Nuclear Magnetic Resonance Studies of a Ca^{2+}-Binding Fragment of Troponin C†

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ABSTRACT: The effect of Ca^{2+} binding on the 270-MHz protein nuclear magnetic resonance spectrum of the largest cyanogen bromide fragment (CB-9) of troponin C (TN-C) has been studied. Assignments of resonances in the aromatic region of both Ca^{2+}-free and Ca^{2+}-bound CB-9 to histidine-125 and tyrosine-109 (TN-C numbering system) have been made from pH titrations, relaxation time measurements, decoupling experiments, and Overhauser enhancements. Both NMR and circular dichroism experiments show evidence of a large conformational change of the CB-9 fragment upon Ca^{2+} binding. From CD titrations of CB-9 with Ca^{2+} and several lanthanide ions, dissociation constants for these metal ions bound to CB-9 have been determined to be $\sim 10^{-5}$ M. A comparison of the NMR spectra of the CB-9 fragment with the NMR spectra of TN-C reported earlier has been made [Seamon, K. B., Hartshorne, D. J., and Bothner-By, A. A. (1977), *Biochemistry 16,* 4039). Making use of published X-ray coordinates for TN-C [Kretsinger, R. H., and Barry, C. D. (1975), *Biochim. Biophys. Acta 405,* 40], a model of the CB-9 fragment was constructed and used to explain the differences in the NMR spectra between the effect of Ca^{2+} binding to CB-9 when compared to TN-C. Evidence for the presence of unfoldable CB-9 in the final preparation has been shown by NMR, and similarities with the TN-C spectrum suggest that a portion of the TN-C preparation may similarly remain unfolded.

The calcium regulation of muscle contraction in vertebrate skeletal muscle results from the interaction of calcium with the protein complex troponin (Ebashi and Endo, 1968). The binding of calcium to troponin presumably causes a conformational change, mediated by tropomyosin, which allows the myosin heads of the thick filament to interact with the actin strands of the thin filament, initiating muscle contraction. Troponin itself is composed of three unique subunits, TN-C,[1] TN-I, and TN-T, of which only TN-C appears to bind Ca^{2+} (Greaser and Gergely, 1971). Troponin C from rabbit skeletal muscle is a protein with a molecular weight of ~18 000 whose amino acid sequence has been determined by Collins et al. (1973, 1977). TN-C bears a strong sequence homology to parvalbumin, a protein isolated originally from fish. Parvalbumin is roughly half the size of TN-C, and Kretsinger (1972) has suggested that the protein is related to TN-C via gene doubling. Parvalbumin binds 2 mol of Ca^{2+} (Pechère et al., 1971; Benzonana et al., 1972) compared to 4 mol of Ca^{2+} in TN-C (Potter and Gergely, 1975), and the crystal structure of the protein isolated from carp has been determined by Kretsinger and Nockolds (1973). The similarity between the primary sequences of TN-C and parvalbumin led Collins et al. (1973) to suggest the possible location in the sequence for the four Ca^{2+} binding sites on TN-C. Kretsinger and Barry (1975) using this similarity in primary sequence proposed an overall structure for TN-C which described the four Ca^{2+} sites suggested by Collins et al. (1973) as repeating "E and F hands."

Troponin C has been found to have two unique Ca^{2+} binding sites, two unique Mg^{2+} sites, and two sites for which both metal ions compete (Potter and Gergely, 1975). In the absence of Mg^{2+}, the two strong Ca^{2+} sites, which also bind Mg^{2+}, have binding constants of 2×10^7 M^{-1}, while the two weaker Ca^{2+} sites have binding constants of 3×10^5 M^{-1}. The binding of Ca^{2+} to TN-C and parvalbumin causes a major conformational change to take place which can be followed by a variety of physical methods, including, fluorescence, CD, NMR, and ultracentrifugation (e.g., see McCubbin and Kay, 1973; Potter et al., 1976; Seamon et al., 1977). The CD studies reveal that the conformational change is primarily a dramatic increase in the α-helix content of the protein.

Recently, Nagy et al. (1976) reported that one of the cyanogen bromide fragments of whole TN-C, originally isolated by Collins et al. (1973, 1977), binds a single Ca^{2+} with a K ~10^5 M^{-1} and mimics the conformational change of TN-C upon Ca^{2+} binding. This fragment, labeled CB-9, is the largest fragment isolated, containing 52 residues and extending from Lys-84 through Hse-135. This region in the sequence of TN-C corresponds to the third Ca^{2+} binding site with the higher association constant, suggested by analogy with the parvalbumin structure and Ca^{2+} binding behavior (Weeds and McLachlin, 1974; Kretsinger and Barry, 1975). This fragment is of special interest, since it contains the single histidine and cysteine residues of whole TN-C and one of the two tyrosine residues of TN-C, Tyr-109, which has been implicated in the binding of a Ca^{2+} ion by tyrosine fluorescence (Kawasaki and Van Eerd, 1975) and by Tb^{3+} fluorescence enhancement (Miller et al., 1975).

It was felt that a detailed NMR study of this fragment, containing a single Ca^{2+} ion binding site, should be of great help in unraveling the more complicated NMR picture of TN-C, containing both the weak and strong Ca^{2+} binding sites (Seamon et al., 1977; Levine et al., 1976, 1977). In addition, there has been a strong recent interest in protein fragments in general, including fragments of Ca^{2+}-binding proteins, and this study offers the first detailed NMR study of the conformation of a fragment in comparison with the native protein

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[1] Abbreviations used: NMR, nuclear magnetic resonance; CB-9, cyanogen bromide cleavage fragment no. 9; DSS, 2,2-dimethyl-2-silapentane-5-sulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); DTT, dithiothreitol; ME, β-mercaptoethanol; HEDS, bis(2-hydroxyethyl) disulfide; EDTA, ethylenediaminetetraacetic acid; CD, circular dichroism; TN-C, troponin C; CNBr, cyanogen bromide.

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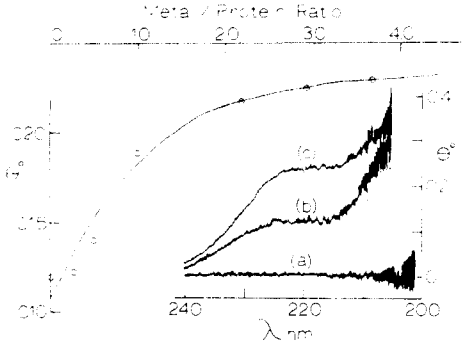

FIGURE 1: Ca^{2+} ion CD spectral titration of CB-9 at λ 222 nm together with the least-squares fit. Inset: CD spectra of (a) base line with buffer in cell, (b) Ca^{2+}-free CB-9 in buffer, (c) Ca^{2+}-saturated CB-9 in buffer. Buffer is 0.1 M KCl and 2.5×10^{-2} M Pipes (pH 6.8). [CB-9] $\simeq 1 \times 10^{-4}$ M. $\Delta[\theta]_{222} = 6200$ deg cm^2 dmol^{-1} mean residue weight change upon Ca^{2+} binding.

(e.g., see Coffee and Solano, 1976; Drabikowski et al., 1977; Derancourt et al., 1978; Pearlstone and Smillie, 1977).

Materials and Methods

Preparation of Bis(2-hydroxyethyl) Sulfide-TN-C. Troponin C, isolated from rabbit skeletal muscle following the methods of Ebashi et al. (1971), Staprans et al. (1972), and Greaser and Gergely (1971), was judged pure by NaDodSO$_4$-gel electrophoresis and amino acid analysis. Protein concentration was determined by amino acid analysis. The free sulfhydryl group of TN-C was protected utilizing the disulfide interchange reaction essentially according to Bradshaw et al. (1967), utilizing bis(2-hydroxyethyl) disulfide instead of cysteine because of its better solubility. A solution of 8 M urea, 0.01 M EDTA, 0.1 M methylamine, and 0.2 M bis(2-hydroxyethyl) disulfide in 0.1 M phosphate buffer at pH 9.4 was deoxygenated by bubbling with N_2 for 20 min. Approximately 600 mg of reduced TN-C was dissolved in this buffer and stirred overnight (~16 h). The bis(2-hydroxyethyl) sulfide-TN-C was separated from the reaction mixture on a 80 × 5 cm Sephadex G-25 column equilibrated with 50 mM NH_4HCO_3 at pH 8. The first peak contained essentially pure protein and was lyophilized.

Preparation of CB-9 Fragment. The bis(2-hydroxyethyl) sulfide-TN-C was dissolved in 60 mL of 70% formic acid containing 5.7 g of CNBr (a 200-fold molar excess) and allowed to react overnight (~16 h) according to the method of Pearlstone et al. (1977). After reaction, a tenfold excess of water was added to the CNBr solution followed by lyophilization. The CB-9 fragment was isolated by dissolving the lyophilized reaction mixture in a minimal volume of 50 mM NH_4HCO_3 followed by chromatography on a 230 × 5 cm Sephadex G-75 column equilibrated previously with 50 mM NH_4HCO_3 (pH 8). The column was eluted with 50 mM NH_4HCO_3 (pH 8) at ~60 mL/h. Amino acid analysis and end-group analysis were used to confirm the purity of the fragment. A sedimentation velocity study on a typical NMR sample using a Beckman Model E ultracentrifuge showed only a single symmetrical peak with a $s^0_{20,w} = 1.36$, suggesting that no aggregation was taking place at 0.5 mM concentrations.

pH Titrations. pH titrations were carried out in the NMR tube using a Radiometer Model 26 or Beckman Expandomatic SS-2 pH meter with an Ingold Model 6030-04 microelectrode. pHs were adjusted with 0.1 M NaOD or 0.1 M DCl. Before each pH reading the electrode was standardized in H_2O buffers, and between each reading the electrode was soaked in 0.1 M HCl to ensure reproducible standardization. pHs reported are meter readings and are not corrected for the deuterium isotope effect.

Metal Ion Analysis. Stock lanthanide solutions were prepared from fired Johnson-Matthey ultrapure oxides, dissolved in a slightly less than stoichiometric amount of concentrated HCl, filtered, and made up to volume using dithionized D_2O. The stock solution was then treated with dithizone to remove residual transition metal ions, and the final concentration of Ln^{3+} was determined by titration with EDTA in pH 6 acetate buffer using Xylenol orange as an indicator. Calcium stock solutions were prepared from reagent-grade $CaCl_2 \cdot 2H_2O$ dissolved in dithizoned D_2O. Final Ca^{2+} concentrations were determined by EDTA titrations using calcein as a fluorescent indicator in basic solution. All metal solutions were lyophilized once from D_2O to reduce the H_2O content prior to final standardization.

NMR Spectra. NMR spectra were obtained using a Bruker HXS 270-MHz NMR spectrometer operating in the Fourier transform mode usually with quadrature detection. Typical spectra were obtained using a 1-s acquisition time, with a 9-μs pulse (~90°), a ±2000-Hz sweep width, and a minimal line broadening of less than 1 Hz. Spin-lattice relaxation times, T_1, were obtained using the standard $(T\text{-}180°\text{-}\tau\text{-}90°\text{-}\text{acquisition})_n$ inversion recovery pulse sequence, with a 5-s delay time between each sequence for the protein T_1 measurements to allow for recovery of at least 95% of the magnetization. Typically, 1000 acquisitions were acquired for each of the 10 points on a T_1 plot. Nuclear Overhauser enhancements were calculated from the equation $\text{NOE} = (A_{\text{irrad}} - A_{\text{control}})/(A_{\text{control}})$, where A_{irrad} and A_{control} are the integrated intensities of the observed peak with the decoupling irradiating radio frequency field on resonance and off resonance, respectively, for the irradiated peak. All decoupling was accomplished in the pulsed homonuclear mode. Protein concentrations were roughly 0.5 mM (~2.5 mg/mL), determined by amino acid analysis. All spectra were obtained at ambient temperature, 27 °C.

CD Spectra. CD spectra were obtained using a Cary 60 ORD spectropolarimeter with CD accessory. All spectra were run at room temperature and referenced to a base line obtained with buffer [25 mM Pipes, 0.1 M KCl (pH 6.8)] in the same cell. Aliquots of stock metal solutions were added directly to the protein solution (~10^{-4} M) in the cell.

Recovery of Protein. Protein that had been used for NMR and CD studies was recovered by passing through a Sephadex G-25 column equilibrated with 50 mM NH_4HCO_3 and lyophilized. Unfortunately, the conformational sensitivity to the presence of Ca^{2+} could *not* be demonstrated after this mild recovery step. Attempts to refold the CB-9 fragment using 8 M urea, EDTA, or high Ca^{2+} concentrations were unsuccessful.

Results

Circular Dichroism. The utility of the CB-9 fragment lies in the spectral sensitivity of the fragment to conformational changes caused by the binding of Ca^{2+}. Nagy et al. (1976) reported that the binding of Ca^{2+} to the CB-9 fragment produces over 90% of the ellipticity change at 222 nm observed for Ca^{2+} binding to whole TN-C. Similar results were obtained in our laboratory. Titration of CB-9 with Ca^{2+} while following the change in ellipticity at 222 nm produces a typical titration curve for a 1:1 association. Figure 1 shows the least-squares fit for the Ca^{2+} titration curve, together with the Ca^{2+}-free and Ca^{2+}-bound spectra. The dissociation constants for the binding of Ca^{2+} to CB-9 as well as for several lanthanide ions were determined by a least-squares fit as follows: $Ca^{2+} = (3.7 \pm 1.9)$

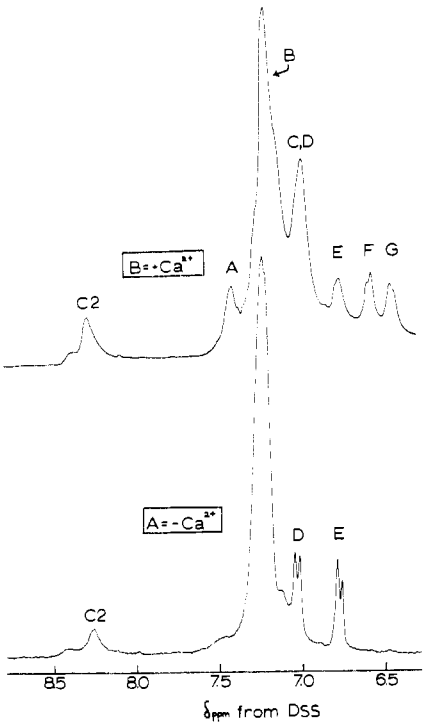

FIGURE 2: NMR spectra of the aromatic region of CB-9: (A) No Ca^{2+}; (B) excess Ca^{2+}. [CB-9] $\simeq 4 \times 10^{-4}$ M in 0.1 M KCl (pH 6.8). The C-2 proton of histidine is labeled. For identity of labels A–G, see the text.

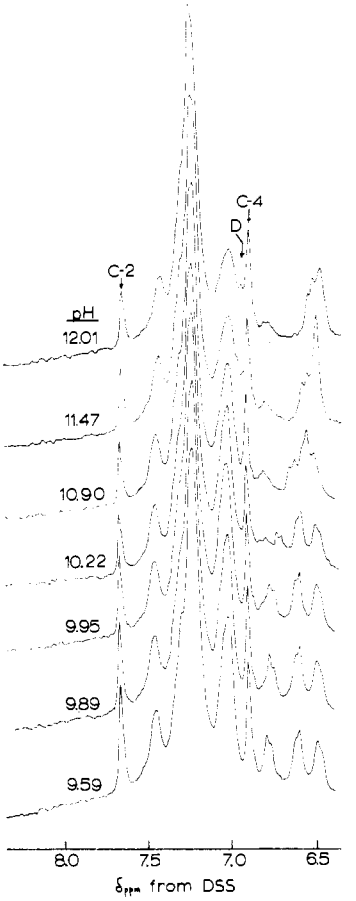

FIGURE 3: Aromatic region of Ca^{2+}-bound CB-9 as a function of pH. [CB-9] $\simeq 4 \times 10^{-4}$ M in 0.1 M KCl. The C-2 and C-4 protons of histidine are labeled. Peak D refers to the same protons as in Figure 2.

$\times 10^{-5}$ M; $La^{3+} = (6.7 \pm 6) \times 10^{-6}$ M; $Pr^{3+} = (2.2 \pm 0.5) \times 10^{-5}$ M; $Gd^{3+} = (2.6 \pm 0.6) \times 10^{-5}$ M; $Yb^{3+} = (8.2 \pm 4.6) \times 10^{-5}$ M; $Lu^{3+} = (1.3 \pm 0.8) \times 10^{-5}$ M. The CD spectra obtained at the end of the titration were essentially the same for all metal ions, indicative of very similar gross conformations for all CB-9–metal ion complexes. The same CD spectral changes upon Ca^{2+} binding were observed in D_2O, as well as in H_2O.

NMR pH Titration and Peak Assignments. The CB-9 peptide fragment from troponin C is of particular interest from an NMR standpoint, since it has no tryptophans, a single tyrosine (Tyr-109) and histidine (His-125), and only three phenylalanines. As a result, the NMR spectrum in the aromatic region is relatively simple. Figure 2 shows the aromatic region of this peptide with and without added Ca^{2+}. It is of note that the Ca^{2+}-free spectrum (Figure 2A) is obtained by dissolving the peptide fragment as isolated in buffer. Addition of EDTA produces no additional change in the spectrum. The Ca^{2+}-free spectrum at pH ~7 has a somewhat broadened C-2 resonance of the single histidine at 8.3 ppm, while the C-4 resonance is at the edge of the phenylalanine region at ~7.15 ppm. The small resonance at 8.45 ppm is due to a small amount of residual formate from the CNBr cleavage. A sharp AA′BB′ pattern from the single tyrosine residue can be seen at 7.06 and 6.83 ppm (labeled D and E, respectively), while the phenylalanine resonances appear as a large unsplit peak centered at ~7.25 ppm (labeled B). The assignment of these peaks is readily apparent from a pH titration over the pH range 5–12. The pK_a of the histidine was determined to be 7.01 ± 0.02 from the titration of the C-2 proton; the pK_a of the tyrosine was determined to be 10.6 ± 0.1 as an average from the titration of the ortho and meta protons. The meta protons titrate 0.16-ppm upfield to 6.90 ppm; the ortho protons titrate 0.3-ppm upfield to 6.53 ppm. There are no upfield peaks analogous to the peaks observed at 6.44 and 6.67 ppm by Seamon et al. (1977) for Ca^{2+}-free TN-C.

The addition of roughly equimolar Ca^{2+} to this sample causes some substantial alterations in this region of the spectrum, as is shown in Figure 2B. With the exception of the histidine resonances, which appear to be insensitive to the Ca^{2+} addition, both the phenylalanine and tyrosine resonances undergo large shifts. The phenylalanine single peak splits into three peaks, one at lower field (~7.47 ppm, labeled A), one at higher field (~7.04 ppm labeled C), and one (the major peak, B) that is essentially unshifted. The phenylalanine resonance which shifted upfield overlies the position of one of the doublets from tyrosine in the Ca^{2+}-free CB-9 spectrum (labeled D at 7.06 ppm). The effect of Ca^{2+} in the tyrosine region is to cause a new AA′BB′ pattern to appear upfield of peak E at 6.64 and 6.51 ppm from DSS, labeled F and G, respectively. A decoupling experiment shows that these two new sets of peaks, F and G, are coupled together and they can be assigned to tyrosine by pH titration, T_1 measurements, and NOE measurements (see below). The new peak positions of the tyrosine resonances in the presence of Ca^{2+} presumably reflect the new environment that this single tyrosine residue has in the *folded* protein fragment. The existence of a residual amount of protein that apparently remains *unfolded* is suggested by the presence of resonance E, which is in the same position as the Ca^{2+}-free spectrum. It is unclear whether or not this unfolded portion of the protein fragment CB-9 binds Ca^{2+} or not.

A pH titration of the Ca^{2+}-bound CB-9 fragment in the region from 5 to 8 yields a simple monoprotic titration curve for the C-2 and C-4 histidine resonances with a $pK_a = 7.08 \pm 0.03$, essentially the same as for Ca^{2+}-free CB-9. At more basic pHs (see Figure 3), the effect of the ionization of the tyrosine

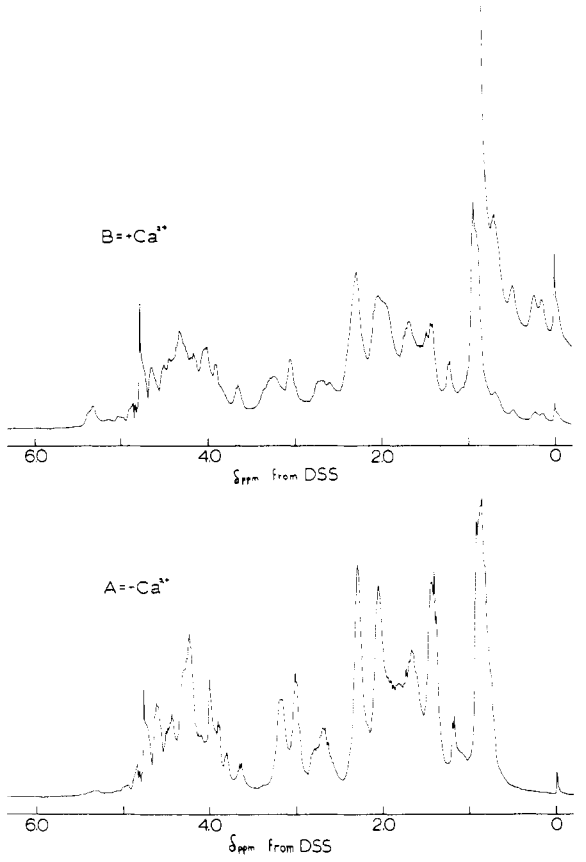

FIGURE 4: NMR spectra of the aliphatic region of CB-9: (A) No Ca^{2+}; (B) excess Ca^{2+}. [CB-9] $\simeq 4 \times 10^{-4}$ M in 0.1 M KCl (pH 6.8).

hydroxyl group can be seen. Resonance F shifts rapidly upfield as the pH is raised, suggesting that it is due to the protons ortho to the hydroxyl group. Resonance G, to which resonance F is coupled, would, therefore, correspond to the protons meta to the hydroxyl group, the reverse of model tyrosine derivatives (Karplus et al., 1973, Martin and Morlino, 1965). Resonance G, however, does not shift appreciably during the titration. The coupled nature of resonances F and G can be seen by their apparent singlet character as they cross over at a pH near 11.47. Similar behavior has been observed for tyrosine-21 of the bovine pancreatic trypsin inhibitor (Snyder et al., 1975, 1976). This reversed assignment is confirmed by the relative relaxation rates of these two resonances [i.e., T_1^{-1} (F-ortho) = 0.57 s^{-1} < T_1^{-1} (G-meta) = 0.77 s^{-1}], with the protons meta to the hydroxyl group relaxing somewhat faster due to the additional interaction with the β-CH_2 protons of the tyrosine. The negative Overhauser enhancements of these two resonances observed during decoupling are also in agreement with this assignment, being greater for resonance F when resonance G is saturated (NOE $\simeq -0.52$) than for G when F is saturated (NOE $\simeq -0.28$). Throughout this work there is no evidence for restricted rotation about the C_β-C_γ bond of tyrosine resulting in nonequivalent ortho and meta protons. Similarly, the relative relaxation rates of the ortho and meta protons of Tyr-109 are similar to those of model tyrosine derivatives, e.g., 0.43 and 0.63 s^{-1} for the ortho and meta protons, respectively, in the dipeptide L-Ala-L-Tyr (B. D. Sykes, unpublished results). This implies that the tyrosine ring lies at the surface of the peptide and is exposed to the solvent, in agreement with chemical evidence for TN-C (McCubbin and Kay, 1975). The relative relaxation rates of the tyrosine protons in Ca^{2+}-free CB-9 are normal with the upfield ortho peak

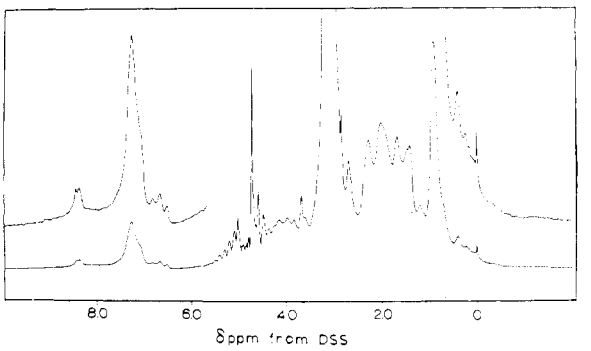

FIGURE 5: NMR spectrum of the La^{3+}-CB-9 complex. [CB-9] $\simeq 4 \times 10^{-4}$ M in 0.1 M KCl and 2.5×10^{-2} M Pipes (pH 6.8).

slower than the downfield meta peak [T_1^{-1} (E-ortho) = 0.71 s^{-1} < T_1^{-1} (D-meta) = 1.17 s^{-1}].

The titration behavior of resonance E at high pH is also of interest. A portion of this peak moves rapidly upfield as the pH is increased, eventually merging into peaks F and G. This behavior is identical to the pH behavior of peak E in Ca^{2+}-free CB-9 and, therefore, suggests that this resonance is due to the ortho protons of the residual unfolded protein. At pH 11.47 and 12.01, the appearance of a shoulder on the upfield side of the phenylalanine resonance C (labeled D in Figure 3) is consistent with the pH titration of the meta protons of Ca^{2+}-free CB-9 (peak D in Figure 2A) and, therefore, suggests that this is the resonance associated with the meta protons of the tyrosine in residual unfolded CB-9. At high pH there is still a small doublet at 6.83 ppm which has not been shifted, which presumably is a phenylalanine proton. The intensity of the ortho tyrosine resonance of the unfolded CB-9 fragment at high pH compared to the tyrosine resonance of the folded CB-9 fragment suggests that between 20 and 30% of the peptide is not folded properly upon addition of calcium. The addition of DTE to these NMR samples did not appreciably alter the amount of folded vs. unfolded tyrosine peaks, suggesting that loss of the SH-protecting group and subsequent dimerization are *not* responsible for the presence of the unfolded fragment.

The aliphatic region of the Ca^{2+}-free peptide is shown in Figure 4A, while the fully Ca^{2+}-bound peptide is shown in Figure 4B. The spectra are similar with the exception of the region 0–1.0 ppm and a general increase in line width in the Ca^{2+}-bound form. The most obvious effect of the protein folding upon Ca^{2+} binding is to shift several resonances upfield, producing a series of five new peaks, one of which is slightly upfield of DSS. These peaks are presumably due to methyl groups which upon folding are placed close to an aromatic ring of one of the phenylalanines or the tyrosine, causing a significant shielding effect. The other region of marked alteration due to Ca^{2+} binding occurs from ~1.7 to 2.0 ppm, presumably due to methylene groups moving into new environments upon protein folding. Similar effects have been observed in the spectra of whole TN-C, with and without Ca^{2+}.

NMR Spectra of CB-9 + La^{3+}. The possibility of using lanthanide ions to probe the structure of the CB-9 fragment in solution was suggested by Leavis et al. (1976), who reported that Tb^{3+} fluorescence was enhanced upon binding. Our CD titration of several lanthanides suggested that the overall conformations of the Ca^{2+}- and Ln^{3+}-bound CB-9 peptide were very similar. The NMR spectrum of the peptide bound to La^{3+}, a diamagnetic lanthanide ion (Figure 5), is indeed very similar to the Ca^{2+}-bound spectrum. However, there are some significant differences in the spectra. The increase in the line

width of the resonances in the La^{3+} spectrum compared to the Ca^{2+} spectrum is readily apparent. The phenylalanine region is no longer well resolved into three peaks, the tyrosine doublet splitting is lost in the La^{3+} spectrum, and the upfield aliphatic methyl peaks in the 0–0.9 ppm region are not as well resolved. In addition to a loss of resolution in the high-field region, there are some significant differences in the position of these upfield methyl peaks. The principal difference between the Ca^{2+} and La^{3+} spectra is that the methyl resonances are not shifted upfield as far in the La^{3+} spectrum as in the Ca^{2+} spectrum. The La^{3+} spectrum only has four upfield methyl peaks split away from the large methyl resonance at ~0.91 ppm compared to five in the Ca^{2+} spectrum. The least-shifted peak is a shoulder on the large methyl peak in the La^{3+} spectrum, whereas in the Ca^{2+} spectrum this peak is well resolved. On the other hand, the next two peaks in the Ca^{2+} spectrum appear to be at the same resonance position in the La^{3+} spectrum. The two furthest upfield peaks in the La^{3+} spectrum appear to be ~0.14 ppm downfield from their counterparts in the Ca^{2+} spectrum. This peak for peak comparison between the La^{3+} and Ca^{2+} spectra is made assuming the same number of methyl resonances in both spectra are being shifted to high field. This would only be the case if the fragment is folding around the two metal ions in a very similar fashion. It is not possible to exclude the case where methyl resonance positions have interchanged relative positions or even that different methyl groups have been shifted to high field in the two metal ion spectra, although this would be unlikely in view of the similarity in the CD spectra and the overall close resemblance of their NMR spectra.

Discussion

The circular dichroism spectra of the Ca^{2+}-free and -bound CB-9 fragment reflect the large conformational change that the fragment undergoes due to Ca^{2+} ion binding. A similar large difference in the NMR spectra of the Ca^{2+}-free and -bound forms of the fragment would be expected. Two large changes are obvious in the spectrum of CB-9 upon Ca^{2+} binding: the appearance of the upfield methyl groups between 0 and 0.9 ppm and the new peaks that appear in the aromatic region. The spectrum also shows a general line broadening in addition to other minor perturbations.

Assignment of resonances to specific amino acid residues would permit the characterization of the conformational changes in more detail. While this is not easily possible in the aliphatic region of the spectrum, the resonances due to the single histidine (His-125) and tyrosine (Tyr-109) can be assigned in the aromatic region. This is straightforward in the case of the Ca^{2+}-free CB-9 from the resonance positions typical of the amino acid in model compounds, the coupling pattern observed, and the titration behavior. In the Ca^{2+}-bound CB-9, the histidine resonance is unperturbed and can, therefore, be assigned using the above criteria. The situation with tyrosine-109 is more complex. The two new, coupled high-field peaks which appear in the aromatic region upon the addition of Ca^{2+} were assigned to the Tyr-109 ring protons in the folded CB-9 fragment primarily on the basis of the pH titration and the similarity of their relaxation behavior (including NOE's) to that expected for tyrosine. The peaks at the resonance position corresponding to Ca^{2+}-free CB-9 were assigned in part to residual CB-9 incapable of binding Ca^{2+} and/or folding properly.

The assignment of peaks F and G to tyrosine ring protons gives a result different from that expected from model compound chemical shifts. A careful perusal of Figure 3, showing the high pH titration of Ca^{2+}-bound CB-9, reveals that while peak F shifts upfield nicely with increasing pH, peak G does not, although peaks F and G are coupled together. If F and G are, in fact, tyrosine protons, then there must be an explanation for the unusual pH behavior of resonance G. One possibility lies in the abnormal chemical shift of peak G. This peak was assigned to the *meta* proton of the tyrosine ring, rather than the ortho proton as would be expected on the basis of its relative chemical shift. The small pH dependence of peak G, the faster relaxation time of G, and the smaller negative Overhauser effect compared to peak F all lead to this assignment. If upon deprotonation of the hydroxyl proton the tyrosine ring moves away from the environment which is causing the meta proton to appear at such a high-field position, then the expected pH upfield shift may be counterbalanced by the change in environment of the meta proton.

Inspection of a model [constructed using the coordinates calculated by Kretsinger and Barry (1975) based on the sequence homology of TN-C with parvalbumin] suggests a reason for the unusual chemical shifts of the Tyr-109 protons in the folded CB-9 fragment. Tyr-109 in the Kretsinger and Barry model is homologous with a phenylalanine in parvalbumin. In parvalbumin, the carbonyl oxygen of the peptide bond of this residue is bound to the Ca^{2+} ion. This places Tyr-109 very close to a rather dense region of negative charge associated with the four ionized carboxyl groups which bind that Ca^{2+} ion. In addition, one of the carboxyls (from Asp-107) could easily swing out from the Ca^{2+} binding site, placing it in close proximity to the meta protons of Tyr-109. These effects would tend to place the meta proton in a highly shielded environment. At the basic pHs where the tyrosine hydroxyl is deprotonated, the resulting negative charge should repel the negative carboxyl groups associated with the binding site, resulting in the chemical shift compensation observed for resonance G in the pH titration.

A comparison of the CB-9 NMR spectrum with the TN-C spectrum reported earlier by Seamon et al. (1977) and Levine et al. (1977) is of interest. They have assigned the two highest field peaks in the aromatic region of Ca^{2+}-free (Seamon et al., 1977) and -bound TN-C at 85 °C (Levine et al., 1977) to phenylalanine protons. Since these peaks appear in approximately the same position as peaks F and G in the Ca^{2+}-bound CB-9 spectrum, an alternative possibility would have been to assign peaks F and G to phenylalanine protons. The difficulty with such an assignment is then to account for the absence of peaks that can be assigned to the tyrosine protons. An explanation is that these resonances are completely buried under other peaks and do not shift appreciably upon titration. However, this is much less likely than the straightforward assignment offered above. In addition, the T_1 values, NOE's, and coupling pattern of peaks F and G are much more typical of tyrosine than of phenylalanine. An explanation for the pH sensitivity of peak F would also have to be found in order to be able to assign this peak to a phenylalanine proton(s).

On the balance it would appear that the two upfield aromatic peaks F and G in the Ca^{2+}-bound CB-9 fragment spectrum must be assigned to the tyrosine ortho and meta protons, respectively, of the folded fragment. Peaks D and E are then due to the tyrosine ring protons associated with a residual amount of denatured peptide which will not refold upon addition of excess Ca^{2+}. Peak E is also shown to overlap another single resonance in the folded protein.

Further differences are found in a comparison of the CB-9 NMR spectrum with the TN-C spectrum. If we regard the appearance of the high-field resonances close to DSS and the splitting of the phenylalanine peak at 7.25 ppm as evidence of protein structure, then it would appear that TN-C has a sig-

nificant amount of tertiary structure even in the absence of Ca^{2+}, whereas the CB-9 fragment has very little. The effect of adding Ca^{2+} to the fragment is to very markedly increase the amount of tertiary structure, as reflected by the CD spectral changes as well as the NMR changes. However, many of the peaks that appear to be associated with Ca binding in the spectrum of the fragment are missing or severely broadened in the spectrum of TN-C. If we compare the Seamon et al. (1977) spectra, which has a complete calcium titration curve for TN-C, we can see several striking differences. Both sets of tyrosine ring protons in Ca^{2+}-free TN-C appear at ~6.9- and 7.2-ppm downfield from DSS. No evidence is found for the appearance of two new high-field tyrosine peaks upon the addition of calcium. The implication of this observation is that the additional peptide length of TN-C is folded back onto the region of the peptide containing Tyr-109 in such a manner so as to prevent the interaction that causes the appearance of these high-field tyrosine resonances in the fragment. If we again look at the TN-C model based on the parvalbumin structure, we can see that Arg-145, which is *not* present in the CB-9 fragment, is part of the residues composing Ca^{2+}-binding site 4, and this region of the protein is folded back onto Ca^{2+} site 3 in such a way that the positively charged group of Arg-145 lies very close to the tyrosine. This juxtaposition of Arg-145 close to Tyr-109 could easily reduce the shielding effects of the carboxyl groups observed in the NMR spectrum of the fragment, resulting in the Tyr-109 NMR resonances appearing at a normal chemical shift for TN-C. Similarly, the absence of the high-field phenylalanine resonances in the CB-9 spectrum that appear in the TN-C spectrum could be due either to the fact that a phenylalanine not contained in the fragment is responsible for those peaks or that the presence of additional portions of the protein is necessary to cause protons on a phenylalanine contained in the CB-9 fragment to shift upfield.

The same kind of effect is observed in the high-field aliphatic region. While five peaks are observed in the Ca^{2+}-bound CB-9 spectrum and none in the Ca^{2+}-free spectrum, only two or three broad peaks are observed in the Ca^{2+}-free TN-C spectrum, and little change is observed in this high-field aliphatic region until the weak binding sites become occupied, whereupon most of these peaks disappear.

A final interesting aspect relative to protein fragments in general is the sensitivity of the preparation with respect to its ability to fold and/or bind Ca^{2+}. This is evidenced by the fact that 20–30% of the original preparation of the CB-9 fragment did not fold. In addition, material recovered by passage over a Sephadex G-25 desalting column lost the ability to fold upon the addition of Ca^{2+} ion. Finally, it should be noted that the line width of the C-2 proton of the single histidine in the fragment is somewhat broad and assymmetric throughout the pH titration range 5–8, even in unfoldable CB-9. The implication that a manifold of states exists for the CB-9 fragment in solution is a logical possibility. It should be pointed out in this regard that both of the NMR studies of TN-C discussed earlier show the C-2 proton of the single histidine in TN-C as a broad asymmetric peak. The similarity of the C-2 resonance in the CB-9 fragment and the whole protein suggests that a manifold of conformations may exist for TN-C as well as for CB-9.

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References

Benzonana, G., Capony, J.-P., and Pechère, J.-F. (1972), *Biochim. Biophys. Acta 278,* 110.

Bradshaw, R. A., Kanarok, L., and Hill, R. L. (1967), *J. Biol. Chem. 242,* 3789.

Coffee, C. J., and Solano, C. (1976), *Biochim. Biophys. Acta 453,* 67.

Collins, J. H., Potter, J. D., Horn, M. J., Wilshire, G., and Jackman, N. (1973), *FEBS Lett. 36,* 268.

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

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

Drabikowski, W., Grabarek, Z., and Barylko, B. (1977), *Biochim. Biophys. Acta 490,* 216.

Ebashi, S., and Endo, M. (1968), *Prog. Biophys. Mol. J. Biol. 18,* 123.

Ebashi, S., Wakabayashi, T., and Ebashi, I. F. (1971), *J. Biochem. (Tokyo) 69,* 441.

Greaser, M. L., and Gergely, J. (1971), *J. Biol. Chem. 246,* 4226.

Karplus, S., Snyder, G. H., and Sykes, B. D. (1973), *Biochemistry 12,* 1323.

Kawasaki, Y., and van Eerd, J. P. (1972), *Biochem. Biophys. Res. Commun. 49,* 895.

Kretsinger, R. H. (1972), *Nature (London), New Biol. 240,* 85.

Kretsinger, R. H., and Barry, C. D. (1975), *Biochim. Biophys. Acta 405,* 40.

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

Leavis, P. C., Nagy, B., and Lehrer, S. S. (1976) *Biophys. J. 16,* 71a.

Levine, B. A., Mercola, D., and Thornton, J. M. (1976), *FEBS Lett. 61,* 218.

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

Martin, R. B., and Morlino, V. J. (1965), *Science 150,* 493.

McCubbin, W. D., and Kay, C. M. (1973), *Biochemistry 12,* 4228.

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

Miller, J. L., Nelson, D. J., Brittain, H. G., Richardson, F. S., Martin, R. B., and Kay, C. M. (1975), *FEBS Lett. 58,* 262.

Nagy, B., Potter, J. D., and Gergely, J. (1976), *Biophys. J. 16,* 71a.

Pearlstone, J. R., and Smillie, L. B. (1977), *Can. J. Biochem. 55,* 1032.

Pearlstone, J. R., Carpenter, M. R., and Smillie, L. B. (1977), *J. Biol. Chem. 252,* 971.

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

Potter, J. D., and Gergely, J. (1975), *J. Biol. Chem. 250,* 4628.

Potter, J. D., Seidel, J. C., Leavis, P., Lehrer, S. S., and Gergely, J. (1976), *J. Biol. Chem. 251,* 7551.

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

Snyder, G. H., Rowan, R., Karplus, S., and Sykes, B. D. (1975), *Biochemistry 14,* 3765.
Snyder, G. H., Rowan, R., and Sykes, B. D. (1976), *Biochemistry 15,* 2275.
Staprans, I., Takahashi, H., Russell, M. P., and Watanabe, S. (1972), *J. Biochem. (Tokyo) 72,* 723.
Weeds, A. G., and McLachlan, A. D. (1974), *Nature (London) 252,* 646.

Evidence from Hydrogen-1 and Carbon-13 Nuclear Magnetic Resonance Studies That the Dissociation Rate of Oxytocin from Bovine Neurophysin at Neutral pH Is Slow†

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ABSTRACT: The interaction of the peptide hormone oxytocin with bovine neurophysins I and II (NPI and NPII) was studied using 270-MHz ^{1}H nuclear magnetic resonance (NMR) spectroscopy and observing the tyrosine-2 aromatic protons of the hormone. Using oxytocin and its diastereoisomer [1-hemi-D-cystine]oxytocin (which does not bind to the neurophysins), we investigated the T_2 (line width) values of these protons as a function of temperature, protein concentration at constant hormone concentration, and protein concentration at a constant hormone protein ratio and the T_1 values of these hormones in the presence and absence of the neurophysins. The results of these studies indicate that at neutral pH most of the effects observed can be accounted for by viscosity changes and suggest that the bound hormone is in slow exchange. This was corroborated by ^{13}C NMR studies. A total synthesis of specifically ^{13}C labeled (90% ^{13}C enrichment) [2-^{13}C]tyrosine (L and DL derivatives) was accomplished, and the enriched amino acid was incorporated into oxytocin and arginine vasopressin by total synthesis. The diastereoisomers of [2-DL-[2-^{13}C]tyrosine]oxytocin were separated and purified by partition chromatography. The interactions of these hormone derivatives with NPI and NPII were investigated at a variety of temperatures and hormone and protein concentrations but at constant pH (6.6). Under all conditions, [2-L-[2-^{13}C]tyrosine]oxytocin (**1**) and [2-L-[2-^{13}C]tyrosine, 8-arginine]vasopressin (**3**) interact strongly with NPI and NPII, and on binding, a 2.2-ppm downfield chemical shift of the labeled carbon atom is observed for both hormones at a 1:1 hormone to protein stoichiometry. These results indicate an equivalence of binding sites for both hormones to both NPI and NPII in the 1:1 complex. On the other hand, [2-D-[2-^{13}C]tyrosine]-oxytocin (**2**) does not significantly interact with the neurophysins. When an excess of labeled hormone **1** is added to NPI (or NPII) (e.g., a 1.4:1 hormone to protein mole ratio), free and bound peaks are observed. The spectra indicate that the hormone is in slow exchange under all conditions studied and that a 1:1 stoichiometry obtains for the oxytocin–neurophysin complex. An upper limit for the overall dissociation rate of about 2 s^{-1} can be estimated from NMR data leading to an upper limit of about 2×10^5 M^{-1} s^{-1} for the association rate. This small rate constant and the substantial chemical shifts associated with the binding process suggest that a substantial conformational change occurs at the tyrosine-2 position when the hormones bind to neurophysins.

Considerable information has been obtained in recent years on the interaction of the neurohypophyseal hormones, oxytocin and arginine (or lysine) vasopressin (AVP or LVP),[1] with a group of related proteins, called neurophysins, found in neurosecretory granules of the posterior lobe of the pituitary gland. From studies with both nonapeptide and tripeptide analogues and derivatives of oxytocin, Breslow and co-workers (Breslow, 1974; Breslow et al., 1971, 1973) found evidence that the three N-terminal residues of the hormones, ½-Cys-1, Tyr-2, and Ile-3 (Phe-3 in AVP and LVP), are critical for binding. Significant support for this hypothesis has come from various laboratories employing nuclear magnetic resonance (NMR) techniques (Glasel et al., 1973; Hruby et al., 1977b; Griffin et al., 1977; Alazard et al., 1974; Blumenstein et al., 1977; Balaram et al., 1973).

An area of disagreement about the oxytocin–NP interaction concerns the dissociation rate constant of the hormone–protein complex. The determination of this rate constant is important for several reasons. This rate is related to the strength of the protein–hormone complex. Also, a knowledge of this rate, as well as the equilibrium constant for interaction, allows a calculation of the overall association rate constant, which reflects the ease (or difficulty) of complex formation. In addition, the exchange rate is of great experimental significance in NMR studies, since a knowledge of this rate, or at least limits on its values, is often necessary for proper interpretation of NMR data. Our previous work (Blumenstein and Hruby, 1977) has

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[1] Standard abbreviations and nomenclature for amino acids, peptides, and peptide derivatives are used throughout. Amino acids, except glycine, are of the L configuration unless otherwise stated. Other abbreviations used include: AVP, arginine vasopressin; LVP, lysine vasopressin; NP, bovine neurophysin; NPI, bovine neurophysin I; NPII, bovine neurophysin II; NMR, nuclear magnetic resonance; Boc, *tert*-butyloxycarbonyl; DCC, dicyclohexylcarbodiimide; TLC, thin-layer chromatography.

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