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Proton Nuclear Magnetic Resonance Determination of the Sequential Ytterbium Replacement of Calcium in Carp Parvalbumin†

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ABSTRACT: The substitution of the paramagnetic lanthanide ion ytterbium for the calcium ions bound to the CD and EF sites of carp parvalbumin results in a series of ^{1}H NMR resonances which are shifted far outside the envelope of the ^{1}H NMR spectrum of the diamagnetic form of the protein. Titrations of Ca^{2+}-saturated parvalbumin with ytterbium (Yb^{3+}) demonstrate that Yb^{3+} sequentially replaces the two bound calcium ions of the protein. Analysis of the ^{1}H NMR data yields the relative affinities of the two sites (CD and EF) for ytterbium with respect to calcium. The dissociation constants for ytterbium are then calculated to be $K_{Yb^{3+}}^{CD} = (4–7) \times 10^{-10}$ M and $K_{Yb^{3+}}^{EF} = (2–6) \times 10^{-10}$ M from the known dissociation constants for calcium [Haiech, J., Derancourt, J., Pechere, J.-F., & Demaille, J. G. (1979) *Biochemistry 18*, 2752–2758]. The approximate equality of these constants is verified by Yb^{3+} titrations of apoparvalbumin.

The X-ray crystallographic structure of the calcium binding protein parvalbumin from carp reveals that each of its two calcium binding sites is completely formed from a contiguous polypeptide sequence folded into the homologous CD and EF hands (Kretsinger & Nockolds, 1973). Each Ca^{2+} binding site contains two turns of helix, a 12-residue loop around the metal, and a second two-turn helix. The loop around the metal contains regularly spaced carboxyl, carbonyl, and hydroxyl ligands. Homologous sequences to parvalbumin (Kretsinger, 1976) can be found in a number of Ca^{2+} binding proteins such as rabbit skeletal troponin C and the myosin alkali light chains (Collins, 1974; Weeds & McLachlan, 1974), the 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB)[1] light chains (Collins, 1976), bovine calmodulin (Vanaman et al., 1977; Stevens et al., 1976), rat testis calmodulin (Dedman et al., 1978), bovine intestinal calcium binding protein (Fullmer & Wasserman, 1977), and the procine intestinal calcium binding protein (Hofmann et al., 1979). The number of times in a given protein the sequence repeats, and the amino acid substitutions therein, can be correlated with the number of metals bound to the protein and their binding strengths, respectively. These findings have led to the proposal that homologous structures, at least at the level of the calcium binding sites, exist for all these proteins.

We have described elsewhere the strategy and development of an NMR methodology whose final goal is the comparison of the structures of calcium binding proteins in solution (Lee et al., 1979; Lee & Sykes, 1980a–d). The technique will enable us to test the hypothesis that all of these calcium binding proteins have homologous structures. The method is based upon the fact that the interaction of calcium binding proteins such as parvalbumin with lanthanides such as ytterbium results in a series of ^{1}H NMR resonances shifted far outside the normal chemical shift range of the diamagnetic form of the protein. These shifted resonances are sensitive monitors of the behavior and geometry of the metal binding sites in solution. Our overall approach is to study carp parvalbumin initially and to use the known X-ray structure of this protein to determine the unknown parameters of the NMR experiment, which are required before the shifts and broadenings can be interpreted in terms of the structure of the protein. With these parameters, and the knowledge of the amino acid substitutions for different proteins, we will then be able to compare the calculated and observed NMR spectra of a new protein as a probe of its structure.

In this paper, we focus on the analysis of the stoichiometry of the binding of Yb^{3+} to carp parvalbumin (p*I* 4.25). We have chosen Yb^{3+} for our study primarily because the contact interaction is the smallest relative to the other lanthanides (Reuben, 1973). In addition, the ratio of the susceptibility line broadening to the pseudocontact shift is relatively small; this results in comparatively high resolution spectra for the shifted peaks (Lee & Sykes, 1980a).

Titrations of Ca^{2+}-saturated parvalbumin with ytterbium demonstrate that there is a sequential replacement of the two protein-bound calcium ions. Analysis of the data allows us to determine, for each of the two sites (CD and EF), the

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[1] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; DSS, sodium 4,4-dimethyl-4-silapentane-1-sulfonate; DTT, dithiothreitol; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine; Hepes, *N*-2-(hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid.

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relative affinities for ytterbium with respect to calcium. The affinities of the two sites for ytterbium can then be calculated from the previously measured calcium affinities.

The problem is that conflicting results exist for the binding of Ca^{2+} to parvalbumin, both qualitatively and quantitatively. The early studies of Benzonana et al. (1972) suggested that the two calcium binding sites of hake and frog parvalbumin have equal dissociation constants for calcium of 1×10^{-7} M. However, these studies were performed in the presence of competing Mg^{2+} ions, and later studies have calculated the dissociation constants to be approximately 2 orders of magnitude smaller (Potter et al., 1977). In addition, several reports suggest that the binding of calcium is cooperative (Benzonana et al., 1972; Cave et al., 1979a) whereas others contradict this hypothesis (Donato & Martin, 1974; Nelson et al., 1977; Sowadski et al., 1978). Recently, Haiech and co-workers (Haiech et al., 1979) have reported that the two calcium sites of hake parvalbumin have different affinities for calcium. We show that the cooperativity vs. independent sites question is not relevant under our experimental protocol, that our experimental results support the conclusions of Haiech et al. (1979), and that the calculated ytterbium dissociation constants for the CD and EF binding sites of carp parvalbumin are $K_{Yb^{3+}}{}^{CD} = (4\text{–}7) \times 10^{-10}$ M and $K_{Yb^{3+}}{}^{EF} = (2\text{–}6) \times 10^{-10}$ M. The approximate equality of these constants is verified by Yb^{3+} titrations of apoparvalbumin. Our results are compared with literature resuls for the binding of other lanthanides (Sowadski et al., 1978; Cave et al., 1979b).

Materials and Methods

NMR Procedures. ^1H NMR spectra were obtained on a Bruker HXS-270 spectrometer operating in the Fourier-transform mode with quadrature detection. Typical instrumental settings for the spectra were acquisition time 0.2 s, sweep width ±10 kHz, spectrum size 8192 data points, and line broadening 5 Hz. No HDO homonuclear decoupling was employed. The sample temperature was 303 K. The chemical shifts were measured relative to the principal resonance of sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) as an internal standard. The standard filters on the HXS-270 spectrometer were replaced with Bessel filters (Ithaco Model 4302). Spectra were taken with a filter width set equal to twice the spectral width, which results in minimum base-line distortion. The data were accumulated with NMR recycle times long relative to the spin–lattice relaxation times (Lee & Sykes, 1980c); therefore, the observed areas represent true intensities. The areas of the resonances were calculated as the product of the line width times the maximum line intensity which is proportional to the area for a Lorentzian line shape.

Sample Preparation. Stock ytterbium solutions were prepared from dried Johnson–Matthey ultrapure oxides as discussed earlier (Birnbaum & Sykes, 1978). The concentrations of Yb^{3+} were determined by titration with EDTA in pH 6 Mes [2-(*N*-morpholino)ethanesulfonic acid] buffer with xylenol orange as an indicator.

Carp parvalbumin (p*I* 4.25) was isolated by the method of Pechere et al. (1971). The purity and identity of this isotype were confirmed by slab gel electrophoresis, ultraviolet absorption spectra, and amino acid analysis. The protein so isolated was Ca^{2+} saturated as evidenced by comparison with the ^1H NMR spectrum of apo- and Ca^{2+}-saturated parvalbumin and by crystallization of the protein, since the apo form does not crystallize (Kretsinger & Nockolds, 1973).

The NMR samples for the Ca^{2+}-saturated protein titrations were prepared by dissolving the lyophilized protein in D_2O buffer, 15 mM Pipes, 0.15 M KCl, 10 mM DTT, and 0.5 mM DSS, pH 6.6. Measurements of the pH were made with an Ingold microelectrode (model 6030-04) attached to a Beckman Expandomatic SS-2 or Radiometer 26 pH meter. The pH values quoted are those observed and are not corrected for the deuterium isotope effect on the glass electrode. Protein concentrations were determined by amino acid analysis after 24 h of acid hydrolysis at 110 °C. The Yb^{3+} titrations were performed by the addition of 5-μL aliquots of Yb^{3+}, prepared at the same pH as the protein sample, pH 6.6. The addition of Yb^{3+} did not affect the final pH of the resulting protein solution.

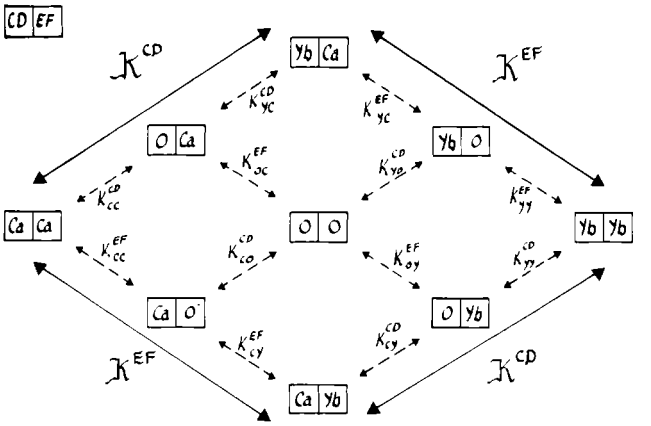

FIGURE 1: Outline of the mechanism for the binding of ytterbium and calcium to parvalbumin. The two squares represent the two metal binding sites of parvalbumin. The left square indicates the CD site, the right the EF site. Y refers to ytterbium; C refers to calcium. The superscripts of the association constants refer to the site being filled; the subscript refers to the final state of metal occupancy. An open circle indicates no metal present.

Demetalized parvalbumin was prepared in the following manner. A 11.3-mg aliquot of parvalbumin was dissolved in 1 mL of 15 mM Pipes buffer, pH 7.0, containing 50 mM EDTA. The solution was gently stirred for 1.5 h and then desalted on a Sephadex G-25 column (1 × 24 cm). The protein fraction was then dialyzed extensively against double-distilled water and lyophilized. The lyophilized protein was dissolved in Chelex-treated and dithizoned 15 mM Pipes, 0.15 M KCl, 15 mM DTT, and 0.5 mM DSS, in D_2O, pH 6.64. The protein was then shown to be apo, within the limits of NMR (≳90%), by comparison of its ^1H NMR spectrum with that of the protein in the presence of EDTA. The absence of NH resonances, in particular, in the ^1H NMR spectrum indicates a partially unfolded state of the protein, resulting from the loss of the bound calcium ions. This phenomenon has been reported elsewhere for another carp isozyme (Birdsall et al., 1979).

Analysis of Ytterbium Titration Data. The possible species present during the titration of calcium-saturated parvalbumin with ytterbium are shown in Figure 1, wherein parvalbumin is represented as two squares, with the right-hand square taken to represent the EF site and the left-hand square the CD site. The dissociation constants are written as K_{mn}^{i} where i = CD or EF and n, m = O, C, or Y for apo, Ca^{2+}, or Yb^{3+}, respectively. The superscript indicates which site the binding is referred to whereas the double subscript indicates the final product of the binding. Thus, the dissociation constant $K_{CY}{}^{CD}$ refers to the reaction

$$\square|Y + C \rightleftharpoons C|Y$$

We will assume that while the binding to a given site may depend upon whether or not there is a metal in the other site it does not depend upon which metal (Ca^{2+} or Yb^{3+}) is present

in the other site. Thus, for example, $K_{CY}^{EF} = K_{YY}^{EF}$. We further note that with the concentrations of metals and protein used (in the millimolar range) for the titration of the Ca^{2+}-saturated parvalbumin, and with the extremely small dissociation constants ($\sim 10^{-9}$ M for Ca^{2+} and $\sim 10^{-10}$ M for Yb^{3+}), there will be negligible concentrations of any of the apo or partially apo species. Thus, the relevant mechanism may be reduced to the four species connected by the solid lines. The relative concentrations of the four species are a function of only two constants, which are the relative affinities of the CD or EF site for Yb^{3+} with respect to Ca^{2+} (K^{CD} and K^{EF}). That is, for example:

$$K_{YC}^{EF} = \frac{[YO][C]}{[YC]} \qquad K_{YY}^{EF} = \frac{[YO][Y]}{[YY]}$$

therefore

$$\frac{[YC][Y]}{[YY][C]} = \frac{K_{YY}^{EF}}{K_{YC}^{EF}} \equiv K^{EF}$$

In this limit, any cooperativity, even if present, is not observable in the titration of the Ca^{2+}-saturated protein.

This simplified scheme was used to analyze the data in Figure 3 of this manuscript. The solid lines in Figure 3 were calculated on a NOVA computer with the only adjustable parameters being K^{CD} and K^{EF}, and the final areas of each of the three peaks analyzed. Thus, any two curves in Figure 3 determine the third within an arbitrary scaling parameter related to the relative areas of the peaks. Details are available as supplementary material.

Results

When a paramagnetic trivalent lanthanide (excluding the isotopic Gd^{3+}) is added to a protein–metal ion binding site, it will cause shifting and broadening of the NMR resonances of nearby nuclei. Since the rates of dissociation of the lanthanide ion from the protein are in the slow exchange limit on the NMR chemical shift time scale (Lee & Sykes, 1980a–d), the resulting shifted and broadened resonances will be observed separate from the resonances of the diamagnetic protein. When Yb^{3+} was added to Ca^{2+}-saturated parvalbumin, the sequential appearance and disappearance of several sets of shifted peaks were observed (Figure 2A,B). Up to a ratio of total metal to total protein (Yb_0/P_0) of $\sim$1:1, one set of peaks appears (Figure 2A), exemplified by the resonance indicated by a triangle in Figure 2A. As the second calcium is replaced at higher Yb_0/P_0 ratios, some of the first set of peaks such as the one indicated by a triangle are not affected. However, other resonances disappear (shifted elsewhere, see resonance labeled with a square) and are replaced by a new set of peaks. In addition, some peaks appear only after binding of the first metal, such as the resonance labeled with a circle. These results indicate immediately that Yb^{3+} sequentially replaces the two Ca^{2+} ions of parvalbumin.

The areas of three shifted resonances, selected to represent the various possible modes of behavior during titration as described above, were corrected for dilution effects and plotted as a function of total Yb^{3+} concentration to total protein concentration (Figure 3). The solid lines in Figure 3 represent the fit to the mechanism proposed in Figure 1, as described under Materials and Methods. The relative dissociation constants of each site for Yb^{3+} with respect to Ca^{2+} obtained from the fitting procedure were $K^{CD} = 0.13$ and $K^{EF} = 0.01$ (see Discussion for the assignment to CD and EF sites). Area measurements were also determined as part of the fitting procedure. The calculated areas of the resonances with observed chemical shifts of 14.55 and 15.17 ppm were 983 ± 57 and 804 ± 24, respectively, whereas the area of the resonance with an observed chemical shift of 29.80 ppm is 304 ± 13. The differences (when scaled to one proton) of the area of these three peaks (328, 268, and 304) represent the combined errors in the experimental measurements and the fitting procedure.

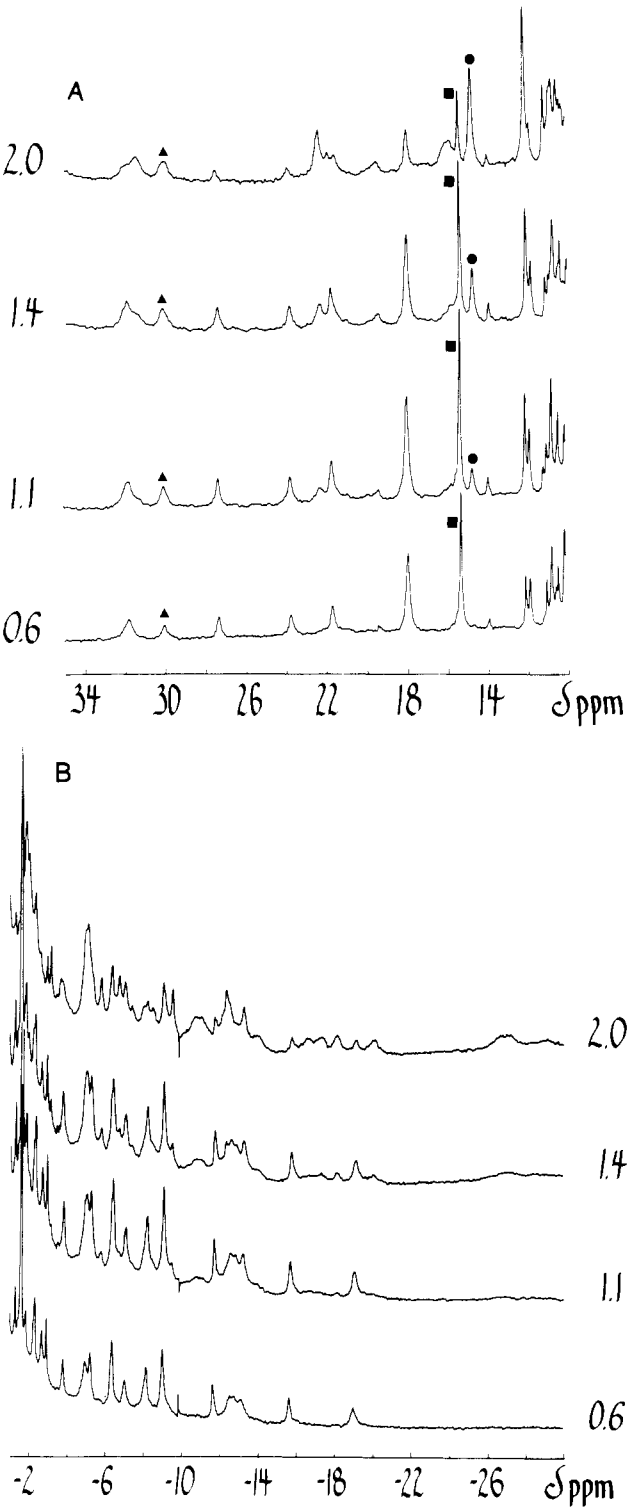

FIGURE 2: ^1H NMR spectrum (270 MHz) of 0.84 mM carp parvalbumin in 15 mM Pipes, 0.15 M KCl, 10 mM DTT, and 0.5 mM DSS in D_2O, pH 6.63, at total $Yb_0^{3+}/protein_0$ ratios of 0.6, 1.1, 1.4, and 2.0. Ambient temperature = 303 K. (A) Downfield region of spectrum; (■, ●, and ▲) peaks plotted in Figure 3. (B) Upfield region of spectrum; spike near −10 ppm is an instrumental artifact.

The titration of the partially apoparvalbumin with Yb^{3+} yielded the same final spectrum at a ratio of $Yb_0/P_0 \approx 2$–3

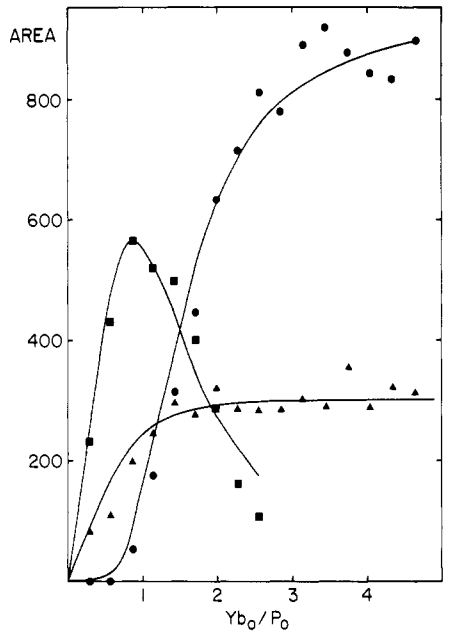

FIGURE 3: Areas of three representative peaks as a function of Yb_0^{3+}/protein$_0$ ratio; (▲, ■, and ●) resonances which have chemical shifts of 29.80, 15.17, and 14.55 ppm, respectively (see Figure 2A).

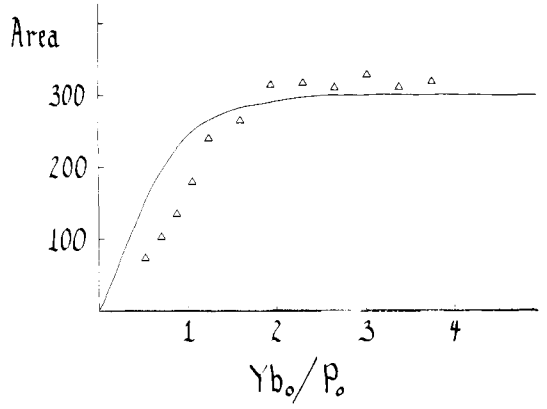

FIGURE 4: Open triangles indicate the area of the resonance in the Yb^{3+} titration of apoparvalbumin which corresponds to the resonance in the Yb^{3+} titration of Ca^{2+}-saturated parvalbumin at 29.80 ppm, which is indicated by filled triangles in Figures 2A and 3. The solid line is the fit from Figure 3 to the area of this resonance in the Yb^{3+} titration of the Ca^{2+}-saturated parvalbumin as a function of Yb_0/P_0. The concentration of apoparvalbumin was 0.85 mM; all other conditions were identical with Figures 2 and 3.

as the titration of the Ca^{2+}-saturated protein. However, some of the intermediate resonances observed were different, and the order of appearance of the resonances plotted in Figure 3 was different. In Figure 4, the appearance of the resonance at 29.80 ppm (represented by the filled triangle in the titration of the Ca^{2+}-saturated protein) is plotted as a function of Yb_0/P_0. It is clear that the appearance of this resonance in the titration of the apoprotein significantly lags behind its appearance in the titration of the Ca^{2+}-saturated protein, plateauing near ~2:1 for apo as opposed to near ~1:1 for the Ca^{2+}-saturated titration.

Discussion

We have outlined elsewhere the strategy for the complete analysis of the lanthanide-induced ^1H NMR chemical shifts of the ytterbium–parvalbumin complex in order to determine the structure of the EF metal binding site of carp parvalbumin in solution (Lee & Sykes, 1980a–d). In this paper, we focus on the stoichiometry of the binding of ytterbium to parvalbumin. Our ^1H NMR studies have enabled us to observe the sequential filling of the two metal binding sites by ytterbium, to characterize the proximity of the shifted resonances to the two metal binding sites, to measure their relative areas, and to determine the relative affinities of the two metal binding sites of parvalbumin for ytterbium. These studies do not, however, tell us which metal binding site of parvalbumin is filled first. We must draw on previous evidence such as the X-ray analysis (Sowadski et al., 1978) and optical studies (Donato & Martin, 1974; Horrocks & Sudnick, 1979) on the partially terbium-substituted protein. The X-ray crystallographic structure of parvalbumin indicates that the protein contains two metal binding sites. The CD site is surrounded by six protein ligands and is solvent inaccessible whereas the EF site is surrounded by five protein ligands and one water molecule. Three studies indicate that lanthanides preferentially bind to the EF site of calcium-saturated parvalbumin. Terbium fluorescence of the partially substituted protein (Donato & Martin, 1974; Nelson et al., 1977) is observed upon irradiation at 259 nm. Phe-57, which is the nearest aromatic ring to either Ca^{2+} ion, is closer to the EF site. Therefore, energy transfer occurs between Phe-57 and the Tb^{3+} bound in the EF site of parvalbumin. The laser-induced fluorescent decay constants of europium- and terbium-substituted parvalbumin (Horrocks & Sudnick, 1979) indicate that there is one water molecule bound to the lanthanide. Again, this evidence indicates that the EF site which contains one water molecule in the first coordination sphere is the site of initial lanthanide replacement. X-ray crystallographic studies of the parvalbumin (Moews & Kretsinger, 1975; Sowadski et al., 1978) indicate that the isomorphous replacement of Ca^{2+} by Tb^{3+} in parvalbumin at low Tb_0/P_0 ratios results in the increase of electron density at the EF site. Additional increase of electron density at the CD site is only observed upon subsequent increases in Tb_0/P_0 ratios. Replacement of both Ca^{2+} ions is observed; however, the EF site is initially occupied. ^{113}Cd NMR results (Drakenberg et al., 1978) also suggest that the lanthanide Gd^{3+} replaces the Cd^{2+} ions bound to the EF site first before the Cd^{2+} ions in the CD site. By analogy to these reports, we conclude that the Ca^{2+} bound in the EF site of parvalbumin is initially replaced by ytterbium.

When the Ca^{2+}-saturated protein was titrated with Yb^{3+}, the sequential appearance and disappearance of sets of shifted peaks were observed. Up to a total metal to total protein ratio of 1:1, one set of resonances, such as the resonance labeled with a triangle, is observed; these resonances correspond to nuclei in the vicinity of the first site filled, the EF site. At higher ratios, some of the first set of peaks, such as the peak labeled with a square, disappear (shift elsewhere) and were replaced by a new set of peaks; these resonances correspond to nuclei in the vicinity of both the EF and CD sites of parvalbumin. In addition, some peaks appeared only after binding of the first metal (see resonance labeled with a circle); these resonances correspond to nuclei either in the vicinity of the CD site only or under the influence of both sites. We are thus able to determine the approximate proximity of each resonance to each of the two metal binding sites.

The measurement of areas of the shifted resonances, obtained as part of the fitting procedure, allows us to discriminate single protons from methyl groups. For example, the resonances labeled with a circle and a square (Figure 2) have calculated areas of 983 ± 57 and 804 ± 24, respectively; the area of the resonance labeled with a triangle has a calculated value of 304 ± 13. These data indicate that the latter resonance corresponds to a single proton whereas the former

Table I: Metal Binding Properties of Parvalbumin

expt	parvalbumin (pI)	genetic lineage[a]	Ca^{2+} in the presence of Mg^{2+}		Ca^{2+} in the absence of Mg^{2+}		Mg^{2+}	other metals
			K_{d_1}[b]	K_{d_2}	K_{d_1}	K_{d_2}		
1	hake (4.36)	β	1.0×10^{-7}	1.0×10^{-7}	ND	ND	ND	ND
2	frog (4.88, 4.50)	(α, β)	1.0×10^{-7}	1.0×10^{-7}	ND	ND	ND	ND
3	carp (4.25)	β	ND[c]	ND	4.0×10^{-9}	4.0×10^{-9}	ND	ND
4	carp (4.25)	β	1.3×10^{-7}	1.3×10^{-7}	ND	ND	9.1×10^{-5}	ND
5	rabbit	α	$<10^{-6}$	$<10^{-6}$	ND	ND	ND	ND
			$<10^{-6}$	$<10^{-6}$	ND	ND	ND	ND
			4.0×10^{-7}	4.0×10^{-7}	1.0×10^{-9}	1.0×10^{-9}	4.3×10^{-6}	ND
6	carp (4.25)	β	ND	ND	4.0×10^{-9}	4.0×10^{-9}	6.7×10^{-5}	ND
7	carp (4.25)	β	ND	ND	ND	ND	ND	$K_{Gd}/K_{Cd} = 1/8$
8	carp (4.25)	β	ND	ND	ND	ND	ND	$K_{Tb}^{CD} = 6.3 \times 10^{-7}$[d]
9	carp (4.25)	β	ND	ND	ND	ND	ND	$K_{Tb}^{EF} = 1.6 \times 10^{-9}$[d]
10	whiting	β	ND	ND	1.4×10^{-6}	5.0×10^{-7}	ND	ND
11	rabbit (5.55)	α	ND	ND	6.6×10^{-9}	6.6×10^{-9}	1.6×10^{-5}	ND
12	frog (4.88)	α	ND	ND	7.8×10^{-9}	7.8×10^{-9}	2.1×10^{-5}	ND
13	frog (4.50)	β	ND	ND	2.2×10^{-9}	2.2×10^{-9}	2.7×10^{-5}	ND
14	hake (4.36)	β	ND	ND	$(3\text{–}5) \times 10^{-9}$	$>1.7 \times 10^{-8}$	3.0×10^{-5}	ND
15	chicken		ND	ND	$\leq 10^{-6}$	$\leq 10^{-6}$	ND	ND
16	carp (4.25)	β	ND	ND	ND	ND	ND	$K_{Gd} = 5.0 \times 10^{-11}$ $K_{Gd} = 5.0 \times 10^{-11}$ $K_{Mn} = 6.0 \times 10^{-4}$

expt	pH	reference	technique	conditions and comments for Ca^{2+} binding measurements
1	6.7	Benzonana et al. (1972)	^{45}Ca-Chelex partition	30 mM imidazole, 60 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA
2	6.7	Benzonana et al. (1972)	^{45}Ca-Chelex partition	
3	7.5	Potter et al. (1977)	equilibrium dialysis	20 mM imidazole, 0.1 M KCl, 0.1 mM EDTA
4	7.0	Potter et al. (1977)	equilibrium dialysis	20 mM imidazole, 0.1 M KCl, 0.1 mM EDTA, 3 mM $MgCl_2$
5	ND	Blum et al. (1977)	^{45}Ca-Chelex partition	
	6.7	Blum et al. (1977)	equilibrium dialysis	50 mM imidazole, 4 °C
	7.4	Cox et al. (1977)	equilibrium dialysis	25 mM Tricine, 80 mM KCl, 1 mM EDTA or EGTA
6	ND	Potter et al. (1978)	ND	ND
7	ND	Drakenberg et al. (1978)	^{113}Cd NMR	ND
8	6.5	Sowadski et al. (1978)	fluorescence	0.1 M KCl-piperazine
9	6.5	Sowadski et al. (1978)	fluorescence	0.1 M KCl-piperazine
10	8.0	White & Closset (1979)	fluorescence	50 mM Tris, 100 mM KCl, 20 °C
11	7.55	Haiech et al. (1979)	UV difference spectra	ND
12	7.55	Haiech et al. (1979)	UV difference spectra	ND
13	7.55	Haiech et al. (1979)	UV difference spectra	ND
14	6.8–8.0	Haiech et al. (1979)	UV difference spectra	0.1 M Hepes, 0.1 M KCl
15	6.7	Heizmann & Strehler (1979)	equilibrium dialysis	50 mM imidazole, 4 °C
16	6.0	Cave et al. (1979b)	proton relaxation enhancement	50 mM cacodylate, 25 °C

[a] The parvalbumins have been separated into the α and β lineages (Goodman & Pechere, 1977). [b] All dissociation constants are measured in molarity. [c] ND indicates the data are not determined or not defined. [d] A dissociation constant of 2×10^{-7} M (Benzonana et al., 1972) was assumed by Sowadski et al. (1978). However, this constant refers to the case where Mg^{2+} is present. Data in this table suggest that there is a decrease by 2 orders of magnitude in the dissociation constant when no Mg^{2+} is present. Since no Mg^{2+} was apparently present during their experiments, the published data of Sowadski et al. (1978) should be corrected by a factor of approximately 10^{-2}.

resonances correspond to methyl groups. We have used these two methyl groups as well as four other shifted methyl groups extensively in our procedure to relate the observed chemical shifts with the three-dimensional structure of the metal binding site (Lee & Sykes, 1980a–d).

The metal binding properties of parvalbumin have previously been investigated by numerous methods. Parvalbumin contains two high-affinity metal binding sites for which both Ca^{2+} and Mg^{2+} compete. In the presence of millimolar concentrations of Mg^{2+}, the dissociation constant of calcium for parvalbumin is 1×10^{-7} M (Benzonana et al., 1972). In the absence of Mg^{2+}, the dissociation constant decreases to 4×10^{-9} M (Potter et al., 1977). Table I lists previously published dissociation constants for a variety of parvalbumins.

Evidence to support both the cooperative and the noncooperative binding of Ca^{2+} to parvalbumin has been reported. Cooperative binding was first suggested (Benzonana et al., 1972) by the observation of a small curvature in Scatchard plots of $^{45}Ca^{2+}$ binding to calcium-free parvalbumins. The NMR observation that the spectrum of the protein with one Ca^{2+} bound contains features of the spectra from both the apoprotein and the fully calcium-bound protein (Parello et al., 1974; Cave et al., 1979a) further supports cooperative binding. However, the selective removal of one calcium from calcium-saturated parvalbumin (Donato & Martin, 1974; Nelson et al., 1977) is inconsistent with strong positive cooperativity.

In our titration of the calcium-saturated form of parvalbumin with Yb^{3+}, we are unable to address the question of cooperativity. Under our conditions of high metal and protein concentration, we will never have any of the apo or partially apo species present. We are able, therefore, to determine only the relative affinities of each of the two metal binding sites for ytterbium with respect to Ca^{2+}.

Haiech and co-workers (Haiech et al., 1979) have demonstrated that the CD and EF sites have different affinities for calcium. The two dissociation constants of hake parvalbumin are $(3\text{–}5) \times 10^{-9}$ M and $(17\text{–}60) \times 10^{-9}$ M. We will assume these same values for the homologous protein carp parvalbumin, another member of the β lineage of parvalbumin (Goodman & Pechere, 1977). We know that EGTA will

remove one Ca^{2+} preferentially from carp parvalbumin (Nelson et al., 1977). If we assume that calcium has the higher affinity for the CD site (Donato & Martin, 1974), then the calculated ytterbium dissociation constants (using our determined values of $K^{CD} = 0.13$ and $K^{EF} = 0.01$) are $K_{Yb^{3+}}{}^{CD} = (4–7) \times 10^{-10}$ M and $K_{Yb^{3+}}{}^{EF} = (2–6) \times 10^{-10}$ M. This indicates that the sequential Yb^{3+} replacement of the two Ca^{2+} ions from Ca^{2+}-saturated parvalbumin is controlled by the Ca^{2+} affinities and implies that the binding of Yb^{3+} to the two sites of parvalbumin, starting from the apo form, will be approximately equal. This is supported by the data for the resonance at 29.80 ppm as shown in Figure 4. We have argued above that this resonance is near to the EF site but unaffected by metals in the CD site. Its area is thus a measure of the sum of the concentrations of species CY + YY in the Ca^{2+}-saturated titration, or OY + YY in the apo titration. In the apo titration, the area of this resonance increases linearly up to a Yb_0/P_0 ratio of ~2:1 (Figure 4). This is consistent with equal affinities of the CD and EF sites for Yb^{3+} in the absence of Ca^{2+}.

The high affinities of the CD and EF sites of parvalbumin for Yb^{3+} ($K \sim 5 \times 10^{-10}$ M) are consistent with previously published results for Tb^{3+} and Gd^{3+}. Sowadski et al. (1978) calculate Tb^{3+} affinities of $K_{Tb^{3+}}{}^{CD} = 6.3 \times 10^{-7}$ M and $K_{Tb^{3+}}{}^{EF} = 1.6 \times 10^{-9}$ M from the fluorescence data of Nelson et al. (1977) by using the same model as that described herein. They assumed, however, Ca^{2+} dissociation constants from Benzonana et al. (1972) of 2×10^{-7} M, which were determined in the presence of 2 mM Mg^{2+}. The data collected in Table I indicate that the dissociation constants for Ca^{2+} are approximately 2 orders of magnitude smaller in the absence of Mg^{2+}. Since the data of Nelson et al. (1977) are in the absence of Mg^{2+}, we feel the calculated constants of Sowadski et al. (1978) are more accurately calculated as $K_{Tb^{3+}}{}^{CD} \simeq 6 \times 10^{-9}$ M and $K_{Tb^{3+}}{}^{EF} \simeq 2 \times 10^{-11}$ M. These numbers bracket our observed constants for Yb^{3+}. Cave et al. (1979b), using proton relaxation enhancement techniques, calculate equal dissociation constants of the CD and EF sites for GD^{3+} of 5×10^{-11} M, which are approximately 1 order of magnitude less than our values for Yb^{3+}. Their primary data, however, as in this study, are the relative dissociation constants for Gd^{3+} with respect to Ca^{2+}, which they measure as 0.05–0.005 M for both sites. They are unable to distinguish between the two sites and choose an approximate Ca^{2+} dissociation constant of 10^{-9} M.

The interpretation of fluorescence measurements by using terbium-bound parvalbumin suggests the existence of a third metal binding site in parvalbumin (Nelson et al., 1977); however, X-ray crystallographic studies (Sowadski et al., 1978) indicate the absence of such a third site. The discrepancy may be due to differences between the solution and solid-state protein structure. The NMR methods presented herein are extremely sensitive to metal binding, because unique resonances are observed corresponding to each of the metals bound to the protein. Our results indicate that up to a total metal to total protein ratio of 4.5 no shifted resonances due to binding at a third site are observed. Shifting and broadening of resonances in the fast exchange limit are possible for weakly bound metals, but under the conditions of the titration no such effects were observed. A third metal binding site may exist near the EF site, but it apparently only binds metals such as Mn^{2+} or Mg^{2+} (Cave et al., 1979b,c). We conclude that there are only two specific metal binding sites in parvalbumin to which the lanthanide ytterbium will bind and that these two sites are the EF and CD binding sites.

In conclusion, we have shown that the ytterbium-induced shifted resonances of carp parvalbumin are sensitive monitors of specific metal binding sites on the protein in solution. We have used these resonances to determine the lanthanide binding affinities for each of the two sites in the presence and absence of Ca^{2+}. The data indicate that the sequential Yb^{3+} replacement of the CD and EF Ca^{2+} ions of parvalbumin (EF displaced first) is the result of differences in Ca^{2+} affinities between the two sites rather than differences in Yb^{3+} affinities.

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Supplementary Material Available

Equations used to fit data in Figure 3 (2 pages). Ordering information is given on any current masthead page.

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Environment of the Tryptophan Residues in a Myosin Head: A Hydrogen–Deuterium Exchange Study†

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ABSTRACT: The structural environment of the tryptophan residues in the myosin head is studied by hydrogen–deuterium exchange of the tryptophan residues in heavy meromyosin and subfragment 1. About seven tryptophan residues per myosin head are found to be buried in the hydrophobic domain(s) of myosin head. The addition of ATP causes almost all the tryptophan residues to become more buried, reflecting that the conformational changes during ATP hydrolysis involve almost all the tryptophan residues in myosin head. This is strongly contrasted by the results obtained so far that only about two tryptophan residues per myosin head are involved in the conformational changes of myosin head during ATP hydrolysis [Morita, F. (1967) *J. Biol. Chem. 242*, 4501–4506; Werber, M. M., Szent-Gyorgyi, A. G., & Fasman, G. D. (1972) *Biochemistry 11*, 2872–2883]. The exchange rate of the tryptophan residues increases at the reaction step M·ADP·P_i (e.g., the majar steady-state intermediate at 25 °C) to M·ADP + P_i. Our previous study on the hydrogen–deuterium exchange of the peptide hydrogens in heavy meromyosin [Yamada, T., Shimizu, H., Nakanishi, M., & Tsuboi, M. (1977) *J. Biochem. (Tokyo) 82*, 139–144] showed, however, that the exchange rate of the peptide hydrogens decreases at the reaction step M·ADP·P_i to M·ADP + P_i. This means that at the reaction step M·ADP·P_i to M·ADP + P_i the tryptophan residues become more exposed while the peptides become more buried in myosin head. These results indicate that at the reaction step M·ADP·P_i to M·ADP + P_i the conformational changes take place in the reciprocal way at the tryptophan domains and at the peptide domains in myosin head.

Muscle contraction takes place with the mutual sliding motion of actin and myosin filaments utilizing the chemical energy of ATP hydrolysis. Although the details of the relation between the ATP hydrolysis scheme and the chemomechanical process of the actomyosin system have not been definitely established yet, it is agreed that Scheme I is directly related with the chemomechanical process of muscle contraction (Inoue et al., 1979; Taylor, 1979). In Scheme I, A and M represent actin and myosin head, respectively. At step a, the dissociation of myosin head from an actin filament occurs concomitantly with the binding of ATP (the formation of the M·ATP complex); at b, the bound ATP is split on myosin head forming the M·ADP·P_i complex; at c, the dissociated myosin head is reassociated with the actin filament; at d, the bound ADP and P_i are released from the actin–myosin complex. In step d, motion (probably rotation) of the myosin head is believed to take place on the actin filament (Huxley, 1969; Huxley & Simmons, 1971).

One ATP binding site and one actin binding site are spatially separated in myosin head (Barany & Barany, 1959; Martonosi, 1975; Highsmith, 1976; Greene & Eisenberg, 1978a; Stein et al., 1979). As the M·ATP and M·ADP·P_i complexes have weaker affinity for actin filaments (Eisenberg et al., 1972; Hoffmann & Goody, 1978; Stein et al., 1979) while free M and M·ADP complex have stronger affinity (Highsmith et al., 1976; Greene & Eisenberg, 1978b; Highsmith, 1978; Mar-

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