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Nuclear Magnetic Resonance Determination of Metal–Proton Distances in a Synthetic Calcium Binding Site of Rabbit Skeletal Troponin C†

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ABSTRACT: The binding of gadolinium to a synthetic peptide of 13 amino acid residues representing the calcium binding loop of site 3 of rabbit skeletal troponin C [AcSTnC(103-115)amide] has been studied by using proton nuclear magnetic resonance (^{1}H NMR) spectroscopy. In particular, the proton line broadening and enhanced spin-lattice relaxation have been used to determine proton-metal ion distances for several assigned nuclei in the peptide–metal ion complex. These distances have been used in conjunction with other constraints and a distance algorithm procedure to demonstrate that the structure of the peptide–metal complex as shown by ^{1}H NMR is consistent with the structure of the EF calcium binding loop in the X-ray structure of parvalbumin but that the available ^{1}H NMR distances do not uniquely define the solution structure.

Calcium has been implicated in varous cellular functions [see reviews by Kretsinger (1979, 1980), Wang & Waisman (1979), and Means et al. (1982)] through its binding to calmodulin (Cheung, 1970; Kakiuchi & Yamazaki, 1970) and troponin C (Ebashi et al., 1968). These two proteins and several others including parvalbumin (Pechère et al., 1971; Benzonana et al., 1972; Nockolds et al., 1972; Coffee & Bradshaw, 1973), S-100 (Isobe & Okuyama, 1978, 1981; Mani et al., 1982), the myosin light chains (Frank & Weeds, 1974; Jakes et al., 1976; Collins, 1976; Kendrick-Jones & Jakes, 1977; Chantler & Szent-Gyorgyi, 1978), and the intestinal calcium binding proteins (Hofmann et al., 1979; Fullmer & Wasserman, 1981) possess two to four EF hand[1] domains (Kretsinger & Nockolds, 1973). These regions are about 30-35 amino acids long and are composed of two α-helical segments flanking a calcium binding loop (Gariépy & Hodges, 1983).

We have been involved in the "molecular dissection" of a model EF hand domain using synthetic analogues of site 3 of rabbit skeletal troponin C (Reid et al., 1980, 1981; Gariépy

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[1] Abbreviations: AcSTnC(103-115)amide, synthetic N-terminal acetylated rabbit skeletal troponin C fragment, residues 103-115, with a C-terminal amide; EF hand, second calcium binding domain of carp parvalbumin; TnC, troponin C; DSS, sodium 4,4-dimethyl-4-silapentane-1-sulfonate; FID, free induction decay; ^{1}H NMR, proton nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid; HPLC, high-pressure liquid chromatography; M_r, molecular weight; 2D, two dimensional.

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et al., 1982). The importance of the N-terminal helix was confirmed by the demonstration that analogues lacking this region showed a thousandfold decrease in their ability to bind calcium (Reid et al., 1981). Reciprocally, a consequence of calcium binding to these sites is the formation of α-helical segments in both the N- and C-terminal regions (Reid et al., 1981; Gariépy et al., 1982). Removal of both helical regions reduces the calcium binding constant of the site to about 10^2 M^{-1}.

The lanthanides have been used as calcium analogues in structural studies of several EF hand containing proteins. The spectroscopic properties of Tb^{3+} and Eu^{3+}, for example, have been used to measure distances between calcium binding sites of carp parvalbumin (Rhee et al., 1981) and of troponin C (Wang et al., 1982a). In the case of carp parvalbumin, the paramagnetic properties of the lanthanide Yb^{3+} induce shifting and broadening of the NMR resonances of nuclei in proximity to the metal. These ytterbium-induced spectral perturbations can then be analyzed in terms of the structure of the metal binding site (Lee & Sykes, 1979, 1980a,b, 1981, 1982, 1983). A key feature of the lanthanides is their stronger affinity for EF domains than calcium, as in the case of Eu^{3+} and Tb^{3+} binding to rabbit skeletal troponin C (Potter & Gergely, 1975; Wang et al., 1981) and Yb^{3+} binding to parvalbumin (Lee & Sykes, 1981). Thus, the binding ability of a short 13-residue fragment representing the calcium binding loop of site 3 of rabbit skeletal troponin C [AcSTnC(103–115)amide] can be enhanced by the use of lanthanides. These metals demonstrate binding affinities to this peptide of the order of 10^4–10^5 M^{-1} (Gariépy et al., 1983). In this paper, we report on the use of the ^1H NMR relaxation probe gadolinium to generate metal–proton distances in the metal–AcSTnC(103–115)amide peptide complex and discuss the use of these distances along with other constraints to predict the structure of the complex in solution.

Theory

When a diamagnetic lanthanide such as lanthanum or lutetium is bound to a peptide, it can alter the relaxation times T_1 and T_2 of nuclei in the peptide. If the paramagnetic lanthanide gadolinium is used in the place of La^{3+} or Lu^{3+}, there will be an additional large contribution to the relaxation rates from the unpaired 4f electrons on the metal ion. It is this difference in relaxation rates between La^{3+} and Gd^{3+} that we wish to extract and analyze in terms of the geometry of the complex.

The Solomon–Bloembergen equations (Solomon, 1955; Bloembergen, 1957a,b) relate the observed increase in relaxation rates of a nucleus in the metal–peptide complex caused by the unpaired electrons on gadolinium to the distance between the nucleus and the metal:

$$\frac{1}{T_1m} = \frac{2\gamma^2\beta^2g^2J(J+1)}{15r^6}\left(\frac{3\tau_c}{1+\omega_I^2\tau_c^2}+\frac{7\tau_c}{1+\omega_s^2\tau_c^2}\right)+\frac{2J(J+1)}{3}(A/\hbar)^2\left(\frac{\tau_e}{1+\omega_s^2\tau_e^2}\right) \quad (1)$$

$$\frac{1}{T_2m} = \frac{\gamma^2\beta^2g^2J(J+1)}{15r^6}\left(4\tau_c+\frac{3\tau_c}{1+\omega_I^2\tau_c^2}+\frac{13\tau_c}{1+\omega_s^2\tau_c^2}\right)+\frac{J(J+1)}{3}(A/\hbar)^2\left(\tau_e+\frac{\tau_e}{1+\omega_s^2\tau_e^2}\right) \quad (2)$$

where ω_I and ω_s are the nuclear and electronic Larmor precession frequencies, g is the Landé g factor, $A/\hbar$ is the scalar electron–nuclear hyperfine interaction constant, J is the total angular momentum for the Gd^{3+} ion ($^7/_2$), β is the Bohr magneton, γ is the nuclear magnetogyric ratio, r is the metal to nucleus distance, and τ_c and τ_e are the correlation times for the dipolar interaction and the contact interaction, respectively.

Two simplifications reduce the apparent complexity of these two expressions: (1) The term ω_s is much greater than ω_I; (2) the scalar terms in both expressions are assumed to be insignificant for gadolinium as demonstrated by temperature variation studies (Bernheim et al., 1959; Dwek et al., 1971; Reuben et al., 1971). The quantity $[\gamma^2\beta^2g^2J(J+1)]/15$ has a value of 2.583×10^{17} s^{-2} $Å^6$ (Marinetti et al., 1976), so that the two expressions can now be rewritten in terms of only two unknowns, τ_c and r:

$$\frac{1}{T_1m} = \frac{5.166 \times 10^{17}}{r^6}\left(\frac{3\tau_c}{1+\omega_I^2\tau_c^2}\right) \quad (3)$$

$$\frac{1}{T_2m} = \frac{2.583 \times 10^{17}}{r^6}\left(4\tau_c+\frac{3\tau_c}{1+\omega_I^2\tau_c^2}\right) \quad (4)$$

and the ratio of the relaxation times is given by the expression

$$\frac{T_1m}{T_2m} = \frac{7}{6}+\frac{4\omega_I^2\tau_c^2}{6} \quad (5)$$

Given that the quantities T_1m and T_2m can be obtained for a selected number of proton resonances from gadolinium relaxation experiments, one can determine the correlation time τ_c of the dipolar interaction from eq 5. This latter value can then be used to solve eq 3 or 4 for the metal to proton distance involved for each nucleus studied.

The quantities $1/(T_1m)$ and $1/(T_2m)$ are obtained in this case under the assumption that the metal binding to the peptide is in the NMR fast exchange limit. In this limit, the relaxation rate $1/T_2$, for example, for peptide nuclei in the peptide–La^{3+} solution is given by

$$\frac{1}{T_2(La^{3+})} = \frac{[P]}{[P]_0}\left(\frac{1}{T_{2P}}\right)+\frac{[PM]}{[P]_0}\left(\frac{1}{T_{2PM}}\right) \quad (6)$$

where T_{2P} and T_{2PM} are the spin–spin relaxation times for the free peptide and the La^{3+}–peptide complex, respectively, and $[P]_0$ is the total peptide concentration.

In the presence of a very small amount of gadolinium such that the fractional saturation of the peptide is unchanged, and with the additional assumptions that the binding constants for La^{3+} and Gd^{3+} are equal, that the complexes are isostructural, and that exchange is fast enough that the NMR spectrum is still in the fast exchange limit for Gd^{3+}, the relaxation rate $1/T_2$ becomes

$$\frac{1}{T_2(La^{3+}+Gd^{3+})} = \frac{[P]}{[P]_0}\left(\frac{1}{T_{2P}}\right)+\frac{[PM]}{[P]_0}\left(\frac{1}{T_{2PM}}\right)+\frac{[PM]}{[P]_0}\left(\frac{[Gd]_0}{[Gd]_0+[Ln]_0}\right)\frac{1}{T_{2M}} \quad (7)$$

$1/(T_2m)$ can then be obtained from the slope of a plot of $1/[T_2(La^{3+}+Gd^{3+})]$ vs. percent peptide bound to Gd^{3+} and similarly for $1/(T_1m)$.

Materials and Methods

Materials. The solid phase peptide synthesis and HPLC purification of AcSTnC(103–115)amide were described earlier (Gariépy et al., 1983). The purity of the synthetic fragment

was confirmed by its elution as a single peak off HPLC ion-exchange (SynChropak AX300, Linden, IN) and reversed-phase (SynChropak RP-P, Linden, IN) analytical columns. The peptide composition was verified by amino acid analysis. Gadolinium chloride and lanthanum chloride were purchased from Alfa Inorganics-Ventron (Beverly, MA).

Metal Ion Analysis. The lanthanum and gadolinium solutions were made up in 100 mM KCl and 0.1 mM DSS and adjusted to pH 6.0 with NaOD or DCl. The metal ion content of these solutions was determined by EDTA titration using xylenol orange as the end-point indicator (Lee & Sykes, 1980).

Preparation of ^1H NMR Samples. The gadolinium titration was performed on a 0.8 mM sample of AcSTnC(103–115)-amide dissolved in 100 mM KCl, 0.1 mM DSS, and 1.3 mM $LaCl_3$ and readjusted to pH 6.0 with a 0.5 N NaOD solution. The sample was titrated by adding aliquots of a 1 mM Gd^{3+} solution to the NMR tube. Note that since most protons are within 10 Å of the metal, only trace amounts of gadolinium (≤5% of the peptide concentration) are required to excessively broaden the observed resonances. It was thus necessary to add an excess amount of lanthanum to the NMR tube to ensure that the small concentrations of Gd^{3+} present in the sample did not fluctuate due to the known interaction of such metals with glass matrices (Jones et al., 1974; Marinetti et al., 1976). The exact peptide concentration was determined by amino acid analysis.

At the initial lanthanum and AcSTnC(103–115)amide concentrations of 1.27 and 0.77 mM, respectively, 98% of the peptide can be calculated to exist in the bound state by using a La^{3+} binding constant of 1.1×10^5 M^{-1} (Gariépy et al., 1983). The percent peptide bound to gadolinium was calculated by using the following relationship:

$$\% \ Gd^{3+} \text{ bound} = \frac{98[Gd^{3+}]_0}{[Gd^{3+}]_0 + [La^{3+}]_0} \quad (8)$$

The terms $[Gd^{3+}]_0$ and $[La^{3+}]_0$ indicate the total concentrations of gadolinium and lanthanum in the NMR tube. This expression assumes that Gd^{3+} and La^{3+} have the same affinity for the peptide and that the amount of Gd^{3+} added is small so that the fractional saturation of peptide by metal is unchanged.

^1H NMR Experiments. The proton NMR spectra for the gadolinium titration experiments were obtained on a Bruker HXS 270-MHz spectrometer operating in a Fourier transform mode and equipped for quadrature detection. The spin–lattice relaxation times (T_1) of seven well-defined proton resonances (Gly-108 doublets at 3.53 and 4.00 ppm, Tyr-109 doublets at 7.03 and 6.82 ppm, Ala-106 and -112 doublets at 1.47 and 1.32 ppm, and an acetyl Asp-103 singlet at 1.95 ppm) were determined from progressive saturation experiments. Typically, the total delay time between spectral acquisitions was varied from 0.21 to 4 s, and 300 free induction decays (FID) were collected for each delay time, using a 9.5-μs (90°) pulse width, a 0.2-s acquisition time, a ±2500-Hz sweep width, and a line broadening value of 1 Hz. Peak heights of the resonances were measured and fit as a function of the total delay time between pulses to the equation

$$M_z = M_0(1 - e^{-t/T_1}) \quad (9)$$

where t is the total delay time, M_0 and M are the maximum and observed resonance peak heights, respectively, and T_1 is the spin–lattice relaxation time for the proton nucleus investigated. The spin–spin relaxation times (T_2) of six well-defined resonance patterns were determined by using a line-shape analysis program to determine $\Delta\nu$ and the relationship

$$\pi\Delta\nu = 1/T_2 \quad (10)$$

where $\Delta\nu$ represents the line width (in hertz) of a particular resonance line at half-height. Spectra used for the line-width measurements were obtained from 4000 acquisitions by using a 0.5-s acquisition time, an 8-μs pulse width, a ±2000-Hz sweep width, and a line-broadening value of 1 Hz.

Spin–lattice relaxation time measurements and two-dimensional ^1H NMR spectra were also obtained at 300 MHz on a Nicolet NT-300WB spectrometer. Spin–lattice relaxation time measurements were made by using a 180°–τ–90° inversion recovery pulse sequence fit to the equation $M = M_0[1 - (1 - \cos\alpha)e^{-t/T_1}]$, where α is the effective flip angle. 2D J-resolved spectra were obtained with the pulse sequence $(90°–t_1/2–180°–t_1/2–t_2)_n$ where t_1 is the evolution period and t_2 is the observation period. The final spectra were the result of 128 blocks of 8K spectra, with resulting digital resolution of 0.4 Hz in the J dimension and 0.6 Hz in the ω_2 direction. The resolution on the final spectrum was improved by Lorentzian to Gaussian transformation in both dimensions. 2D COSY spectra were obtained with the pulse sequence $(90°–t_1–90°–t_2)_n$. The resulting spectra contained 512 points in both dimensions, covering a sweep width of ±1200 Hz. A total of 200 scans were taken for each t_1 value, and the resolution in the final spectra was improved by use of a Lorentzian to Gaussian transformation in both dimensions.

Propagation of Error in the Distance Measurements. The uncertainty surrounding the T_2m estimates is the result of the indeterminate error associated with the slope measurements from $1/T_2$ vs. percent Gd^{3+} bound plots. A 90% confidence interval was thus calculated for each T_2m value determinated, taking in account the standard deviation associated with each linear regression and the number of data points used to construct each plot (Hoel, 1971). A similar approach for the measurement of the uncertainty associated with T_1m values was difficult because of the small number of data points involved in these plots (reduced degree of freedom in the Student's t test distribution) and the large standard deviation associated with the regression analysis of $1/T_1$ vs. percent Gd^{3+} bound as compared to the plots involving T_2 values.

The standard deviation obtained from averaging the observed $T_1m/(T_2m)$ values was used to estimate the error surrounding the averaged correlation time value. The uncertainty associated with distance measurements was then calculated from the errors surrounding the T_2m and τ_c estimates. The distances calculated from T_1m values represent gross estimates having an uncertainty of more than 1 Å.

Distance Geometry Calculations. We used the programs described by Crippen (1981) and Havel et al. (1983) to calculate three-dimensional coordinates for the peptide subject to the distance constants from NMR. Conventional bond length–bond angle data were obtained from P. A. Kollman (private communication). We used the TEMPLATE program [written by Weiner et al. (1983)] for initial entry of the peptide connectivity and for the distance constraints. Calculations were performed on the VAX 11/750 computer in the UCSF Computer Graphics Laboratory.

RESULTS

Assignment of Resonances. Figure 1 presents the ^1H NMR spectrum of the peptide in the presence (B) and absence (A) of La^{3+}. Assignments in the La^{3+}–peptide complex were made by using 2D J-resolved and 2D COSY spectra. The assignments are listed in Table I. The assignments relevant to this work are indicated above the spectrum of the La^{3+}–peptide complex (Figure 1B). The presence of lanthanum permits the

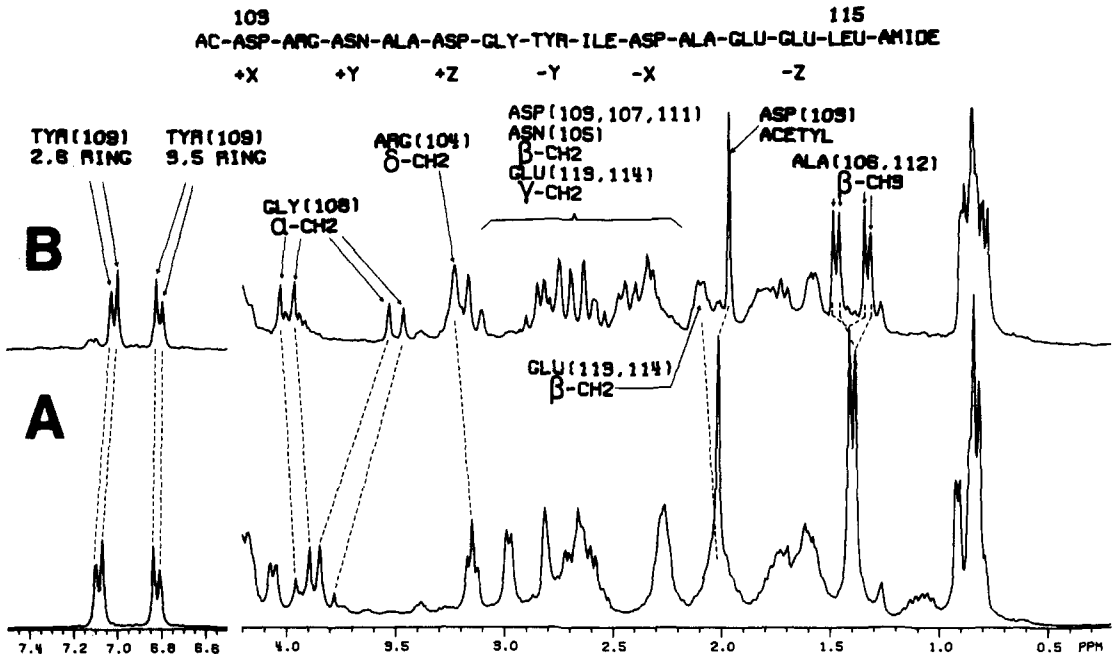

FIGURE 1: Assignment of AcSTnC(103–115)amide ^{1}H NMR resonances. [Peptide] = 0.77 mM in 100 mM KCl and 0.1 mM DSS, pH 6.0. (A) ^{1}H NMR spectrum of the apopeptide. (B) ^{1}H NMR spectrum of the peptide in the presence of 1.27 mM $LaCl_3$. The primary sequence of the synthetic fragment is listed above spectrum B. The letters X, Y, Z, –Y, –X, and –Z represent the calcium coordinating ligands of the peptide.

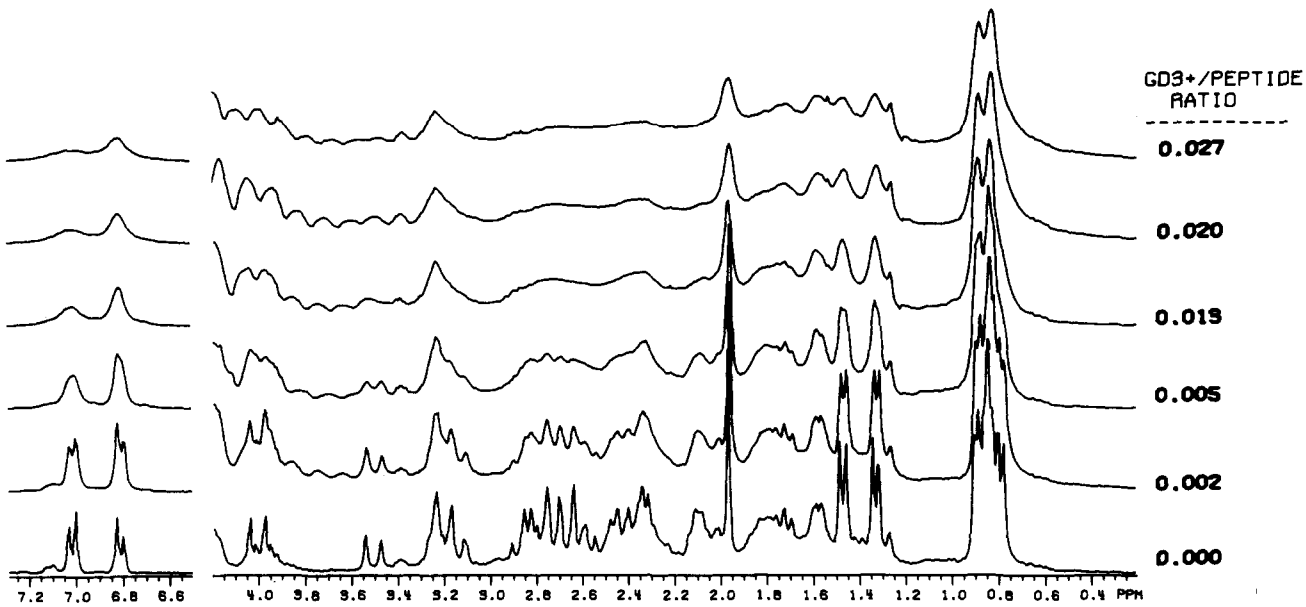

FIGURE 2: Gadolinium titration of AcSTnC(103–115)amide in the presence of excess lanthanum. [Peptide] = 0.77 mM in 100 mM KCl, 0.1 mM DSS, and 1.27 mM $LaCl_3$, pH 6.0. The Gd^{3+}/peptide ratio represents the ratio of gadolinium bound to the peptide following eq 8. Experimental parameters and precautions used are described under Materials and Methods.

resolution of the β-CH_3 doublets of alanine-106 and -112 (1.32 and 1.47 ppm regions), the acetyl group singlet of Asp-106 (1.95 ppm), the α-CH_2 doublets of Gly-108 (3.53 and 4.00 ppm, respectively), the δ-CH_2 protons of Arg-104 (the broad singlet at 3.23 ppm), and the ring proton doublets of Tyr-109 (2,6 protons, 7.03 ppm; 3,5 protons, 6.82 ppm) and also permits an improved definition of β-CH_2 and γ-CH_2 resonances associated with the aspartic acid, asparagine, and glutamic acid side chains ($\approx$2.1–2.9 ppm envelope).

Gadolinium Titration. The Gd^{3+} titration of the La^{3+}–peptide solution is presented in Figure 2 and illustrates the broadening effect induced by gadolinium on the proton resonances of AcSTnC(103–115)amide. The changes in resonance line width due to the presence of this paramagnetic metal can be related to the metal–proton distance involved (see eq 2, 7, and 10). Since the β-CH_2 protons of aspartic acids-103, -107 and -111, of asparagine-105, and of glutamic acid-114 (2.2–2.9 ppm region) are parts of side chains involved in metal chelation (Figure 1, sequence), these particular protons should lie closer to the metal than the methyl groups of alanine-106 and -112 (doublets centered at 1.32 and 1.47 ppm), the acetyl group of aspartic acid-103 (singlet at 1.95 ppm), or the tyrosine ring protons (doublets situated at 6.82 and 7.03 ppm). This assumption is verified readily by the observation of the greater broadening of the resonances associated with the coordinating side chains as compared to other proton resonances seen in Figure 2.

The spin–lattice relaxation times (T_1) associated with individual resonances shown in Figure 1 were determined at each addition of gadolinium to the NMR tube. The measured $1/T_1$

Table I: Assignment of ^1H NMR Resonances in the La^{3+}–AcSTnC(103–115)amide Complex

chemical shift (ppm)	assigned resonance	chemical shift (ppm)	assigned resonance
0.78	γ-CH_3 Ile-110	3.53	α-CH_2 Gly-108
0.83	δ-CH_3 Ile-110	3.77	α-CH Asx a
0.92	δ-CH_3 Leu-115	3.97	α-CH Ala I
1.29	γ-CH_2 Ile-110	4.00	α-CH Gly-108
1.32	β-CH_3 Ala I	4.04	α-CH Ala II
1.47	β-CH_3 Ala II	4.20	α-CH Glu B
1.60	γ-CH_2 Ile-110	4.22	α-CH Ile-110
1.61	γ-CH Leu-115	4.27	α-CH Glu A
1.66	β-CH_2 Leu-115	4.48	α-CH Tyr-109
1.76	β-CH Ile-110	4.48	α-CH Asx b
		4.48	α-CH Asx c
1.84	γ-CH_2 Arg-104	4.48	α-CH Arg-104
1.88	β-CH_2 Arg-104		
1.95	Ac Asp-103		
2.10	β-CH_2 Glu A		
2.32	β-CH_2 Glu B	6.82	3,5 Tyr-109
2.56	β-CH_2 Asx b	7.03	2,6 Tyr-109
2.57	γ-CH_2 Glu A		
2.65	β-CH_2 Asx c		
2.70	β-CH_2 Tyr-109		
2.85	β-CH_2 Asx a		
2.92	γ-CH_2 Glu B		
3.17	β-CH_2 Tyr-109		
3.21	α-CH Leu-115		
3.23	δ-CH_2 Arg-104		

Table II: Relaxation Times[a] and Calculated Proton–Metal Distances[b]

proton species	T_1m (ms)	T_2m (ms)	$T_1m/(T_2m)$	r (Å)
Asp-103				
acetyl CH_3	1.0	0.43	2.32	8.2 ± 0.4
Ala-106, 112				
β-CH_3, 1.32 ppm	0.83	0.50	1.66	8.4 ± 0.3
β-CH_3, 1.47 ppm	0.60	0.44	1.36	8.3 ± 0.2
Tyr-109				
3,5 ring protons	0.56	0.38	1.47	8.0 ± 0.2
2,6 ring protons	0.23	0.19	1.21	7.2 ± 0.4
Gly-108				
α-CH, 3.53 ppm	0.80	0.15	5.33	6.9 ± 1.0
α-CH, 4.00 ppm	1.2			~9[c]
Arg-104				
δ-CH_2	1.6			~9[c]

[a] Determined by linear least-squares fitting of $1/T_1$ and $1/T_2$ values as a function of percent peptide bound to gadolinium. [b] Represent metal–proton distances calculated from the relaxation times obtained. [c] The metal–proton distances were estimated from eq 3 by using a τ_c value of 0.48 ns and the determined T_1m value.

values were then plotted as a function of the percent of the peptide bound to gadolinium, and a T_1m value at a 1 to 1 ratio of Gd^{3+} to peptide was extrapolated from a least-squares fit of the data. Similarly, $1/T_2$ values calculated from line-width measurements for the well-defined resonances were plotted as a function of the percent of peptide bound to Gd^{3+} and yielded a linear relation from which a value of T_2m at a 1 to 1 ratio of gadolinium to peptide could be extrapolated. Table II lists relaxation times calculated for the resonances assigned in Figure 1.

Note that in the determination of T_2m values the spectral line-broadening contribution of 1 Hz was subtracted from all line-width estimates, as described under Material and Methods. However, this factor and any other contributions affecting the initial line width should not influence the extrapolated T_2m value since it is the slope characterizing the linear relation between $1/T_2$ observed and the percent peptide bound to Gd^{3+} that dictates the final T_2m value calculated. Also, the increase in line width for a given resonance due to Gd^{3+} bound to the peptide will represent a direct T_2m measurement only in the case where the peptide exchange rate between its metal-free and metal-bound state is fast on the NMR time scale (i.e., fast exchange). We have assumed a fast exchange process between the bound and free states of the peptide in light of the following facts:

(1) Differential line broadening is observed for various resonances so that exchange cannot be in the slow exchange limit except for the closest nuclei.

(2) The $T_1m/(T_2m)$ ratio remains constant for a variety of resonances (Table I), except for the Gly-108 α-CH protons. However, if the value of the exchange lifetime were becoming significant for the closer Gly protons, the value of $T_1m/(T_2m)$ should be reduced and not increased. The deviation in the value $T_1m/(T_2m)$ for the Gly-108 α-CH proton (3.5 ppm) reflects experimental errors associated with the T_1m and T_2m values, especially the T_2m value since this resonance broadens the most and is difficult to measure.

(3) The largest frequency shift observed during the peptide–lanthanum titration was 0.315 ppm (Gly-108 α-CH; Gariépy et al., 1983). This implies that the lifetime of the metal-bound state is small compared to the reciprocal maximum observed frequency difference (<2 ms).

The major source of uncertainty in the determination of T_1m and T_2m values is associated with the measurement of slopes from $1/T$ vs. percent Gd^{3+} bound plots. For example, a slope calculated from a regression analysis of three or four points as in the case for the T_1 extrapolation is less reliable than that from an analysis involving 8–10 data points as in the case of a T_2 measurement.

Calculation of Metal–Proton Distances. An average $T_1m/(T_2m)$ value of 1.6 ± 0.4 was determined from the summation of individual values listed in Table II. The value of 5.33 obtained for the Gly-108 3.5 ppm resonance was dropped from the computation of this average $T_1m/(T_2m)$ term on the basis of its statistical significance (Dean & Dixon, 1951). A correlation time (τ_c) value of 0.48 ± 0.06 ns was then computed from eq 5 and is in good agreement with a value of 0.6 ns [$(M_r \times 10^{-12})/2.4$ s for $M_r \sim 1500$; Cantor & Schimmel, 1980] predicted for our peptide. Note that the correlation time τ_c is related to τ_M (the lifetime of a nucleus in the bound state), τ_s (the electron spin relaxation time), and τ_R (the rotational correlation time of the Gd^{3+}–peptide complex) by the equation

$$\frac{1}{\tau_c} = \frac{1}{\tau_M} + \frac{1}{\tau_s} + \frac{1}{\tau_R}$$

Since $\tau_s >> \tau_R$ in the case of Gd^{3+} and Eu^{2+} (Nieboer, 1975) and τ_M is large in comparison to τ_R ($\sim 10^4$–10^6 times), the correlation time value reflects the shortest term of the equation, i.e., τ_R.

Metal–proton distances were calculated from eq 4 by using T_2m values. When such a value was not available, the distance was estimated by using eq 3 and the corresponding T_1m value. The calculated distances are listed in Table II.

Distance Geometry Results. We tried several sets of constraints. All included the bond length, bond angle, and chirality information from standard peptide parameters and the NMR-derived distances. If no other data were used, the distance geometry program returned quite variable collapsed structures consistent with an underdetermined data set. We next explored how well the NMR distances fit the parvalbumin crystal geometry. With backbone distances from the X-ray result, the distance geometry program readily fit the NMR results. We conclude that at this stage the NMR data are consistent with the crystallographic model.

Discussion

Our interest has focused on the use of lanthanides to study the structural requirements and folding constraints in calcium binding proteins such as troponin C. One approach is to investigate the geometry of a single calcium binding site in solution in the presence of lanthanide ions. We have synthesized a 13-residue fragment representing site 3 of rabbit skeletal troponin C by solid phase peptide synthesis and made use of ^{1}H NMR techniques to demonstrate the metal ion specificity of this site (Gariépy et al., 1983). Our results have indicated the importance of both the metal charge and radius on the metal binding affinity of the site and showed that the conformation adopted by this synthetic peptide is a function of the metal used (Gariépy et al., 1982, 1983). In this report, we have attempted to reconstruct the conformation adopted by this synthetic fragment in the presence of lanthanides using gadolinium broadening measurements obtained for a selected number of proton resonances.

In the ^{1}H NMR spectrum of the La^{3+}–AcSTnC(103–115)amide complex presented in Figure 1, the peptide adopts a conformation where an improved resolution of key resonances is observed (i.e., β-CH_3 of alanine-106 and -112, β-CH_2 of glutamic acid-113 and -114). The addition of the relaxation probe gadolinium to the lanthanum-bound peptide broadens the observed resonances. Note that regions of the peptide spectrum undergo broadening at different rates. One can qualitatively conclude from this titration series that the β-CH_2 groups of the aspartic acids, asparagine, glutamic acids, and tyrosine residues as well as the γ-CH_2 groups of glutamic acids broaden faster than the β-CH_3 groups of alanine-106 and -112, for example, and are thus closer to the metal than these latter methyl groups. Their proximity to the metal correlates with their roles as calcium ligands.

The first assumption in the use of lanthanides as calcium substitutes is that their binding to these EF domains is analogous to calcium. This assumption may not be necessarily true since lanthanides prefer to form complexes having a coordination number of 8–9 rather than 6 as in the case of calcium (Nieboer, 1975; Einspahr & Bugg, 1977; dos Remedios, 1981). Variations in the geometry adopted by our synthetic peptide were observed by ^{1}H NMR in the case of lanthanum and lutetium additions (Gariépy et al., 1983). Also, Leavis et al. (1980) pointed out that the intrinsic fluorescence of tyrosine-109 in site 3 of rabbit skeletal troponin C is enhanced upon calcium and magnesium binding but remains unaffected in the case of terbium, lanthanum, neodymium, or holmium binding. These results were rationalized in terms of subtle differences in the tertiary structure of TnC induced by these ions. However, the ability of calmodulin to bind to troponin I or calcineurin and to stimulate phosphodiesterase activity is retained when terbium is used as a calcium analogue (Wallace et al., 1982). Further, no large structural changes were observed in the X-ray studies of thermolysin with a series of lanthanide ions substituting for Ca^{2+} (Matthews & Weaver, 1974), or in the NMR studies of parvalbumin with Yb^{3+} substituted for Ca^{2+} in the EF site (Lee & Sykes, 1983). One can thus conclude that variations in the protein geometry near the metal binding site do occur and are dependent on the properties of the cation present, but the overall structure of the protein remains comparable to the calcium-bound form.

The association constant of Gd^{3+} to the peptide was assumed comparable to the one calculated for La^{3+} ($K_{La^{3+}} = 1.1 \times 10^5$ M^{-1}; Gariépy et al., 1983). Added proof for this premise came from fluorescence measurements on a 12-residue-long synthetic fragment of site 2 of rabbit skeletal troponin C where the Tb^{3+} association constant was estimated to be (2–3) $\times 10^5$ M^{-1} (Kanellis et al., 1983).

Nonspecific binding is possible at the end of the gadolinium titration, where the concentration of free lanthanum is about 0.5 mM while the concentration of unbound gadolinium is less than 7 μM. Free carboxyl groups represent the major class of ligands that would bind La^{3+} or Gd^{3+} from this pool of unbound lanthanides. The presence of an amide group at the C-terminus of the peptide eliminates one source of carboxyl group. However, the side chain of glutamic acid-113 is not a calcium binding ligand, based on the crystal structure of carp parvalbumin (Kretsinger & Nockolds, 1973), and represents a possible source of free ligand. The concentration of this ligand at the end of the gadolinium titration was 0.74 mM. Using the binding constants of La^{3+} and Gd^{3+} to acetate ion ($K_{La^{3+}}$, 1.05×10^2 M^{-1}; $K_{Gd^{3+}}$, 1.46×10^2 M^{-1}; Kolat & Powell, 1962), where the acetate ion represents a model ligand for the side chain of glutamic acid-113, one can calculate that less than 5% of the available carboxyl ligand would exist in a lanthanum-bound state and less than 0.01% in a gadolinium-bound state. These results consequently demonstrate that nonspecific binding does not represent a significant factor in the gadolinium broadening experiments in terms of a contribution to the T_1m and T_2m estimates.

Distance Restrictions. In order to reconstruct the geometry of the peptide AcSTnC(103–115)amide in solution, one should establish all available distance restrictions imposed on the site. These restrictions fall into three categories: (1) One category is the set of distances calculated from the relaxation time measurements. (2) The rapid broadening of the β-CH_2 resonances of aspartic acids, asparagine, glutamic acids, and tyrosine residues as well as the γ-CH_2 resonances of glutamic acids suggests that their distances to the metal are less than 7 Å, which represents a lower limit on measured distances. (3) The oxygen atoms coordinating the lanthanide Gd^{3+} can be assumed to lie at a distance of 2.5 ± 0.3 Å from the metal as indicated from the crystal structure of a variety of lanthanide complexes (Alberston, 1968; Grenthe, 1969, 1971, 1972).

The NMR data presently available are unable to provide a unique structural determination for the peptide–metal ion complex. However, we have shown by computer calculation that the X-ray structure of parvalbumin forms a good starting point for understanding the NMR results. Of course, this calculation does not demonstrate the uniqueness of the proposed structure. This must await further experimental data.

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Registry No. AcSTnC(103–115)amide, 84648-71-5; La, 7439-91-0; Gd, 7440-54-2; Ca, 7440-70-2.

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