Proton Nuclear Magnetic Resonance Studies of the Interaction of the Lanthanide Ions Ytterbium and Lutetium with Apo- and Calcium-Saturated Porcine Intestinal Calcium Binding Protein†

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ABSTRACT: Proton nuclear magnetic resonance (^{1}H NMR) has been employed to study the effects of the lanthanide ions Lu^{3+} and Yb^{3+} on both the apo- and Ca^{2+}-saturated forms of the porcine intestinal calcium binding protein (porcine ICaBP). Titration of the apoprotein with Lu^{3+} showed that 2 mol equiv of Lu^{3+} bound sequentially to the protein in the absence of Ca^{2+}. The two dissociation constants of the apoprotein for Lu^{3+} were determined to be $K_{d1} < 10^{-6}$ M and $K_{d2} = (2.3 \pm 1.2) \times 10^{-4}$ M. The addition of only 1 mol equiv of Lu^{3+} to the apoprotein resulted in ^{1}H NMR spectral perturbations that were very similar to those observed previously for the addition of 2 mol equiv of calcium [Shelling, J. G., Sykes, B. D., O'Neil, J. D. J., & Hofmann, T. (1983) *Biochemistry 22*, 2649–2654]. The binding of the second mole of Lu^{3+} led to few further changes in the ^{1}H NMR spectra, indicating that this second mole of lanthanide had little effect on the overall conformation of porcine ICaBP. At a $[Lu^{3+}]/[ICaBP]$ ratio greater than 2, it was apparent that the protein began to aggregate as all of the resonances became significantly broadened. As with Lu^{3+}, titration of the apoprotein with Yb^{3+} showed the sequential binding of 2 mol equiv of Yb^{3+} to the protein. The two dissociation constants of the apoprotein for Yb^{3+} were determined to be $K_{d1} < 10^{-7}$ M and $K_{d2} = (5.0 \pm 1.6) \times 10^{-5}$ M. The addition of Yb^{3+} to the Ca^{2+}-saturated protein resulted in the displacement of only 1 mol equiv of Ca^{2+}, with a relative Yb^{3+}/Ca^{2+} dissociation constant ratio of 0.023 $\pm$ 0.015. This ratio, along with the known dissociation constant of the protein for Ca^{2+}, results in a value of $K_{d1} \sim 2 \times 10^{-9}$ M for Yb^{3+}. The lanthanide-shifted ^{1}H NMR resonances, being sensitive structural indicators, showed that the species with Yb^{3+} in one site and Ca^{2+} in the other site differed in structure from the species with Yb^{3+} in one site and no metal ion in the other site.

High-affinity calcium binding proteins with molecular weights near 10000 have been found in the small intestines of several mammalian species (Kallfelz et al., 1967; Drescher & Deluca, 1971; Hitchman et al., 1972; Wasserman et al., 1978). The biosynthesis of these proteins is dependent upon vitamin D, and their probable function may be indirectly involved in the transport of calcium across the intestine (Wasserman et al., 1978; Wasserman, 1980). Porcine intestinal calcium binding protein (pICaBP)[1] is a compact, globular protein (M_r 8799) whose amino acid sequence of 78 residues has been determined (Hofmann et al., 1979). Porcine ICaBP is highly homologous to the bovine ICaBP (Fullmer & Wasserman, 1977, 1981); the X-ray crystal structure of the minor A form of the bovine protein is known (Szebenyi et al., 1981). An examination of the evolutionary relationships among CaBP's shows that the porcine intestinal CaBP is distantly related to parvalbumin, calmodulin, and troponin C (Barker et al., 1977; Gariépy & Hodges, 1983). The amino acid composition of pICaBP is unique in that there are no His, Met, Cys, and Trp residues, but it is typical of CaBP's in that it has a high proportion of acidic residues (22%) and a segment (residues 55–69) whose sequence is very similar to the EF calcium binding site of carp muscle parvalbumin (Hofmann et al., 1979). A second portion of the sequence (residues 15–30) shows a lesser homology to the CD calcium binding site of carp parvalbumin, an important difference being the insertion of Ala at residue 17 and of Pro at residue 23.

The porcine ICaBP binds 2 mol of Ca^{2+} per mole of protein, with $K_{d1} \simeq K_{d2} = (1\text{–}5) \times 10^{-7}$ M (Dorrington et al., 1978; Chiba et al., 1983; Hitchman & Harrison, 1972; Bryant & Andrews, 1984; Shelling, 1984). Several studies suggested that Ca^{2+} bound randomly to the binding sites in pICaBP (O'Neil et al., 1984; Shelling et al., 1983; Shelling, 1984), while recent Ca^{2+} NMR results suggest that the affinity for Ca^{2+} of the C-terminal EF hand site is slightly higher than that for the N-terminal pseudo-EF hand site (Vogel et al., 1985). Sequential binding has been reported for bICaBP (Dalgarno et al., 1983). Both the tyrosine and one or more of the phenylalanine residues have been shown to be perturbed when pICaBP binds metal ions, but there is little effect on the overall conformation of the protein, suggesting that the dramatic changes observed in aromatic optical activity arise from local effects near the binding sites (Dorrington et al., 1978; O'Neil

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[1] Abbreviations: CaBP, calcium binding protein; ICaBP, intestinal calcium binding protein; pICaBP, porcine intestinal calcium binding protein; bICaBP, bovine intestinal calcium binding protein; NMR, nuclear magnetic resonance; LSR, lanthanide-shifted resonance(s); ppm, parts per million; DSS, sodium 4,4-dimethyl-4-silapentane-1-sulfonate; FID, free induction decay; UV, ultraviolet; CD, circular dichroism; $[M]_0$, total metal ion or protein concentration; EDTA, ethylenediaminetetraacetic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

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et al., 1984). Similar conclusions have been made for bICaBP (Dalgarno et al., 1983).

There have been several studies of the interaction of ICaBP with lanthanide ions. Far-UV circular dichroism studies indicated that, as with calcium, there was no change in the overall conformation of the protein upon lanthanide binding (Dorrington et al., 1978). These authors also stated that the porcine protein displayed about the same affinity for calcium and the lanthanides, although no data were shown to this effect. Subsequent Tb^{3+} luminescence studies have shown that Tb^{3+} binds sequentially to two sites on the apoprotein, with $K_{d1} < 10^{-7}$ M and $K_{d2} \simeq 10^{-5}$ M (O'Neil et al., 1984). The first mole of Tb^{3+} fills the C-terminal site, and the second mole fills the N-terminal site. The binding of Tb^{3+} to the second site was found to be less effective as the salt concentration was raised. Anomalous binding curves at $[Tb^{3+}]/[ICaBP]$ ratios greater than 1, at higher protein concentrations, suggested that self-association of the protein occurred under these conditions.

The binding of several divalent cations and the lanthanides to the highly homologous bICaBP has also been studied (Fullmer & Wasserman, 1977). The addition of various metal ions to bICaBP, in the presence of calcium, indicated that the displacement of bound calcium decreased with the divergence of the charge density (charge/ionic radius in angstroms) of the added cation from that of Ca^{2+}. Similar trends were shown for the protection of the protein against cleavage by trypsin. For the lanthanide series in the presence of calcium, La^{3+} showed the largest degree of calcium displacement and protection against trypsin cleavage and Yb^{3+} displayed the smallest. In the absence of any bound calcium, La^{3+} was the only lanthanide ion which afforded any protection against trypsin cleavage. Similarly, recent work has shown that the differences in the affinities of the lanthanides and calcium for the two metal ion sites of parvalbumin are also proportional to the charge density of these ions. These differences were shown to be the result of variation in the k_1 and k_{-1} rate constants across the lanthanide series (Corson et al., 1983; Williams et al., 1984). These results suggest that metal ions other than Ca^{2+} may have different effects on the local structure of the protein.

Fluorescence studies on bICaBP have shown that Tb^{3+} also binds to two sites on the apoprotein with high affinity (Jones et al., 1980). The K_d values differed by at least a factor of 20, with both values being $<8.5 \times 10^{-5}$ M. In the presence of excess calcium ($[Ca^{2+}]/[ICaBP] > 2$), Tb^{3+} was still able to bind to the protein (Jones et al., 1980). X-ray crystallographic studies have shown that the addition of Nd^{3+} and Tb^{3+} to the calcium-saturated crystal resulted in the displacement of only the C-terminal calcium ion. These crystallographic results indicated that the N-terminal site was not available to the solvent (Szebenyi et al., 1981; Jones et al., 1980). These authors suggested that the N-terminal site might be a structural site which, under physiological conditions, remains saturated with calcium, while the C-terminal site might be a regulatory site which binds or releases Ca^{2+} as the intracellular levels of calcium vary.

This paper describes the use of ^1H NMR to study the conformational changes in pICaBP caused by lanthanide binding and the stoichiometry of lanthanide binding in both the presence and absence of calcium. Two lanthanides are used, Yb^{3+} and Lu^{3+}. Lutetium is diamagnetic, and therefore, the changes that occur in the ^1H NMR spectrum can be directly compared with those induced by Ca^{2+}. Yb^{3+} is paramagnetic and causes lanthanide-induced shifts in the ^1H NMR specturm. These shifted resonances provide very sensitive structural probes (Lee & Sykes, 1983) as well as separate spectra for all of the intermediates involved in the metal ion titrations, so that the metal affinities can be accurately measured (Lee & Sykes, 1981; Corson et al., 1983; Williams et al., 1984).

Materials and Methods

Protein Samples. Porcine intestinal CaBP was purified and prepared as described previously (Shelling et al., 1983). The only exception to this procedure was that both the D_2O used to exchange the apoprotein and the deuterated NMR sample buffers were pretreated with preexchanged Chelex-100 (~200 μL of Chelex per 10 mL of buffer in D_2O) prior to use (Shelling, 1984) in order to get rid of the Ca^{2+} contamination observed previously (Shelling et al., 1983). The protein concentrations were determined by UV spectrometry using the following extinction coefficients: $\epsilon^{0.1\%}_{280nm} = 0.170$ mL mg^{-1} cm^{-1} and $\epsilon^{0.1\%}_{276.9nm} = 0.190$ mL mg^{-1} cm^{-1}.

Stock Metal Solutions. Stock Yb^{3+}, Lu^{3+}, and Ca^{2+} solutions were prepared by weight from the corresponding chlorides, which had been dried overnight at 80 °C under bench vacuum. The final concentrations of these solutions, which were made up in the pretreated sample buffer, were determined by titration with EDTA, as outlined previously (Shelling et al., 1983, 1984; Shelling, 1984).

NMR Spectra. The NMR spectra were obtained on a Bruker HXS-270 NMR spectrometer operating in the Fourier transform mode and equipped with quadrature detection. The ambient temperature for the samples was 299 K, and all of the samples were temperature equilibrated 10–15 min prior to acquisition. The parameters used to acquire the lutetium data were the same as those described previously for calcium (Shelling et al., 1983). For the ytterbium data, the parameters were changed to 8K data points, sweep width ±20 000 Hz, filter width 40 000 Hz (Bessel), and pulse width 7 μs (~70°). Chemical shift values are relative to the major resonance of DSS, which was measured as an internal standard, or measured separately under identical conditions (30 mM imidazole-d_4 and 20 mM KCl, pH 6.5). For the spectra which are resolution enhanced, the FID was apodized by double-exponential multiplication.

Results

The results of the ^1H NMR monitored titration of apopICaBP with the diamagnetic lanthanide ion Lu^{3+} are shown in Figure 1. The binding of Lu^{3+} causes perturbations in the spectrum of the protein which are most easily observed in the aromatic and upfield-shifted methyl regions of the spectrum. As was observed previously for Ca^{2+} (Shelling et al., 1983), the NMR spectral changes characterizing the metal exchange are in the NMR slow exchange limit as indicated by the fact that separate resonances are present for the apoprotein and metal-bound protein when less than stoichiometric amounts of metal ion have been added.

As Lu^{3+} is added to the apoprotein up to a $[Lu^{3+}]/[ICaBP]$ ratio of 1, the broad envelope of the phenylalanine resonances (7.0–7.3 ppm) sharpens up and increases in intensity while the intensity of the apoprotein Tyr 2,6 resonance at 7.56 ppm decreases with concomitant increase of intensity of the resonance at 7.5 ppm. The Tyr 3,5 resonance at 6.76 ppm becomes resolved into a clear doublet as Lu^{3+} is added because of the decrease in intensity of an overlapping Phe resonance in the spectrum of the apoprotein. The other upfield-shifted phenylalanine resonances (from 6.4 to 6.7 ppm), and the upfield-shifted methyl resonances, are also perturbed. The nature of these changes in the spectrum of the apoprotein upon the

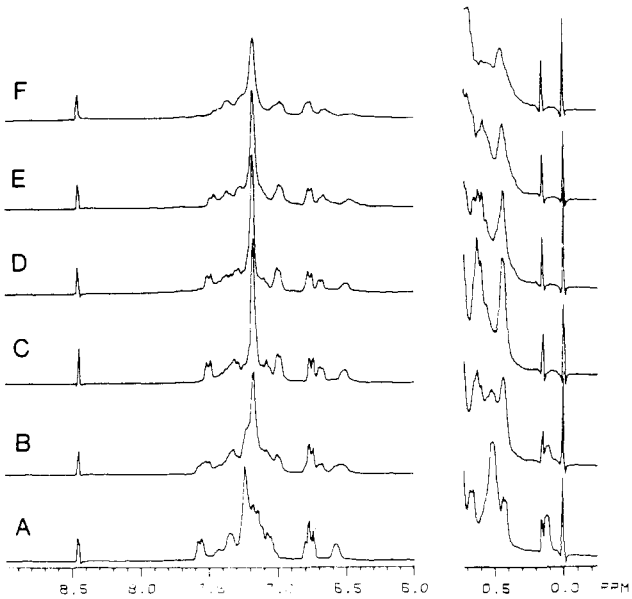

FIGURE 1: ^{1}H NMR spectra at 270 MHz (upfield and downfield regions) during a titration of apo-ICaBP with Lu^{3+}. The sample was 0.54 mM ICaBP, 15 mM Pipes, and 20 mM KCl, pH 6.5. The $[Lu^{3+}]_0/[ICaBP]_0$ ratio values were (A) 0.00, (B) 0.54, (C) 0.98, (D) 1.52, (E) 1.98, and (F) 4.85. The spectra represent 8320 acquisitions and are resolution enhanced with a Lorentzian to Gaussian conversion. The signals at 0.115 and 8.45 ppm are from small-molecule impurities.

addition of 1 mol equiv of Lu^{3+} was essentially the same as those changes previously observed for the addition of 2 mol equiv of Ca^{2+} (Shelling et al., 1983).

At $[Lu^{3+}]/[ICaBP]$ ratios ≥ 1, there was little further change in the spectrum, aside from a general broadening of all of the resonances, resulting in a net decrease in their intensity. This was reflected in both the aromatic and the upfield-shifted methyl resonances, as shown in Figure 1. This broadening was very small for metal to protein ratios of 1–2, but at larger ratios, the entire spectrum began to broaden significantly, and the sample itself became fairly viscous, although it remained clear. The decrease in the intensity of the phenylalanine envelope as a result of the broadening in these resonances can be clearly observed in Figure 2A, which shows the effects of the sequential binding of 2 mol of Lu^{3+} to the protein and then the generalized broadening in the presence of excess lanthanide. A plot of the area of the upfield-shifted phenylalanine resonance at 7.01 ppm as a function of $[Lu^{3+}]_0/[ICaBP]_0$ yeilds the data shown in Figure 2B. The solid line represents a curve computed on the assumption that the apoprotein binds 2 mol of lutetium sequentially. The computed values for the dissociation constants were $K_{d1} = (4.9 \pm 6.7) \times 10^{-6}$ M and $K_{d2} = (2.3 \pm 1.2) \times 10^{-4}$ M. The value of K_{d2} agrees fairly well with the Tb^{3+} value derived previously for pICaBP (O'Neil et al., 1984), but it was apparent that the K_{d1} was too small to be accurately determined by ^{1}H NMR in this protein concentration range, as outlined elsewhere (Shelling, 1984).

Figure 3A,B shows two regions of the ^{1}H NMR spectrum during a titration of apo-ICaBP with increasing concentrations of Yb^{3+}, where changes in the intensity of the lanthanide-shifted resonances are observed as a function of increasing ytterbium concentration. As was noted above for Lu^{3+}, these resonances could be saturated without any observable change in their chemical shift value, indicating that they were in the slow exchange limit. The resulting spectra show that a set of shifted resonances increase in intensity from $[Yb^{3+}]/[ICaBP]$ ratios of 0 to 1 and then decrease in intensity between

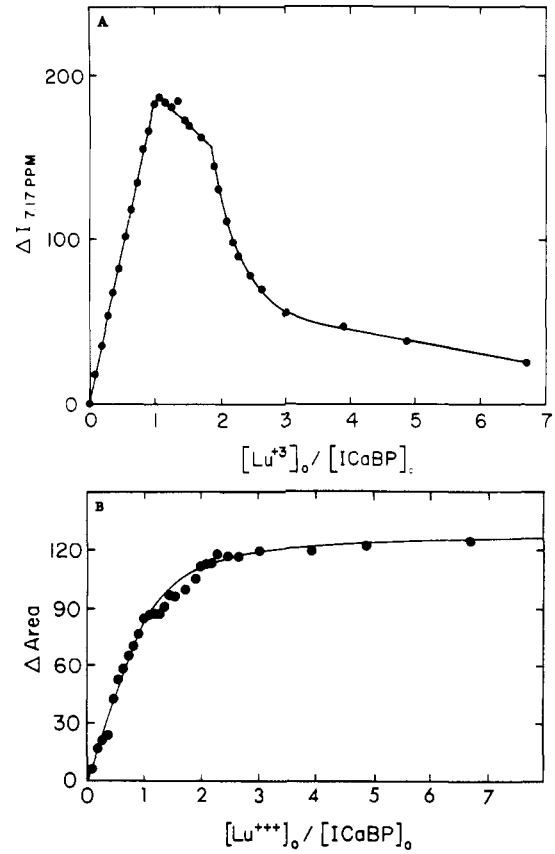

FIGURE 2: (A) Plot of the change of intensity (in arbitrary units) of the 7.17 ppm resonance of phenylalanine as a function of the $[Lu^{3+}]_0/[ICaBP]_0$ ratio. The data were taken in part from the spectra shown in Figure 1. (B) Plot of the change in area (in arbitrary units) of the 7.01 ppm resonance of phenylalanine as a function of the $[Lu^{1+}]_0/[ICaBP]_0$ ratio. The resonance was integrated over the range of 7.04–6.93 ppm. The data were taken in part from the spectra shown in Figure 1. The solid line represents the curve which was computed on the assumption that apoprotein binds 2 mol of lutetium sequentially, with $K_{d1} < K_{d2}$.

[Yb]/[ICaBP] ratios of 1 and 2, being replaced by a new set of resonances which have different shifts and are generally broader. For example, resonances at 10 and −4 ppm rise to a maximum in spectrum c of Figure 3A and in spectrum c of Figure 3B, respectively ([Yb]/[ICaBP] $\sim$ 1) but are completely gone in spectra f of Figure 3A,B ([Yb]/[ICaBP] $\sim$ 3). On the other hand, resonances such as those at 24.5, 17.5, and −5.5 ppm do not appear until [Yb]/[ICaBP] > 1 (spectra d of Figure 3A,B) and are maximal after [Yb]/[CaBP] > 2–3 (spectra e and f of both Figure 3A and Figure 3B). These results very clearly show the sequential binding of 2 mol of Yb^{3+} to the protein and provide accurate probes of the intermediates involved in the titration which can be used to determine the affinities for the metal ion. A plot of the area under the resonance at 15.4 ppm as a function of $[Yb^{3+}]_0/[pICaBP]_0$ yielded the data shown in Figure 4. The fit of these data to a mechanism of the form $P + M \overset{K_{d1}}{\rightleftharpoons} PM + M \overset{K_{d2}}{\rightleftharpoons} PM_2$ yielded the resulting computed values of $K_{d1} < 10^{-7}$ M and $K_{d2} = (5.0 \pm 1.6) \times 10^{-5}$ M. While the dissociation constants thus determined are macroscopic constants, they are equal to the microscopic dissociation constants in this case where the affinities are widely different (Ferguson-Miller & Koppenol, 1981).

Figure 5A,B shows portions of the ^{1}H NMR spectrum during a titration of calcium-saturated pICaBP as a function

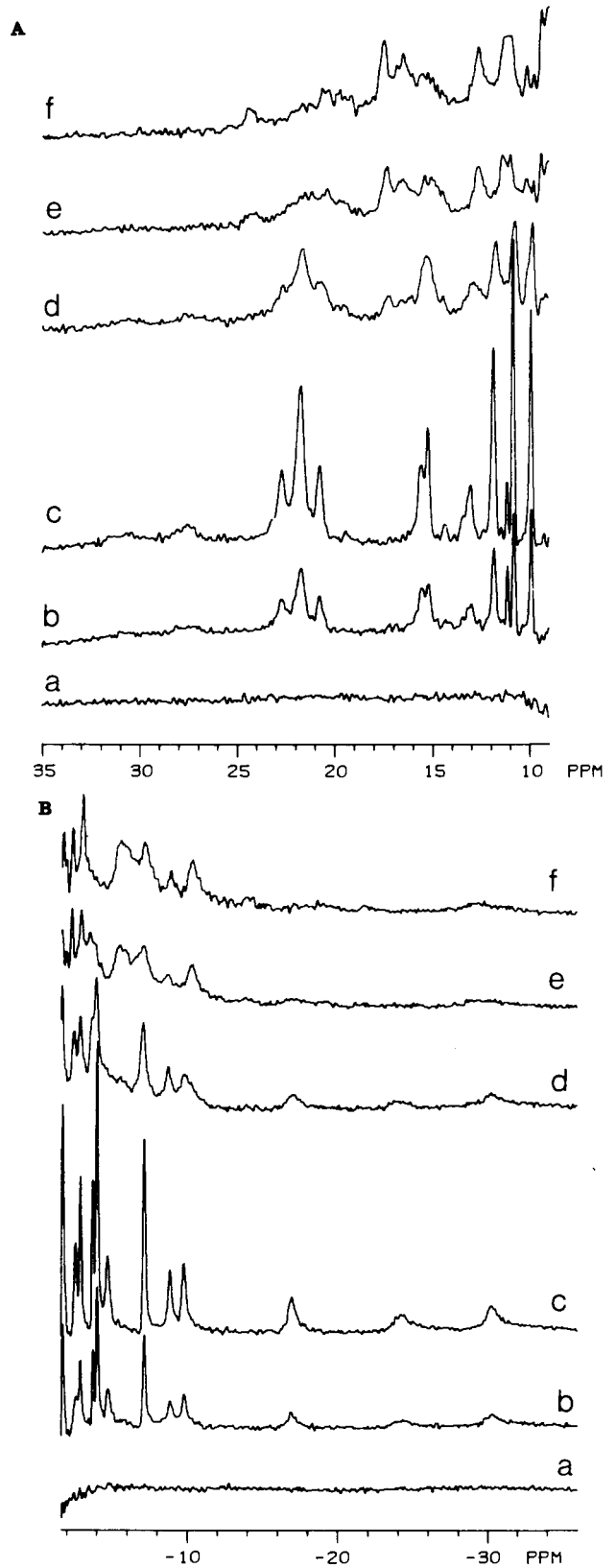

FIGURE 3: ^{1}H NMR spectra during the titration of apo-pICaBP with Yb^{3+}. The LSR in the range of (A) 35–9 ppm and (B) −2 to −36 ppm are shown. The $[Yb^{3+}]_0/[pICaBP]_0$ ratios were (a) 0, (b) 0.43, (c) 1.03, (d) 1.50, (e) 2.12, and (f) 3.06. The sample was 1.0 mM ICaBP, 30 mM imidazole-d_4, and 20 mM KCl, pH 6.5. The spectra represent 20 000 acquisitions. The spectra have been resolution enhanced by a Lorentzian to Gaussian conversion.

of increasing concentrations of Yb^{3+}. In this case, one set of lanthanide-shifted resonances appears and increases in intensity up to a [Yb]/[ICaBP] ratio of ~1 and then levels off. The

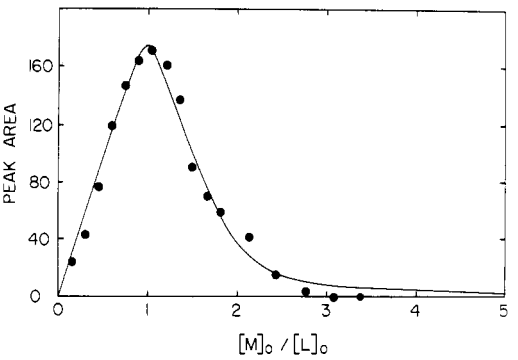

FIGURE 4: Plot of the area (in arbitrary units) of the 15.4 ppm LSR as a function of the $[Yb^{3+}]_0/[apo\text{-}pICaBP]_0$ ratio. The data were taken in part from the spectra shown in Figure 3. The solid line represents the curve computed on the basis of the mechanism $P + M \rightleftharpoons PM + M \rightleftharpoons PM_2$.

effects on the intensities and line widths of the LSR when proceeding from a $[Yb^{3+}]/[ICaBP]$ ratio of 1–3 appeared to be negligible,[2] suggesting that the protein bound only 1 equiv of ytterbium in the presence of calcium and that no significant protein aggregation occurred under these conditions. These results agree with those obtained from a KCl titration (0.02–0.5 M) of Yb^{3+}-substituted ICaBP in that they indicate that no significant protein aggregation occurs in the presence of excess calcium (Shelling, 1984). A plot of the sum of the areas under the resonances at 19.3, 14.5, and −17.5 ppm as a function of $[Yb^{3+}]_0/[2Ca^{2+}\text{–}ICaBP]_0$ is shown in Figure 6, and a computed value for the relative dissociation constant for the Ca^{2+} site which is replaced by Yb^{3+} was determined as $K_d(Yb^{3+})/K_d(Ca^{2+}) = 0.023 \pm 0.015$. Given that $K_{d1} \simeq K_{d2} \simeq 1 \times 10^{-7}$ M for Ca^{2+} (Shelling, 1984), the K_d of the high-affinity Yb^{3+} site of this protein is $\sim 2 \times 10^{-9}$ M.

Discussion

The results of the addition of Lu^{3+} and Yb^{3+} to the apoprotein showed clearly that apo-pICaBP binds 2 mol of lanthanide sequentially. On the basis of the Tb^{3+} luminescence data reported previously (O'Neil et al., 1984), we assume that these lanthanide ions also bind to the C-terminal site first. The first mole equivalent of Lu^{3+} affected the ^{1}H NMR spectra in essentially the same manner as that observed for the addition of 2 mol equiv of Ca^{2+} which bind independently and with comparable affinities (Shelling, 1984; Vogel et al., 1985), suggesting that the binding of metal ions to the C-terminal site on the protein is responsible for the majority of the spectral changes observed. The similarity between the spectral effects of Lu^{3+} and Ca^{2+} also indicates that the binding of these two different metal ions to the protein results in the same types of overall structural changes, in agreement with earlier CD data (Dorrington et al., 1978). This is interesting in view of the results outlined previously for bICaBP where La^{3+} was the lanthanide ion which appeared to most closely mimic the effects of Ca^{2+} on the highly homologous bICaBP, with respect to protection against cleavage by trypsin and Ca^{2+} displacement (Fullmer & Wasserman, 1977).

It was apparent that the binding of Lu^{3+} to apo-pICaBP did not result in significant broadening of the spectra until the

[2] The only exception to this is that the resonance at −13 ppm and the small shoulder at −16 ppm which appear in spectrum d in Figure 5B ([Yb]/[ICaBP] = 1.43) are relatively constant in intensity between [Yb]/[ICaBP] = 1.61 and [Yb]/[ICaBP] = 1.98 (spectrum e of Figure 5B) and then completely disappear by [Yb]/[ICaBP] = 2.34. The origin of these resonances is unknown, and they were not observed in other experiments (Shelling, 1984).

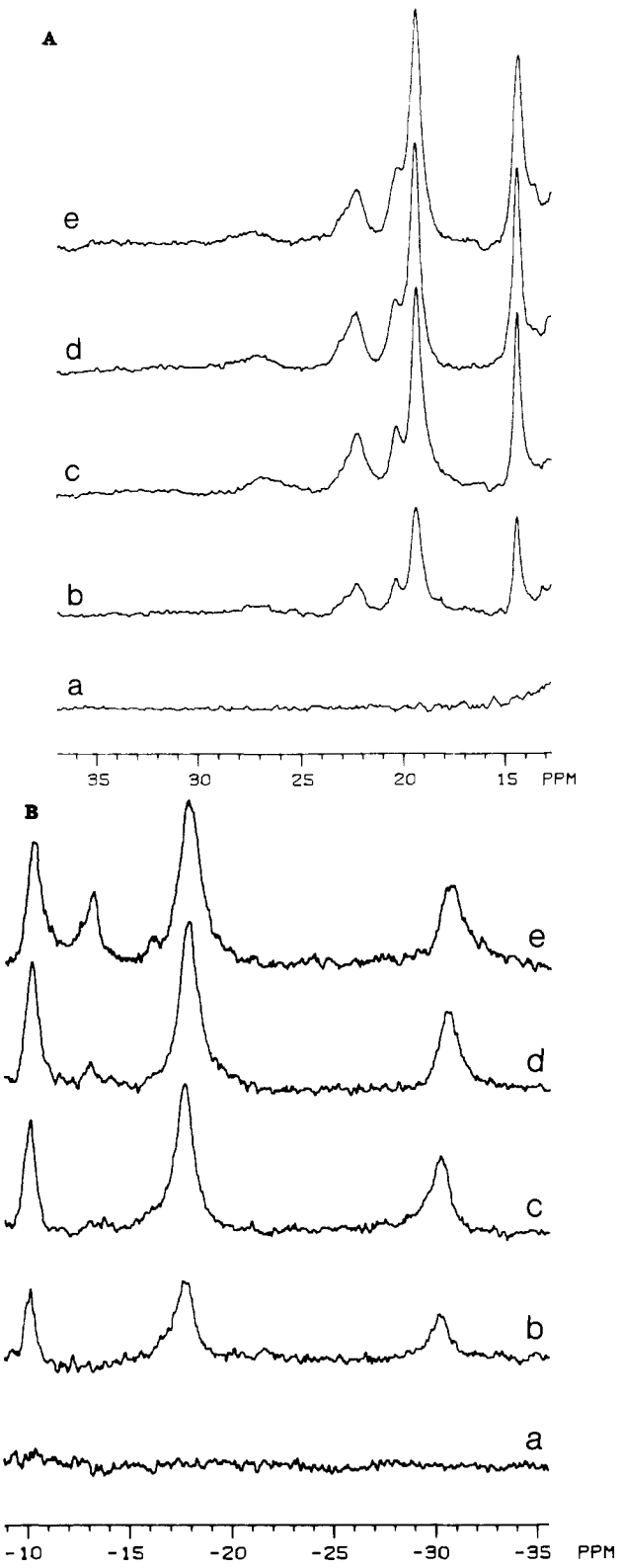

FIGURE 5: ^{1}H NMR spectra during the titration of calcium-saturated pICaBP with Yb^{3+}. The LSR in the range of (A) 37–13 ppm and (B) −9 to −36 ppm are shown. The $[Yb^{3+}]_0/[2Ca^{2+}\text{–pICaBP}]_0$ ratios were (a) 0, (b) 0.44, (c) 0.92, (d) 1.43, and (e) 1.98. The sample was 1.0 mM ICaBP, 30 mM imidazole-d_4, and 20 mM KCl, pH 6.5. The spectra represent 20 000 acquisitions.

ratio of [lanthanide]/[ICaBP] was greater than 2. The overall broadening of the spectrum after this point is most likely due to aggregation, as evidenced by the fact that the sample became somewhat viscous. It is interesting to note that several investigations have shown that, for the apoprotein, the presence of greater than or approximately equal to stoichiometric levels

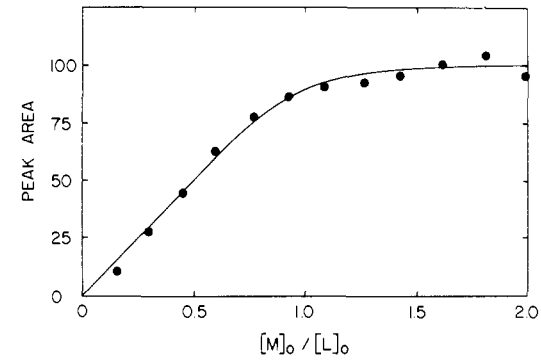

FIGURE 6: Plot of the sum of the change in area (in arbitrary units) of the resonances at 19.3, 14.5, and −17.5 ppm vs. the $[Yb^{3+}]_0/[2Ca^{2+}\text{–pICaBP}]_0$ ratio. The data were taken in part from the spectra shown in Figure 5.

of Tb^{3+} results in anomalous binding curves for both pICaBP (O'Neil et al., 1984) and other CaBP's such as calmodulin (Wallace et al., 1982) and parvalbumin (Donato et al., 1974). It is possible that aggregation was the source of such anomalous data. Alternatively, it could have been due to nonspecific binding of excess lanthanide, as was previously observed for calcium binding to pICaBP (Shelling et al., 1983). The value of K_{d2} determined for the Lu^{3+} titration of the apoprotein was larger than those cited for Ca^{2+} (Hitchman & Harrison, 1972; Chiba et al., 1983; Shelling, 1984; Bryant & Andrews, 1984), while the value of K_{d1} appeared to be less than or comparable with the Ca^{2+} value. These observations differ from some results previously reported for the apo-pICaBP (Dorrington et al., 1978) but do, however, agree well with the recently determined values for the binding of Tb^{3+} to ICaBP (O'Neil et al., 1984) and are in complete agreement with the ^{43}Ca and ^{113}Cd NMR results of Vogel et al. (1985).

The addition of Yb^{3+} to apo-pICaBP resulted in LSR due to the paramagnetism of this lanthanide as was observed previously for parvalbumin (Lee & Sykes, 1982). The nature of the changes in the intensities of some of these shifted resonances as a function of the $[Yb^{3+}]_0/[\text{ICaBP}]_0$ ratio paralleled those observed with Lu^{3+}. The intensities of the first set of resonances characterizing the Ca–Yb–pICaBP intermediate increased up to a ratio of 1; however, at ratios greater than 1, these decreased in intensity and were replaced by the resonances characterizing the 2Yb–pICaBP species. This second set of resonances appeared generally broader than the first set of resonances, presumably due to the aggregation discussed above and the combined line broadening from the two Yb^{3+} ions.

The addition of Yb^{3+} to Ca^{2+}-saturated pICaBP again resulted in LIS which increased in intensity up to a $[Yb^{3+}]_0/[\text{pICaBP}]_0$ ratio of 1, after which there were no further significant changes in the spectra.[3] We have concluded from this observation that Yb^{3+} can still bind to the protein in the presence of 2 mol equiv of Ca^{2+} but that it can only displace the calcium which is bound to the C-terminal site. From the relative dissociation constants determined, it was apparent that Yb^{3+} had a higher affinity than Ca^{2+} for the first site filled. This relative K_d determination has allowed us to determine the dissociation constant of Yb^{3+} for the C-terminal site with a much greater degree of accuracy than we could for Lu^{3+}. The conclusion that Yb^{3+} did not displace the calcium bound

[3] All of the other shifted resonances outside of the chemical shift regions plotted in Figure 5A,B had the same dependence on the concentration of added Yb^{3+}. The only exception was the two anomalous resonances indicated in footnote 2 which represent impurities of unknown origin not present in all samples.

to the N-terminal site on the protein is supported by the known $K_{d2} \simeq 9 \times 10^{-8}$ M of this site for calcium and the $K_{d2} \simeq 5 \times 10^{-5}$ M value derived for the binding of Yb^{3+} to this site in the apoprotein. This conclusion is also supported by our observations that the second mole equivalent of Yb^{3+} added to the Ca^{2+}-saturated protein at both high (0.5 M) and low (20 mM) concentrations of KCl was shown to have little effect on the spectra (Shelling, 1984), particularly in light of the fact that an increase in the salt concentration has been shown to decrease the affinity of the second site for Tb^{3+} in the apoprotein (O'Neil et al., 1984). Due to the high degree of homology between pICaBP and bICaBP, we would expect that the C-terminal Ca^{2+} would be the first to be replaced by a lanthanide for the bovine protein as well. From the results outlined above, we suggest that Nd^{3+} could not displace the calcium bound at the N-terminal site in the crystal of the minor A form of bICaBP because this site has a lower affinity for lanthanides than it does for Ca^{2+} and not necessarily because this site was inaccessible to the solvent as was suggested by previous work (Szebenyi et al 1981; Jones et al., 1980).

There was no evidence of aggregation when Yb^{3+} was added, in excess, to the Ca^{2+}-saturated protein unlike that evident for the apoprotein when the $[Lu^{3+}]_0/[ICaBP]_0$ ratio was greater than 1. The chemical shifts and line widths of LSR are extremely susceptible to changes in the environment, and aggregation would perturb these resonances. It thus appears that either the presence of Ca^{2+} in the second site of the protein and/or the interaction of calcium (the 1 mol equiv of Ca^{2+} displaced from the first site) with some third site stabilizes the protein against aggregation. Since recent studies have shown that the La–Ca–pICaBP species precipitates in the presence of excess lanthanide ions (Vogel et al., 1985), the latter explanation seems more likely. It is interesting to note that the X-ray data show a third calcium ion is found to be present on the surface of the protein (Szebenyi et al., 1981).

One interesting observation for the spectra of $2Yb^{3+}$–ICaBP and Yb^{3+}–Ca^{2+}–ICaBP shown in spectra c of Figure 3A,B spectra c of Figure 5A,B is that although they are very similar, they are not identical. This result suggests that, although the coordination of either Yb^{3+} or Ca^{2+} to the N-terminal site produces no significant overall structural changes in the protein, there are slight structural changes which are specific to the type of metal ion coordinated to that site. It is, however, important to emphasize that the chemical shifts of LSR are extremely susceptible to even very slight alterations in structure (Lee & Sykes, 1983) and, without further data, it is impossible to determine the type and/or magnitude of the structural differences between these two forms of the protein. Similar site–site interactions have been observed with ^{113}Cd NMR (Vogel et al., 1985).

We are aware that one must be careful in choosing a lanthanide analogue for a particular system (Chantler, 1983; Oikawa et al., 1980; Shelling, 1984), but our Lu^{3+} results indicate that this lanthanide produces the same types of structural changes as calcium on pICaBP. Lu^{3+} and Yb^{3+} have comparable ionic radii (Shannon, 1976) and very similar charge/density ratios. This should result in their both having similar dissociation constants for pICaBP (Corson et al., 1983), as we have observed, and also similar modes of binding. We hope to extend this work to further probe the structural characteristics and metal ion effects of the two calcium binding sites of pICaBP.

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Registry No. Yb, 7440-64-4; Lu, 7439-94-3; Ca, 7440-70-2.

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500-MHz Proton NMR Studies of the Medium-Dependent Conformational Preference of Prostaglandin $F_2\alpha$ Analogues†

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ABSTRACT: The complete assignments of the ^1H NMR spectra of 2–10 mM D_2O solutions of prostaglandin $F_2\alpha$ ($PGF_2\alpha$), its C-15 epimer, and analogues bearing a *gem*-dimethyl group at C-16 or C-17 are presented. $PGF_2\alpha$ and its 1,9- and 1,15-lactones were similarly studied in $CDCl_3$ solution. The assignments follow from extensive scalar decoupling and difference NOE spectra and the examination of a specifically deuterated analogue. These studies also define the conformation (including cyclopentane pseudorotational preference) from C-5 through C-16 in each system. The macrolides show little or no conformational freedom at C-4 → C-1, but extensive rotational averaging occurs in the terminal portions of both side chains in the monocyclic compounds. The conformational features so determined are contrasted to those seen in crystal structures and those postulated to occur upon binding to $PGF_2\alpha$-recognizing receptors. The NMR data run counter to the DeTitta hypothesis that changes in the orientation of the C-13,14 π-bond nodal plane relative to the cyclopentane ring and the C-15–O bond are recognition determinants at $PGF_2\alpha$-specific receptors and account for the medium-dependent chiroptical spectral changes previously reported.

The prostaglandins (PGs),[1] a branch of the arachidonate cascade, are ubiquitous in human tissues and display diverse and often opposing physiologic effects even though they all are remarkably similar in structure. At least 13 distinct classes of PG receptors must be postulated[2] on the basis of pharmacologic and structure–activity relationship studies. Most of the receptors show remarkable selectivity in their ability to recognize specific PGs with minimal cross-reactivity with other types of prostaglandins. A detailed understanding of the conformational preferences of the different classes of prostaglandins appears to be essential as a basis for ascertaining the stereostructural requisites for these biorecognition phenomena.

To date, X-ray crystallography (DeTitta et al., 1980, and references cited therein), CD spectroscopy (Leovey & Andersen, 1975a; Andersen et al., 1976), and NMR have been the tools applied in the study of PG structures. The very first PG crystal structure, of a $PGF_1\beta$ derivative (Abrahamsson, 1963), revealed close proximity and specific alignment of the two side chains. Such "aligned side-chain" or "hairpin" models (Rabinovitch et al., 1971; Andersen & Ramwell, 1974) have been the basis of much of the SAR analysis of PGs to date. Three "hairpinlike" $PGF_2\alpha$ structures observed in the solid state are shown in Figure 1. However, PG structures are a priori highly flexible arrays, and computer conformational modeling confirms this. For example, Murakami & Akahori (1977) noted that their analysis yielded 210 conformers of PGE_1 falling within 3 kcal/mol of the most stable one. This conformational versatility results from the pseudorotational possibilities of the cyclopentane ring (Figure 2) combined with rotameric equilibria in the unrestrained side chains. Nonetheless, X-ray crystallographers have, in the discussion of PG biorecognition mechanisms, stated "...with confidence that the crystallographically observed conformations of prostaglandins are the biologically relevant ones at the receptor site" (Langs et al., 1977). If PGs are as flexible as suggested by molecular dynamic calculations, neither solid phase nor free solution conformations are a trustworthy guide to the receptor-bound states. Elucidation of the conformation of PG in dilute aqueous media is, however, directly pertinent to the receptor recognition phenomenon. Changes in aqueous media conformational preference of analogue structures will, of necessity, be related to potency. For a series of analogues which all bear the requisite pharmacophore moieties and which can attain the conformation required at the receptor without violating the

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[1] Abbreviations: PG, prostaglandin; CD, circular dichroism; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; DSS, 4,4-dimethyl-4-silapentane-1-sulfonate; FID, free induction decay; SAR, structure–activity relationship; SPT, selective population transfer; NOESY, NOE spectroscopy via the 2D cross-relaxation experiment; 2D, two dimensional; EDTA, ethylenediaminetetraacetic acid; GC/MS, gas chromatography/mass spectroscopy; S/N, signal to noise; FT, Fourier transform.

[2] Prostanoid SAR's that have led to this conclusion and a classification of the receptors by action mediated and type of PG recognized can be found in Andersen (1985).

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