) *J. Biol. Chem. 253*, 5971–5974.

Nockolds, C. E., Kretsinger, R. H., Coffee, C. J., & Bradshaw, R. A. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 581.

Pechère, J.-F., Capony, J.-P., & Ryden, L. (1971) *Eur. J. Biochem. 23*, 421.

Potter, J. D., & Gergely, J. (1975) *J. Biol. Chem. 250*, 4628–4633.

Potter, J. C., Hsu, F.-J., & Pownall, H. J. (1977) *J. Biol. Chem. 252*, 2452–2454.

Privalov, P. L., & Khechinashvili, N. N. (1974) *J. Mol. Biol. 86*, 665–684.

Reid, R. E., & Hodges, R. S. (1980) *J. Theor. Biol. 84*, 401–444.

Reid, R. E., Clare, D. M., & Hodges, R. S. (1980) *J. Biol. Chem. 255*, 3642–3646.

Reid, R. E., Gariépy, J., Saund, A. K., & Hodges, R. S. (1981) *J. Biol. Chem. 256*, 2742–2751.

Seamon, K. B. (1980) *Biochemistry 19*, 207–215.

Seamon, K. B., Hartshorne, D. J., & Bothner-By, A. A. (1977) *Biochemistry 16*, 4039–4046.

Vogt, H.-P., Strassburger, W., Wollmer, A., Fleischhauer, J., Bullard, B., & Mercola, D. (1979) *J. Theor. Biol. 76*, 297–310.

Walsh, M., Stevens, F. C., Oikawa, K., & Kay, C. M. (1978) *Biochemistry 17*, 3924–3930.

Wolff, D. J., Poirier, P. G., Brostrom, C. O., & Brostrom, M. A. (1977) *J. Biol. Chem. 252*, 4108–4117.

Wong, P. Y.-K., & Cheung, W. Y. (1979) *Biochem. Biophys. Res. Commun. 90*, 473–480.