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Structural Differences in the Two Calcium Binding Sites of the Porcine Intestinal Calcium Binding Protein: A Multinuclear NMR Study†

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ABSTRACT: Cadmium-113 and calcium-43 NMR spectra of Cd^{2+} and Ca^{2+} bound to the porcine intestinal calcium binding protein (ICaBP; M_r 9000) contain two resonances. The first resonance is characterized by NMR parameters resembling those found for these cations bound to proteins containing the typical helix–loop–helix calcium binding domains of parvalbumin, calmodulin, and troponin C, which are defined as EF-hands by Kretsinger [Kretsinger, R. H. (1976) *Annu. Rev. Biochem. 45*, 239]. The second resonance in both spectra has a unique chemical shift and is consequently assigned to the metal ion bound in the N-terminal site of ICaBP. This site is characterized by an insertion of a proline in the loop of the helix–loop–helix domain and will be called the pseudo-EF-hand site. The binding of Cd^{2+} to the apo form of ICaBP is sequential. The EF-hand site is filled first. Both binding sites have similar, but not identical, affinities for Ca^{2+}: at a Ca^{2+} to protein ratio of 1:1, 65% of the ion is bound in the EF-hand site and 35% in the pseudo-EF-hand site. The two sites do not appear to act independently; thus, replacement of Ca^{2+} or Cd^{2+} by La^{3+} in the EF-hand site causes changes in the environment of the ions in the pseudo-EF-hand site. In addition, the chemical shift of Cd^{2+} bound to the EF-hand site is dependent on the presence or absence of Ca^{2+} or Cd^{2+} in the pseudo-EF-hand site. These data show the existence of interactions between the two calcium binding sites that may be of a cooperative nature. ^1H NMR studies demonstrate that the changes in the tertiary structure of ICaBP induced by saturating levels of Ca^{2+} and Cd^{2+}, respectively, are virtually indistinguishable. ^1H NMR studies also confirm the different modes of binding of the two cations.

The levels of the intracellular messenger Ca^{2+} undergo reversible changes that depend on hormonal or nerve impulses. A unique class of calcium binding proteins translates transient Ca^{2+} increases into metabolic or mechanical responses. For example, Ca^{2+}–calmodulin alters the efficiency of various metabolic pathways (Klee & Vanaman, 1982), Ca^{2+}–troponin C regulates contraction in skeletal and cardiac muscle (McCubbin & Kay, 1980), and parvalbumin appears to aid

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in the relaxation of fast skeletal muscles (Heizmann et al., 1982). The calcium binding sites in all these proteins have a similar structure, which has been defined as the EF-hand (Kretsinger, 1975). This structural unit is made up of two helices separated by a calcium binding loop that is 12 amino acid residues long and is wrapped around the metal ion in a characteristic fashion (Kretsinger, 1976). In most of these proteins two EF-hands are arranged structurally in a related manner. This feature stabilizes the overall protein structure and increases Ca^{2+} affinity of each site over that of single isolated sites [Andersson et al., 1983; for reviews, see Seamon & Kretsinger (1983) and Vogel et al. (1983)].

An interesting member of this class of proteins—the troponin C superfamily—is the mammalian ICaBP[1] (M_r 9000). A comparison of the amino acid sequences of the porcine protein with the sequences of other members of this class showed that there is one normal EF-hand located in the carboxy-terminal part of the molecule. However, in the second, amino-terminal calcium binding site the loop is two amino acids longer owing to the insertion of an alanine residue and a proline residue (Hofmann et al., 1979). This unique pseudo-EF-hand has also been found in the bovine ICaBP (Fullmer & Wasserman, 1981) and in the rat ICaBP (Desplan et al., 1983). The 2.3-Å crystal structure of the "minor A" form of the bovine protein showed that the helix–loop–helix arrangement had been preserved and that the overall fold of the protein was in fact very similar to that of parvalbumin (Szebenyi et al., 1981). The refined structure shows that the insertion of the two amino acids into the loop has led to an entirely different liganding of the calcium (Szebenyi & Moffat, 1983). In particular, whereas three side-chain carboxyl groups function as ligands for the calcium in the EF-hand, the calcium in the pseudo-EF-hand apears to be bound to only one side-chain carboxyl group. The other ligands are provided by backbone carbonyl groups.

Spectroscopic studies of metal ions bound to this protein should provide further insight into the structure of each calcium binding site and into the interrelations between them. Unfortunately, no calcium isotopes with spectroscopic properties are known. Hence, we have made use of the luminescent properties of the lanthanide ions Tb^{3+} and Eu^{3+} (O'Neil et al., 1984, and unpublished results). Although it has been shown by ^1H NMR that the lanthanide ion Lu^{3+} induces essentially the same spectral perturbations as Ca^{2+} (Shelling, 1984; Shelling et al., 1985), it has become apparent recently that the trivalent lanthanide ions often bind to EF-hand sites in a manner that is different from Ca^{2+} binding (Corson et al., 1983; Wang et al., 1984; Drakenberg et al., 1985). Fortunately, the magnetic properties of the ^{43}Ca isotope make it possible to perform NMR studies with this nucleus. Since this is a quadrupolar nucleus, broad resonances and consequently poorly resolved spectra are often obtained. Thus, in many instances the $^{113}Cd^{2+}$ ion (spin $^1/_2$) has been used for NMR experiments as a spectroscopically more convenient probe of calcium binding sites [for reviews, see Vogel et al. (1983) and Vogel & Forsén (1985)].

Here, we wish to report on such $^{43}Ca^{2+}$ and $^{113}Cd^{2+}$ NMR studies of porcine ICaBP. These studies were complemented with ^1H NMR studies in order to compare the effects of Ca^{2+} and Cd^{2+} on the overall structure of the protein in the presence of these different cations.

Materials and Methods

ICaBP was prepared from the mucosa of the duodenum and jejunum of 4–6-week-old piglets as described (Hitchman et al., 1972) except that a small amount of ^{125}I-labeled ICaBP was added to the tissue homogenate in order to facilitate detection during purification. Protein purity was assessed by amino acid analysis and absorption spectroscopy (O'Neil et al., 1982).

The apoprotein was prepared as follows. ICaBP (50–120 mg) was incubated in 10 mM Tris–150 mM KCl–10 mM EDTA, pH 8.7 (5–15 mL), for 1 h at 4 °C. The protein was then isolated on a column of Sephadex G-25, fine (450 × 52 mm) (Pharmacia Fine Chemicals AB, Uppsala, Sweden), packed over Chelex 100 (50 × 52 mm) (Bio-Rad, Richmond, CA) equilibrated with 10 mM Tris–10 mM KCl–10 mM EDTA, pH 8.7 The column was developed with Chelex 100 treated 10 mM Tris–10 mM KCl, pH 8.7, buffer. The fractions containing the protein (about 40–50 mL) were pooled and freeze-dried. The Sephadex G-25/Chelex 100 column was then washed and equilibrated with 3 L of Chelex 100 treated 10 mM $NaClO_4$ at pH 8.7. The freeze-dried apoprotein was dissolved in water, applied to the column, and eluted with the Chelex 100 treated 10 mM $NaClO_4$, pH 8.7 solution. The apoprotein thus obtained was free of calcium and EDTA, as shown by CD spectra (Dorrington et al., 1978) and NMR spectra, respectively. Isotopically enriched ^{43}Ca (60%) and ^{113}Cd (90%) were purchased from Oak Ridge, TN, and Matreco AB, Södertälje, Sweden. All other chemicals used were of the highest available purity.

Protein concentrations were determined spectrophotometrically at 277 nm; a molar absorption coefficient of 1681 M^{-1} cm^{-1} (O'Neil et al., 1982) was used. The concentrations of Ca^{2+} and Cd^{2+} stock solutions were determined by atomic absorption spectroscopy.

^{43}Ca NMR measurements were performed with home-built solenoidal probes (17-mm tubes) on a Nicolet 360 WB spectrometer. Routinely, 40-μs (90°) pulses were used with a spectral width of ±10 kHz, an acquisition time of 25 ms, and a spectrometer dead time of 50 μs.[2] In order to obtain satisfactory signal to noise ratios, 2×10^5–2×10^6 scans were averaged in the block-averaging mode (total time, 2–20 h). To measure line widths and resonance areas, we fitted Lorentzian lines to the spectra by using routine Nicolet-1280 software. All spectra are referenced to a 0.01 M $CaCl_2$ standard (0 ppm). Normally an additional line broadening of 25 Hz was introduced by exponential multiplication of the free induction decay. ^1H NMR spectra were obtained on the same spectrometer at a frequency of 361 MHz, with standard 5-mm NMR tubes, 10-μs (90°) pulses, and a delay between pulses of 2 s. The residual HDO resonance in these spectra was suppressed by a selective 5-ms pulse.

^{113}Cd NMR measurements were performed at 56.5 MHz on the home-built UL6T spectrometer (Drakenberg et al., 1983) with 17-mm home-built solenoidal probes. The standard acquisition parameters were 10-μs (45°) pulses, a spectral width of ±10 kHz, and a delay between pulses of 0.5 s. We collected 2.5×10^4–5×10^4 scans in order to obtain spectra

[1] Abbreviations: CaM, calmodulin; ICaBP, intestinal calcium binding protein; α-Lac, bovine α-lactalbumin; Parv, parvalbumin; TnC, cardiac and skeletal troponin C; EDTA, ethylenediaminetetraacetic acid; CD, circular dichroism; NMR, nuclear magnetic resonance; Tris, tris(hydroxymethyl)aminomethane.

[2] In fact, to avoid acoustic ringing, a spectrometer dead time of 250 μs was necessary. However, such a long dead time often caused phasing problems that became obvious when the spectra were fitted with Lorentzian lines. Thus, we acquired our data with a dead time of 50 μs, and afterward we adjusted the first four points (dwell time, 50 μs) of both parts of the free induction decay before Fourier transformation.

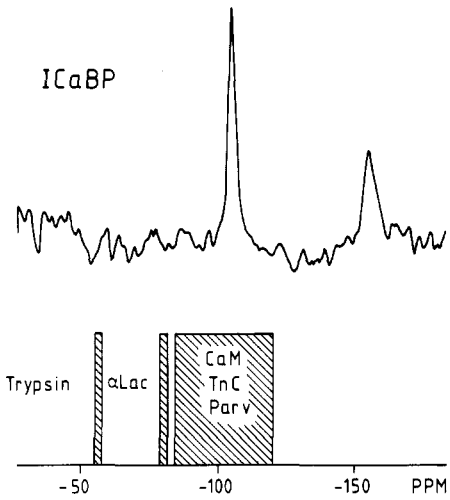

FIGURE 1: Cadmium-113 NMR spectrum obtained for a solution containing 1.5 mM 2Cd–ICaBP at pH 8 and 4 °C. Also shown are the ranges of chemical shifts measured for $^{113}Cd^{2+}$ bound to several other proteins [based on data from Cavé et al. (1982), Drakenberg & Vogel (1983), and Vogel et al. (1983)]. Note that the resonance from the EF-hand is at −105 ppm at the temperature of this experiment.

with a good signal to noise ratio. The total time varied between 4 and 8 h. All spectra were artificially broadened by 30 Hz, unless stated otherwise. Resonance areas were determined by integration.

All protein samples for NMR measurements were dissolved in 99% D_2O for field-frequency locking and homogenizing of the spectrometers. The samples contained between 50 and 100 mM $NaClO_4$; the pH in all experiments was between 7.0 and 7.5, and the temperature was 23 °C, unless otherwise indicated.

Results and Discussion

Cadmium-113 NMR Studies. Figure 1 shows the ^{113}Cd NMR spectrum of Cd^{2+}-saturated ICaBP at 5 °C. Two resonances are discernible; the higher field resonance (−155 ppm) is not observed when the spectrum is obtained at 23 °C, suggesting that the exchange rate for Cd^{2+} bound to this site is such that this resonance is broadened beyond detection at the higher temperature. From a comparison with the chemical shifts measured for Cd^{2+} bound to other calcium binding proteins (Figure 1) it appears that the downfield resonance corresponds to that of an EF-hand. Thus the upfield resonance probably represents $^{113}Cd^{2+}$ bound to the pseudo-EF-hand. Further evidence for this assignment is shown in Figure 2. When apo-ICaBP is titrated with $^{113}Cd^{2+}$, at first only the resonance at −104 ppm appears and increases in intensity up to 1 equiv of Cd^{2+} (Figure 2A). This resonance shifts from −104 to −110 ppm while the second equivalent of Cd^{2+} is bound (Figure 2B). Thus the binding of Cd^{2+} to the two sites is sequential, and the EF-hand site is filled first. CD measurements of Cd^{2+} binding to ICaBP also indicate that the sites are filled sequentially (Eng & Hofmann, unpublished observations). The nature of Cd^{2+} binding is thus similar to that of Mn^{2+} binding (Bryant & Andrews, 1984) and that of the binding of the lanthanides Tb^{3+} (O'Neil et al., 1984), Eu^{3+} (unpublished observations), and Lu^{3+} and Yb^{3+} (Shelling, 1984; Shelling et al., 1985), which is also sequential. A broadening of the resonance of the Cd^{2+} in the EF-hand is associated with the change in its chemical shift when the pseudo-EF-hand site is being filled by Cd^{2+}. The largest broadening is observed around 1.5 equiv (Figure 2C). Assuming that at this point 50% of the second equivalent is in the protein-bound form, one can calculate the off-rate for Cd^{2+}

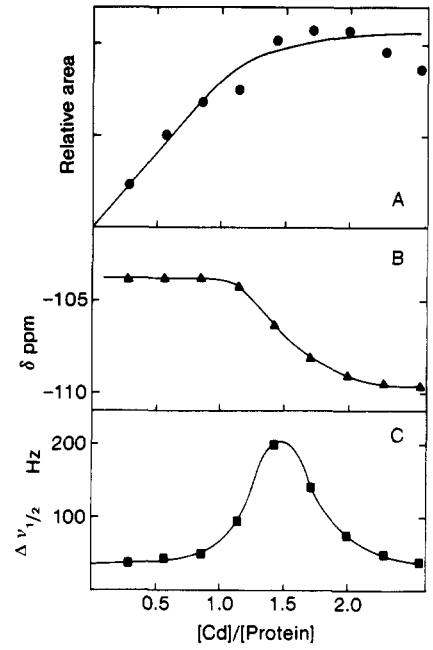

FIGURE 2: Cadmium titration experiment of a solution containing 2 mM apo-ICaBP at pH 7.2 and 20 °C. The following parameters of the ^{113}Cd resonance for Cd^{2+} bound to the EF-hand site are plotted against the Cd^{2+} to protein ratio: (A) relative intensity; (B) chemical shift; (C) line width.

bound to the pseudo-EF-hand as $k_{off} = 700\ s^{-1}$ at 23 °C by use of the equations

$$k_{off} = 1/\tau$$

and

$$\tau = 2\Delta\nu_{1/2}/\pi(\delta\nu)^2$$

(where τ is the metal ion exchange rate, $\Delta\nu_{1/2}$ the maximum broadening, and $\delta\nu$ the chemical shift difference between the two exchanging species). By contrast, the k_{off} for the Cd^{2+} bound to the EF-hand site cannot be accurately determined from our measurements, but it is less than $20\ s^{-1}$.

In a metal ion competition experiment performed at 5 °C in which a solution containing ICaBP and 2 equiv of $^{113}Cd^{2+}$ was titrated with Ca^{2+}, we observed that Ca^{2+} first displaced the Cd^{2+} bound to the pseudo-EF-hand site and then that bound to the EF-hand site (data not shown). Thus the ratio of $K_{D(Cd)}/K_{D(Ca)}$ for the pseudo-EF-hand site exceeds that for the EF-hand site. We also performed a competition experiment, in which Ca^{2+} was titrated into an ICaBP solution containing only 1 equiv of $^{113}Cd^{2+}$. The results are shown in Figure 3. The first equivalent of Ca^{2+} binds to the pseudo-EF-hand site. As a consequence the $^{113}Cd^{2+}$ ion bound to the EF-hand site experiences an upfield shift. The Ca^{2+} ion bound to the pseudo-EF-hand is in slow exchange, and thus the resonance at −104 ppm decreases and simultaneously the resonance at −110 ppm increases in intensity. This is shown in more detail in the insert in Figure 3. Additional Ca^{2+} displaces the Cd^{2+} from the EF-hand site. A least-squares fitting of the decrease in signal intensity above 1 equiv of Ca^{2+} that is depicted in Figure 3 (insert) indicates that $K_{D(Cd)}/K_{D(Ca)} = 4$ for this site. For the pseudo-EF-hand site this ratio is unfortunately so large that our data do not allow us to calculate it. A similar metal ion competition experiment was performed at 5 °C in which La^{3+} was titrated into a solution containing ICaBP and 2 equiv of $^{113}Cd^{2+}$. Cd^{2+} ions bound to both sites were displaced at equal rates. Interestingly, only 1 equiv of La^{3+} was needed to displace both protein-bound Cd^{2+} ions. This could be determined spectrally by measuring the decrease

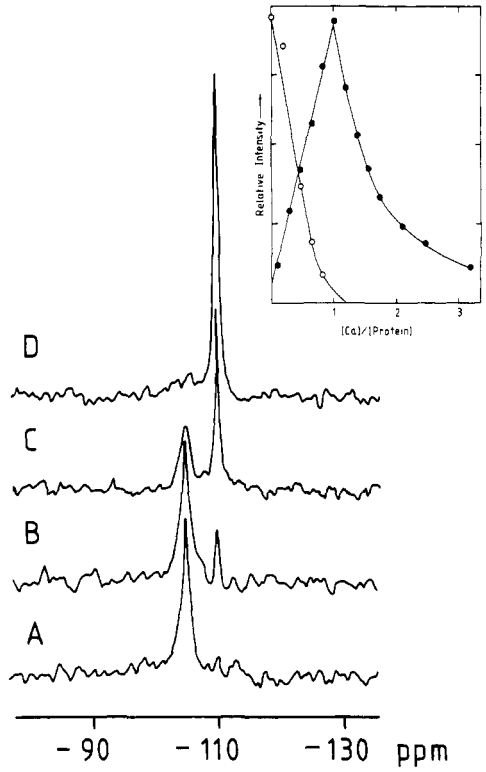

FIGURE 3: Cadmium-113 NMR spectra obtained when a solution (2 mM, pH 7.5, 20 °C) of Cd–ICaBP is titrated with Ca^{2+}: (A) no Ca^{2+}; (B) 0.1 equiv of Ca^{2+}; (C) 0.5 equiv of Ca^{2+}; (D) 0.9 equiv of Ca^{2+}. Note that the resonance at −104 ppm is broader than that at −110 ppm. (Insert) Decrease and increase in the intensities of the ^{113}Cd NMR resonances as a function of added Ca^{2+}: (○) −104 ppm resonance; (●) −110 ppm resonance.

in intensity of both protein-bound resonances as well as the increase of the free Cd^{2+} resonance. Apparently La^{3+} binding to the EF-hand changes the conformation for the pseudo-EF-hand and greatly reduces its affinity for Cd^{2+} (see also below).

Calcium-43 NMR Studies. In ^{43}Ca NMR experiments of each of the EF-hand proteins parvalbumin, troponin C, and calmodulin, single resonances for all the protein-bound $^{43}Ca^{2+}$ ions were observed around 10 ppm (Andersson et al., 1982). In contrast, the ^{43}Ca NMR spectrum of ICaBP contains two overlapping resonances. One of these is centered around 6 ppm, the other one around −8 ppm (Figure 4). From the comparison with the chemical shifts measured for the other proteins it appears likely that the 400 Hz wide downfield resonance corresponds to Ca^{2+} bound in the EF-hand site, so that the broader upfield resonance (700–800 Hz) must correspond to the pseudo-EF-hand. The T_1 values measured for these two resonances with the inversion–recovery method were 1.3 and 0.7 ms, respectively. Using the equations for quadrupolar relaxation of a $^{43}Ca^{2+}$ ion (spin $^7/_2$) bound to a protein described elsewhere (Halle & Wennerström, 1981), we calculated a rotational correlation time $\tau_c = 2 \times 10^{-9}$ s for the Ca^{2+} in both sites, a value similar to the τ_c expected for the overall motion of a globular protein of the size of ICaBP. Thus Ca^{2+} is rigidly held, and the bound ion does not rotate in either of the two sites with motions in the nanosecond time range. The quadrupole coupling constants χ, which are a measure of the symmetry of the environment of the Ca^{2+} ion, were calculated as 0.9 and 1.3 MHz, respectively, providing further support for the notion that the downfield-shifted resonance corresponds to the more symmetric EF-hand site, while the more asymmetric site is the pseudo-EF-hand site. The exchange rate for Ca^{2+} bound to both sites is less than 10 s^{-1}; i.e., slow exchange conditions prevail. We have also attempted to determine the pK_a for both sites. Unfortunately, protein precipitation below pH 4.5 prevented us from obtaining an accurate value. However, since we observed no resonance for free Ca^{2+} in a spectrum recorded at pH 5.0, we conclude that the $pK_a < 4.5$ for both sites.

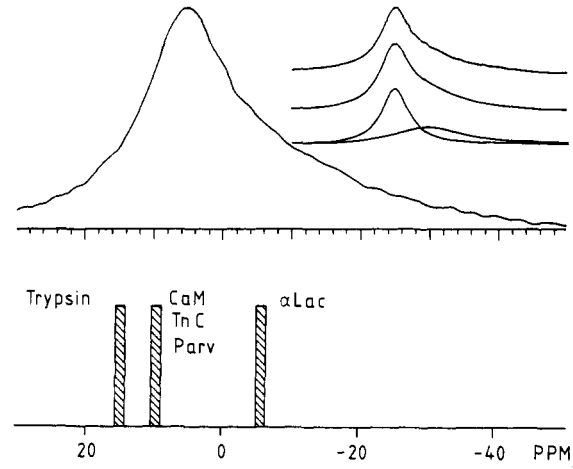

FIGURE 4: Calcium-43 NMR spectrum of 2 mM Ca–ICaBP at pH 7.5. The insert shows from top to bottom the original spectrum, the simulated spectrum, and the two Lorentzian lines used in the simulation. The chemical shifts for several other calcium binding proteins [based on data from Andersson et al. (1982) and Drakenberg & Vogel (1983)] are shown at the bottom.

Upon titration of apo-ICaBP with $^{43}Ca^{2+}$ both resonances were observed from the beginning of the titration (0.25 equiv of $^{43}Ca^{2+}$). From this we conclude that the binding of Ca^{2+} is not strictly sequential, unlike that of Cd^{2+} (see above). However, at about 1 equiv of Ca^{2+} the area ratio for the two resonances was 65:35 (Figure 4), whereas at a calcium to protein ratio of 2 the area ratio became 60:40.[3] This change in ratio suggests that the affinity for Ca^{2+} in the EF-hand site is slightly higher than that for the ion in the pseudo-EF-hand site.

Figure 5 shows the effects of a titration of Ca–ICaBP with La^{3+}. La^{3+} obviously displaces the Ca^{2+} from the EF-hand site. At 1 equiv of La^{3+} (Figure 5E) all the La^{3+} appears to be bound to the EF-hand site with all the $^{43}Ca^{2+}$ now in the pseudo-EF-hand site. During the titration the resonance due to $^{43}Ca^{2+}$ in the pseudo-EF-hand site had narrowed from 800 to 400 Hz. No change in its chemical shift is detectable. At lanthanum to protein ratios higher than 1:1 precipitation was observed [see also Shelling (1984) and Shelling et al. (1985)]. Hence we were not able to determine a value for the relative binding constant of La^{3+} over Ca^{2+} for the pseudo-EF-hand site. Our assignments of the two $^{43}Ca^{2+}$ resonances are supported by other studies of lanthanide binding to ICaBP: Tb^{3+} binding to porcine ICaBP as studied by fluorescence enhancement is sequential, with the EF-hand site as the high affinity site (O'Neil et al., 1984); addition of Nd^{3+} to crystals of 2Ca–ICaBP preferentially displaced the Ca^{2+} bound to the EF-hand site (Szebenyi et al., 1981); the binding of Lu^{3+} and Yb^{3+} to apo-ICaBP as studied by ^1H NMR is sequential, with the two binding constants differing by 2–3 orders of magnitude and with these lanthanides displaying a greater affinity (100

[3] At 2 equiv of Ca^{2+} to 1 equiv of ICaBP one should ideally obtain a 1:1 intensity ratio. The fact that this is not observed experimentally reflects the difficulties in obtaining quantitative ^{43}Ca NMR spectra. This is due in part to the spectrometer dead time, which reduces the intensities of broader resonances more than those of narrow resonances, and in part to the fact that the 90° pulse of 40 μs is long compared to the T_1 values for both ^{43}Ca resonances.

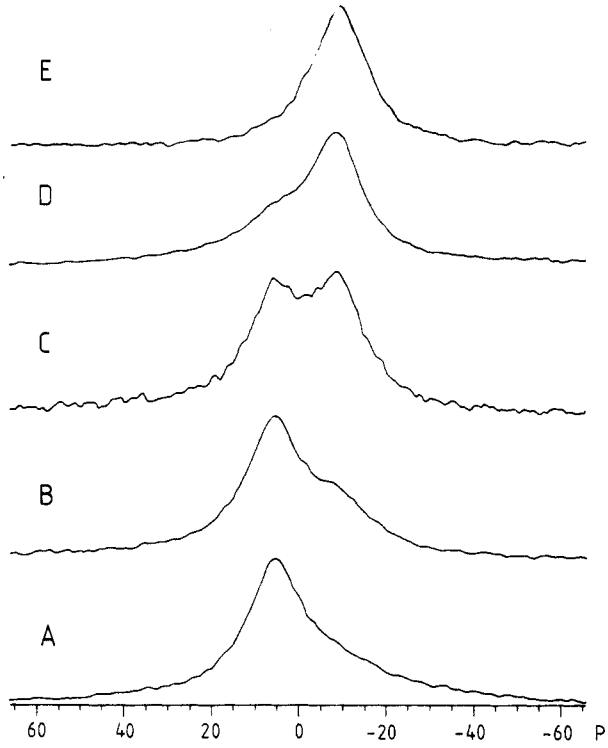

FIGURE 5: Calcium-43 NMR spectra of 2 mM Ca–ICaBP, pH 7.5, in the presence of increasing amounts of La^{3+}: (A) no La^{3+}; (B) 0.2 equiv of La^{3+}; (C) 0.5 equiv of La^{3+}; (D) 0.7 equiv of La^{3+}; (E) 1.0 equiv of La^{3+}. Different numbers of scans were used in the acquisition of these spectra; the plots are normalized to the intensity of the highest resonance.

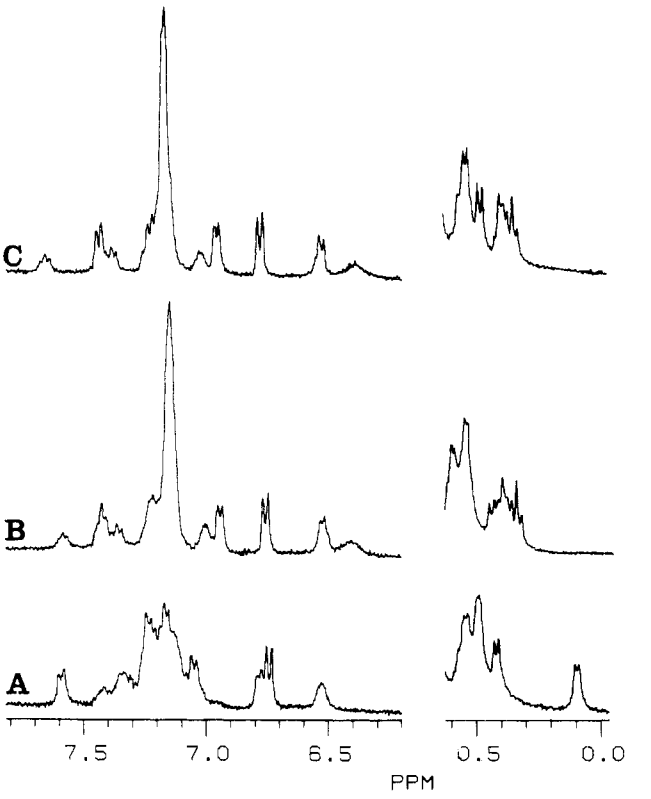

FIGURE 6: Proton NMR spectra of (A) apo-ICaBP, (B) 2Cd–ICaBP, and (C) 2Ca–ICaBP. The solutions contained 0.9 mM ICaBP, pH 7.5. Only the aromatic and the upfield-shifted methyl resonances are shown. Note the large differences between the apoprotein (A) and the metal ion saturated protein (B, C).

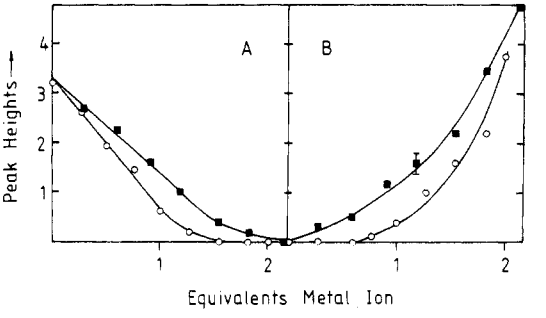

FIGURE 7: Peak heights of two proton NMR resonances at 0.1 (A) and 0.35 ppm (B) as a function of added Ca^{2+} (■) and Cd^{2+} (○).

times) than Ca^{2+} for the EF-hand site and a smaller affinity (200–300 times) than Ca^{2+} for the pseudo-EF-hand site (Shelling, 1984; Shelling et al., 1985); Yb^{3+} can only displace one Ca^{2+} from calcium-saturated porcine ICaBP (Shelling et al., 1985); and Eu^{3+} excitation spectroscopy shows that this lanthanide binds about 100 times more tightly than Ca^{2+} in the EF-hand site (unpublished observations). All these experiments are in good agreement with our observations.

In another ^{43}Ca NMR experiment we titrated Cd–ICaBP with $^{43}Ca^{2+}$. Up to 1 equiv of $^{43}Ca^{2+}$ only the 700–800-Hz broad upfield resonance at −8 ppm was observed, showing that the added calcium binds into the pseudo-EF-hand site. The addition of more $^{43}Ca^{2+}$ led to the appearance of the low-field resonances for free Ca^{2+} and for protein-bound Ca^{2+} that had displaced Cd^{2+} from the EF-hand site. These results confirm the conclusions drawn from the analogous experiment (shown in Figure 3) in which ^{113}Cd NMR was measured during the titration of Cd–ICaBP, namely that Ca^{2+} binds first in the pseudo-EF-hand site of Cd–ICaBP and then displaces Cd^{2+} from the EF-hand site.

Proton NMR Studies. In order to check that addition of Cd^{2+} and Ca^{2+} to the apoprotein induced similar conformational changes, we performed ^1H NMR studies. The results in Figure 6 show that the spectra of 2Ca–ICaBP and 2Cd–ICaBP are virtually indistinguishable. In two-dimensional homonuclear chemical shift correlated spectra (COSY) we observed the same proton–proton coupling pattern for the single tyrosine and for several of the phenylalanine residues in the Ca^{2+}- and Cd^{2+}-saturated proteins as other authors had seen in the Ca^{2+} protein (Shelling et al., 1983; Dalgarno et al., 1983). However, in titration experiments it became apparent that the binding of Cd^{2+} was sequential; i.e., the changes of certain resonances in the spectra were associated with the first equivalent of Cd^{2+}, whereas others were only sensitive to the second equivalent of Cd^{2+}. Moreover, resonances affected by the second Cd^{2+} were in fast to intermediate exchange, whereas those affected by the first equivalent of Cd^{2+} were in slow exchange. In contrast, only slow exchange was apparent in an experiment in which ^1H NMR was used to follow Ca^{2+} titration. In order to show the different sequential effects of Ca^{2+} and Cd^{2+} additions on the ^1H NMR spectrum of ICaBP, we have plotted titration curves for a few selected resonances (Figure 7). Panel A shows a resonance that according to the results obtained in the Cd^{2+} titration is only sensitive to the filling of the EF-hand site, whereas panel B shows the changes in peak height of a resonance that is apparently sensitive only to the filling of the pseudo-EF-hand site. We also plotted several other resonances from the aromatic region of the spectrum (not shown); their changes were, however, more complex than those of the resonances shown in Figure 7. This complexity is to be expected for resonances that are sensitive to the filling of both binding sites [see, e.g., Lee & Sykes (1981)].

From a comparison of the ^1H NMR spectra obtained at 1

equiv of Cd^{2+} and 1 equiv of Ca^{2+}, respectively, we deduced that, in keeping with our results from the ^{43}Ca NMR experiments, the pseudo-EF-hand site of the Ca^{2+} form is about 35% filled at this metal ion to protein ratio. This can also be seen from the data in Figure 7.

Conclusions

^{113}Cd and ^{43}Ca NMR spectra of ICaBP shown in Figures 1 and 4 clearly demonstrate that one of the two calcium binding sites in this protein behaves spectrally like the EF-hand sites of calmodulin, troponin C, and parvalbumin. The other site, which has been shown by X-ray crystallography to have a very different ligand pattern (Szebenyi et al., 1981), showed upfield-shifted $^{113}Cd^{2+}$ and $^{43}Ca^{2+}$ resonances with hitherto unobserved chemical shifts. In fact, the shift for the $^{113}Cd^{2+}$ ion bound to the pseudo-EF-hand is the highest ever measured in a ^{113}Cd NMR spectrum [cf. Ellis (1983)]. Although ^{43}Ca and ^{113}Cd chemical shifts have been measured for whole series of metal ion chelators and other model compounds (Ellis, 1983; Drakenberg & Forsén, 1983), no discernible correlation between the chemical shift and the nature of the ligand patterns of calcium binding sites has unfortunately been found at present. Since only one carboxylate group appears to be coordinated to the Ca^{2+} in the pseudo-EF-hand as compared to three carboxylates in the EF-hand (Szebenyi et al., 1981), the charge distribution will be rather unsymmetric in the former site. It is possible that this is related to the unusual upfield chemical shift of Ca^{2+} and Cd^{2+} ions bound to this site.

The unique nature of the pseudo-EF-hand site of ICaBP has allowed us, for the first time, to resolve resonances for $^{43}Ca^{2+}$ bound to two different sites in the same protein. Therefore, we have been able to study metal ion competition as well as interactions between the two sites by both ^{43}Ca and ^{113}Cd NMR. For the EF-hand site we can rank the metal ions in order of decreasing affinity as $La^{3+} > Ca^{2+} > Cd^{2+}$; for the pseudo-EF-hand site Ca^{2+} clearly has a higher affinity than either Cd^{2+} or La^{3+}. It is unlikely that the pseudo-EF-hand site is a "structural site" whose Ca^{2+} ion does not exchange, as had been suggested on the basis of the Ca^{2+} displacement by Nd^{3+} in the crystals of bovine ICaBP (Szebenyi et al., 1981). Rather, our results, as well as those from studies with other lanthanides (O'Neil et al., 1984; Shelling, 1984; Shelling et al., 1985), show that the pseudo-EF-hand has a lower affinity for the lanthanides than for Ca^{2+}.

In this study we have also obtained various lines of evidence which suggest that each site is aware of the status of the other. For example, $^{43}Ca^{2+}$ bound in the pseudo-EF-hand site has a different line width in a ^{43}Ca NMR spectrum depending on whether there is a Ca^{2+} or a La^{3+} ion in the EF-hand (Figure 5). Similarly, the pseudo-EF-hand site does not bind Cd^{2+} anymore when the EF-hand site is occupied by La^{3+}. Furthermore, $^{113}Cd^{2+}$ bound to the EF-hand has a different chemical shift when the pseudo-EF-hand site is unoccupied or when Ca^{2+} or Cd^{2+} are bound there (Figures 2 and 3). The network of hydrogen bonds connecting the two Ca^{2+} binding loops in the crystal structure of ICaBP is presumably responsible for relaying this information between the two sites (Szebenyi & Moffat, 1983). This intersite communication can be interpreted as a manifestation of some form of allosteric interaction that causes the two calcium binding sites to have very similar calcium dissociation constants (Bryant & Andrews, 1984) despite their marked differences in structure. Further experiments are, however, required to show conclusively whether or not the interactions between the two sites lead to cooperative changes in the intrinsic binding constants of the two sites.

The existence of positive cooperative interaction between the two calcium binding sites of related pairs of EF-hand sites has previously been inferred from various studies of other proteins of the TnC superfamily (Andersson et al., 1983; Klee & Vanaman, 1982; Szebenyi & Moffat, 1983; Teleman et al., 1983). However, this study and the results of Shelling et al. (1985) provide the first direct spectroscopic evidence for intersite communication. The Ca^{2+} titration experiments described here, as monitored by ^{43}Ca and ^{1}H NMR, indicate that, although the rates of binding of Ca^{2+} are very similar for both sites, it appears that the binding of this ion is nevertheless ordered with the EF-hand site being filled first. The relevance of this and of the interactions between the two binding sites to the biological function of ICaBP is unclear since no function for this protein has yet been established.

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Registry No. Ca, 7440-70-2; Cd, 7440-43-9; La, 7439-91-0.

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Hydrolysis of Adenosine 5′-Triphosphate by the Isolated Catalytic Subunit of the Coupling ATPase from *Rhodospirillum rubrum*†

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ABSTRACT: The purified isolated β subunit of the coupling ATPase from *Rhodospirillum rubrum* (RF_1) has an intrinsic ATPase activity, albeit only about 0.1% of that of the intact enzyme. The activity can be separated from intact RF_1 by gel filtration and is not sensitive to bathophenanthroline–Fe, azide, or adenylyl imidodiphosphate, but it is as sensitive as the intact enzyme to aurovertin and efrapeptin. ATPase activity of the isolated subunit, but not of intact RF_1, is rapidly lost by standing at or above room temperature in the absence of either MgATP or dithiothreitol. Hydrolysis of ATP by the β subunit requires Mg^{2+} or Ca^{2+} and occurs with a K_m of around 10 μM, with V_m = 0.6 mol (mol of protein)$^{-1}$ min^{-1}. RF_1 shows a similar K_m and V_m for MgATP, but CaATPase activity occurs with a K_m some 100-fold greater and a V_m 5000-fold higher. The β subunit lacks the very tight (K_d = 10 nM) catalytic ATP binding site and also the cooperativity of catalysis, both of which characterize hydrolysis by intact RF_1. It is shown that product release does not limit ATP hydrolysis by the β subunit, as it does by intact RF_1, and it is suggested that the bond splitting step itself limits the rate of hydrolysis by this subunit.

The coupling ATPase (F_1-ATPase, ATP synthase) from a variety of sources has a common structure ($\alpha_3\beta_3\gamma\delta\epsilon$) and functions to couple ATP synthesis to a transmembrane proton flux and/or to couple ATP hydrolysis to a transmembrane proton pump. The catalytic sites of this enzyme appear to lie on the β subunits [for a review, see Amzel & Pedersen (1983)].

Rapid ATP hydrolysis by F_1 requires cooperation between the three β subunits. Binding of ATP to the three catalytic sites shows strong negative cooperativity, K_d for binding a first ATP molecule being several orders of magnitude less than for subsequent ATP molecules (Cross & Nalin, 1982). Conversely, hydrolytic activity at the three sites shows considerable positive cooperativity, hydrolysis of the first bound ATP being slow until the other active sites are filled, and product release also being stimulated by ATP binding to the second and third active sites (Cross et al., 1982; Gresser et al., 1982).

The remarkably tight binding of ATP to the first catalytic site of F_1 ($K_d \approx 10$ nM; Harris et al., 1981; Cross & Nalin, 1982) is of considerable theoretical importance, since the energy gained on binding ATP is an important factor in shifting the ADP, P_i/ATP equilibrium on the enzyme toward ATP. This is integral to a "conformational" (Boyer 1977) or "binding energy" (Harris, 1981) model for ATP synthesis on F_1. Since the α subunits of F_1 affect cooperativity between the β subunits (Wise et al., 1981, 1984) and also bear very tight, noncatalytic, nucleotide binding sites themselves (Dunn & Futai, 1980), it is advantageous to use an F_1 enzyme lacking the subunits to study the catalytic site.

One fruitful approach has been to study an F_1 molecule mutant in its α subunits, as has been done for the *Escherichia coli* enzyme (Wise et al., 1981, 1984). It is also possible to isolate functional β subunits from the rest of the F_1 molecule (Yoshida et al., 1977; Futai, 1977; Verschoor et al., 1977; Douglas et al., 1979). Here we use the β subunit isolated from the photosynthetic bacterium *Rhodospirillum rubrum*, which can be isolated directly from coupled membranes by treatment

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