Metal-ion binding to parvalbumin

A ^{113}Cd -n.m.r. study of the binding of different lanthanide ions

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^{113}Cd -n.m.r. studies were used to investigate the binding of the lanthanide ions La^{3+} , Gd^{3+} , Tb^{3+} , Yb^{3+} and Lu^{3+} to parvalbumins. It was shown that lanthanide ions with a smaller ionic radius bind sequentially to Cd^{2+} -saturated parvalbumin, whereas those with a larger ionic radius bind with similar affinity to both the CD site and the EF site. The smallest ion, Lu^{3+} , does in fact not compete significantly with Cd^{2+} for the CD site in carp parvalbumin, but appears to bind only to the EF site. This preference of the smaller lanthanide ions for the EF site was used to assign the n.m.r. signals for protein-bound ^{113}Cd . By using Cd n.m.r. and Tb^{3+} fluorescence it was also shown for α -lineage parvalbumin from pike that these proteins possess a third site that can bind lanthanide ions. This site is, however, much weaker than in the β -lineage parvalbumins. It was used to assign the ^{113}Cd resonances from protein-bound Cd^{2+} ions in the spectrum of pike pI5.0 parvalbumin.

X-ray-crystallographic studies performed on carp pI4.25 parvalbumin have elucidated the tertiary structure of this protein (Kretsinger & Nockolds, 1973; Coffee *et al.*, 1972). These studies showed that two primary sites, designated CD and EF, are symmetrical and that they co-ordinate Ca^{2+} ions through oxygen ligands from four carboxylic groups and one peptide carbonyl group. The sixth ligand is provided by a serine-residue side chain for the CD site and by a water molecule for the EF site. Kretsinger (1976) has pointed out that the EF site should be considered as eight-co-ordinated, since two of the carboxylate groups participate with both oxygen atoms in the co-ordination. In contrast, the CD site is six-co-ordinate. The X-ray studies have also shown that Tb^{3+} ions have a preference for the EF site over the CD site (Moews & Kretsinger, 1975; Sowadski *et al.*, 1978). Furthermore, Lee & Sykes (1981) have observed that Yb^{3+} ions bind sequentially to the primary sites of the Ca^{2+} -loaded carp pI4.25 parvalbumin. They proposed that the EF site was the first one to be saturated by Yb^{3+} ions, in analogy with the X-ray studies.

The X-ray studies were not able to characterize

an external, secondary, site that was evidenced by optical studies with Tb^{3+} and Eu^{3+} as luminescence probes (Nelson *et al.*, 1977; Horrocks *et al.*, 1980; Horrocks & Collier, 1981).

Previous ^{113}Cd -n.m.r. studies of metal-ion binding to parvalbumins have revealed that the two sites give rise to separate ^{113}Cd -n.m.r. signals with shifts between -80 and -100 p.p.m. (Drakenberg *et al.*, 1978; Cavé *et al.*, 1982). It therefore appeared possible to use ^{113}Cd -n.m.r. in order to study selectivity of lanthanide-ion binding to the CD and EF sites. In the present paper we describe such studies on carp pI4.25 parvalbumin, belonging to the β -lineage, and pike pI5.0 parvalbumin, belonging to the α -lineage of the parvalbumin family. These two proteins have the same co-ordinating groups in the primary metal-ion-binding sites. However, thus far only the parvalbumins belonging to the β -phylogenetic lineage have been shown to contain the third, secondary, site, which is located close to one of the two primary sites (Cavé *et al.*, 1982). We also present evidence for the existence of this site in parvalbumins belonging to the α -phylogenetic lineage. This has been of help in identifying the CD and EF sites in ^{113}Cd -n.m.r. spectra of the α -parvalbumins as we have previously shown for the β -parvalbumins (Cavé *et al.*, 1982).

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Materials and methods

Carp pI4.25 parvalbumin and pike pI5.0 parvalbumin were prepared as described by Cavé *et al.* (1982) and stored as freeze-dried powder until use. Calcium was removed from the parvalbumins by using the Chelex procedure (Wolff *et al.*, 1977). Cadmium was added from a stock solution of $^{113}\text{Cd}(\text{ClO}_4)_2$ prepared from ^{113}CdO (Oak Ridge National Laboratory, Oak Ridge, TN, U.S.A.) with an isotopic enrichment of 96%. The concentrations of the parvalbumin solutions (1–3 mM) were determined by u.v. spectroscopy by using the relationship $\epsilon_{258} - \epsilon_{268} = 102 N \text{ M}^{-1} \cdot \text{cm}^{-1}$, where N is the number of phenylalanine residues in the protein molecule (Cavé *et al.*, 1979a).

The ^{113}Cd -n.m.r. spectra were recorded on a laboratory-built UL-6T spectrometer (Drakenberg *et al.*, 1983). Standard acquisition parameters were: flip angle 45° ($8 \mu\text{s}$), pulse delay 0.6 s, spectral width 20000 Hz, number of real data points 8000 and number of transients 25000–100000. The ^{113}Cd -n.m.r. chemical shifts are given relative to the shift of an aqueous 0.1 M- $\text{Cd}(\text{ClO}_4)_2$ solution, with high-field shifts given negative values.

Results

^{113}Cd -n.m.r. study of carp parvalbumin (pI4.25)

^{113}Cd -n.m.r. spectra of Cd^{2+} -saturated parvalbumins display two resonances, one from the CD site and the other from the EF site (Drakenberg *et al.*, 1978). When the $^{113}\text{Cd}^{2+}$ -saturated protein is titrated with various lanthanide ions (La^{3+} , Gd^{3+} , Tb^{3+} , Yb^{3+} and Lu^{3+}) the lanthanide-ion competition appears clearly as decreases in the intensities of the ^{113}Cd -n.m.r. signals.

Fig. 1 depicts the decrease in the intensities of the two original ^{113}Cd resonances, 1 and 2 (arbitrarily labelled from low to high field), as well as the variation in intensities of novel resonances for protein-bound $^{113}\text{Cd}^{2+}$ as a function of added equivalents of lanthanides (R). La^{3+} , the largest ($R_0 = 0.115 \text{ nm}$), and Lu^{3+} , the smallest ($R_0 = 0.093 \text{ nm}$), are both diamagnetic, and thus the interpretation of results obtained with these two ions is not complicated by paramagnetic effects, as it is for the other lanthanide ions. Titration with La^{3+} results in a simultaneous decrease in the intensities of both resonances. A new resonance labelled 2' appears in parallel with the resonance from free Cd^{2+} ions. Resonance 2' has a shift of -100 p.p.m. , which, like the shift of signal 2, does not vary with La^{3+} concentration and is therefore assigned to the same site as resonance 2. The shift of resonance 1 depends on the La^{3+} concentration and has shifted upfield by approx. 4 p.p.m. before the signal intensity be-

Fig. 1. Relative intensities of the ^{113}Cd -n.m.r. resonances from Cd^{2+} bound to carp (pI4.25) parvalbumin as a function of added La^{3+} or Lu^{3+} .

The solution contained 2 mM- PaCd_2 in 50 mM-sodium cacodylate buffer, pH 6.1. $\circ$, Site 1 in PaCd_2 ; $\bullet$, site 2 in PaCd_2 ; $\square$, site 1 in PaCdLu ; $\blacksquare$, site 2 in PaCdLu .

comes too low to be observable. The intensity of resonance 2' goes through an intensity maximum for $R = 1$. On the assumption that resonances 2 and 2' are due to the same site in PaCd_2 and PaCdLa respectively (where Pa represents parvalbumin), we find that La^{3+} has a slight preference for site 1.

The titration with Lu^{3+} also results in a simultaneous decrease in resonances 1 and 2, and a new resonance labelled 1' appears approx. 1 p.p.m. downfield from resonance 1. Resonances 1 and 1' are shifted upfield with increasing amounts of Lu^{3+} and are assigned to the same site, whereas resonance 2 does not shift (see Fig. 2). The intensity of resonance 1' increases up to $R = 1$ and stays almost constant for higher ratios (Fig. 1). Again on the assumption that resonances 1 and 1' are due to the same site in PaCd_2 and PaCdLu respectively, we conclude that Lu^{3+} has a strong preference for site 2 in PaCd_2 .

Fig. 2. ^{113}Cd -n.m.r. spectra of 2mM- Cd^{2+} -saturated carp (pI4.25) parvalbumin in 50mM-sodium cacodylate buffer, pH6.1, as a function of added Lu^{3+} . The Lu^{3+} /parvalbumin ratios are indicated in the Figure.

The titration with Gd^{3+} results in large and selective broadening of resonance 1. The intensity of resonance 2 decreases faster than that of resonance 1. In earlier experiments performed at lower frequency and lower S/N ratios, the broadening of signal 1 was overlooked and as a result was erroneously interpreted as a decrease in intensity (Drakenberg *et al.*, 1978). The addition of large excess of Mg^{2+} (about 300equiv.) suppresses the broadening induced by 0.15equiv. of Gd^{3+} on resonance 1 and shifts this resonance to high field, as previously described for similar amounts of Mg^{2+} (Cavé *et al.*, 1979b, 1982), showing that Mg^{2+} and Gd^{3+} compete for the same binding site and that the ratio in the binding constants, $K^{\text{Gd}}/K^{\text{Mg}}$, can be estimated to be between 100 and 1000.

The titration with either Tb^{3+} or Yb^{3+} results in simultaneous decrease of resonances 1 and 2 and appearance of a new resonance labelled 1', which goes through an intensity maximum at about $R = 1$. The chemical shift of resonance 2 stays con-

stant throughout both titrations, whereas the shift of both resonances 1 and 1' depends on the amount of added lanthanide ions. For small amounts of lanthanide ions ($R < 0.5$) signal 1 shifts upfield, as for La^{3+} and Lu^{3+} , and continues to shift that way also for $R > 0.5$ for Yb^{3+} . For Tb^{3+} the shift variations on resonances 1 and 1' are reversed for $R > 1$. On the same assumption as above with regard to the assignments of the resonances we find that both Tb^{3+} and Yb^{3+} have a preference for site 2 in PaCd_2 . This preference is more pronounced in the case of Yb^{3+} , but, however, not as pronounced as for Lu^{3+} .

^{113}Cd -n.m.r. study of pike parvalbumin (pI5.0)

$^{113}\text{Cd}^{2+}$ -saturated pike pI5.0 parvalbumin was titrated with La^{3+} , Gd^{3+} , Yb^{3+} , Lu^{3+} and Mn^{2+} . Fig. 3 shows the variation in the ^{113}Cd resonances as a function of added La^{3+} or Lu^{3+} . Titration with La^{3+} results in very similar decreases in resonances 1 and 2. Two additional resonances, labelled 1' and 2', appear. The intensity of signal 1' is about twice that of signal 2'. The chemical shift of resonances 1 and 1' remain constant, whereas resonances 2 and 2' are shifted slightly to higher field with increasing La^{3+} concentration, and therefore signals 1 and 1' are assigned to one of the two strong sites in the protein and signals 2 and 2' are assigned to the other site. We thus find that La^{3+} has a slight preference for site 2 in PaCd_2 .

Titration with Lu^{3+} results first in a decrease in the intensity of resonance 1, which has almost disappeared at $R = 1$. For higher Lu^{3+} concentrations signal 2 starts to decrease in intensity and shifts to higher field. No extra signal due to Cd^{2+} bound to the protein appears during this titration (Fig. 4). This experiment shows that Lu^{3+} has a strong preference for site 1.

Titration with Gd^{3+} results in a large and selective broadening of resonance 2. The addition of 0.3equiv. of Gd^{3+} causes a broadening of 45Hz, which is about half that observed for resonance 1 from carp parvalbumin under the same conditions. The addition of Gd^{3+} does not affect either the chemical shift or the line width of resonance 1, which is in agreement with previous observations for resonance 2 from carp parvalbumin (Drakenberg *et al.*, 1978). Similarly the intensity of resonance 1 from pike parvalbumin, like that of resonance 2 from carp parvalbumin, decreases faster than that of the other resonance, which is broadened by Gd^{3+} in both proteins.

Titration with either Tb^{3+} or Yb^{3+} leads to a faster decrease in the intensity of resonance 1 than in that of resonance 2. The chemical shift of resonance 1 does not vary in any of these titrations. Resonance 2, however, shifts by approx. 4p.p.m. to high field after the addition of 2equiv. of Yb^{3+}

Fig. 3. Relative intensities of the ^{113}Cd -n.m.r. resonances from Cd^{2+} bound to pike (pI5.0) parvalbumin as a function of added La^{3+} or Lu^{3+}

The solution contained 2mM- PaCd_2 in 50mM-sodium cacodylate buffer, pH6.1. $\circ$, Site 1 in PaCd_2 ; $\bullet$, site 2 in PaCd_2 ; $\square$, site 1 in PaCdLa ; $\blacksquare$, site 2 in PaLaCd .

and by approx. 2p.p.m. to low field after the addition of 2equiv. of Tb^{3+} . No additional resonances could be observed in any of these two cases, in agreement with the Lu^{3+} competition but opposite to the results obtained with La^{3+} . Both Tb^{3+} and Yb^{3+} show a preference for site 1, Yb^{3+} more so than Tb^{3+} , though not as much as Lu^{3+} .

Titration with Mn^{2+} results in significant broadening of both resonances (Fig. 5). However, resonance 2 broadens twice as much as resonance 1. These broadenings are much less than what was previously observed for the carp protein (Cavé *et*

Fig. 4. ^{113}Cd -n.m.r. spectra of 2mM- Cd^{2+} -saturated pike (pI5.0) parvalbumin in 50mM-sodium cacodylate buffer, pH6.1, as a function of added Lu^{3+} . The Lu^{3+} /parvalbumin ratios are indicated in the Figure.

Fig. 5. Excess broadening of the ^{113}Cd -n.m.r. resonances from a 3mM solution of Cd^{2+} -saturated pike (pI5.0) parvalbumin in 50mM-sodium cacodylate buffer, pH6.1, as a function of added Mn^{2+} . $R = \text{Mn}^{2+}/\text{Pa}$.

al., 1982). It may also be noted that the broadening induced by Mn^{2+} is much more nearly linearly dependent on R than is that induced by Gd^{3+} for both proteins.

Fig. 6. Tb^{3+} -fluorescence study of Tb^{3+} binding to pike (pI5.0) parvalbumin

The parvalbumin at 2.5 mM (curve A) and 0.01 mM (curve B) in 50 mM-piperazine buffer containing 0.1 M-KCl was titrated with $Tb(NO_3)_3$. The fluorescence intensity in arbitrary units is plotted versus $R = Tb^{3+}/Pa$.

Tb^{3+} fluorescence

The Tb^{3+} fluorescence emission at 545 nm that is observed when the aromatic chromophore of pike pI5.0 parvalbumin is irradiated at 259 nm is shown in Fig. 6 as a function of Tb^{3+} concentration. The titration curve obtained for the highest concentration of protein (2.5 mM) is similar to that previously reported for the carp parvalbumin (Nelson *et al.*, 1977). Note that quenching of the fluorescence takes place at higher Tb^{3+} concentrations. At lower pike protein concentration (0.01 mM) a titration curve of different shape is observed, as depicted in Fig. 6(b). Here the fluorescence intensity does not decrease at higher Tb^{3+} concentrations. Under similar conditions the carp protein, however, still presents the quenching effect (results not shown) previously described (Nelson *et al.*, 1977).

Discussion

Assignment of the ^{113}Cd -n.m.r. resonances

In a preliminary ^{113}Cd -n.m.r. study on carp parvalbumin we made a tentative assignment of the two observed ^{113}Cd -n.m.r. resonances to the CD and EF sites (Drakenberg *et al.*, 1978). This assignment was, however, based on an erroneous interpretation of the Cd^{2+} - Gd^{3+} competition experiment, and has unfortunately also been used in a ^{113}Cd -n.m.r. study of a series of α -lineage and β -lineage parvalbumins (Cavé *et al.*, 1982). With the better data now at hand for the competition between Cd^{2+} and various lanthanide ions, especially Tb^{3+} , we wish to re-evaluate the assignments.

It has been shown that Tb^{3+} , at low concentrations, in crystals selectively replaces Ca^{2+} in the EF site before replacing the Ca^{2+} in the CD site in carp parvalbumin (Sowadski *et al.*, 1978). Furthermore, it has been shown that there is no selectivity in the Ca^{2+} - Cd^{2+} competition (Drakenberg *et al.*, 1978), which has led us to the conclusion that Tb^{3+} will show a preference for the EF site in PaCd₂ as well as in PaCa₂. We therefore make a tentative assignment of the high-field ^{113}Cd resonance ($\delta = -99.5$ p.p.m.) in carp parvalbumin to the EF site. We note that, even though this metal ion is thought to be accessible to solvent (Kretsinger, 1976), its ^{113}Cd -n.m.r. chemical shift does not depend on pH or metal-ion concentration, whereas the chemical shift of the other ^{113}Cd resonance depends strongly on excess metal ions. This assignment is therefore in agreement with the suggestion by Rhee *et al.* (1981) that the third metal-ion-binding site is situated close to the CD site and shares the carboxylic groups from Asp-53 and Glu-59 with the CD site. On the assumption that the CD-site ^{113}Cd resonance is the one that is sensitive to excess metal ions whether there is a Cd^{2+} ion or a lanthanide ion in the EF site, we can also assign resonance 2' from the La^{3+} competition to the EF site in PaLaCd and resonance 1' from the Lu^{3+} competition to the CD site in PaCdLu.

There is no X-ray-crystallographic structure available for any α -lineage parvalbumin; however, there seems to be a general agreement that the structure is well conserved within the parvalbumin family of proteins. On the basis of this assumed structural similarity we may deduce that the observed variation in the chemical shift in one of the ^{113}Cd resonances from Cd^{2+} bound to pike parvalbumin as a function of added lanthanide ions is caused by ion binding to the third site, close to the CD site, as in the β -lineage parvalbumins. For the pike protein we have observed that it is the low-field ^{113}Cd resonance that is not affected by the addition of lanthanides and therefore tentatively assigned to the EF site. Similarly we can assign resonances 1' and 2' in the La^{3+} competition to the EF and CD sites respectively in the mixed complexes. This is the only example where we have observed all four possible ^{113}Cd -n.m.r. resonances, i.e. two from PaCd₂ and one each from PaCd^{EF}La^{CD} and PaLa^{EF}Cd^{CD}.

Lanthanide-ion- Cd^{2+} competition

Figs. 1 and 3 show that, for both the β -lineage parvalbumin from carp and the α -lineage parvalbumin from pike, La^{3+} as well as Lu^{3+} will compete favourably with Cd^{2+} for at least one of the two primary sites. According to our assignments above, Lu^{3+} will bind preferentially to the EF site in carp parvalbumin and does not compete

very effectively with Cd^{2+} bound to the CD site, whereas La^{3+} shows a slight preference for the CD site. Lee & Sykes (1981) and Corson *et al.* (1983a) have found, by using proton n.m.r., that Yb^{3+} and Lu^{3+} bind preferentially to the EF site of the Ca^{2+} -saturated protein. However, they also observed a strong competition between these ions and Ca^{2+} for the CD site. This difference in the competition for the CD site is most probably due to the fact that in the present work we have studied the competition between lanthanide ions and Cd^{2+} rather than Ca^{2+} ions. Cd^{2+} ions are known to bind more strongly to parvalbumins than do Ca^{2+} ions (Cavé *et al.*, 1979a, 1981).

Tb^{3+} and Yb^{3+} show competition with Cd^{2+} similar to that shown by Lu^{3+} for the two primary sites. There is only a small variation in how effective they are in competing with the Cd^{2+} ions for the CD site. Tb^{3+} is the most and Lu^{3+} the least effective. This is in qualitative agreement with the data reported by Corson *et al.* (1983a). They found that $K_b^{\text{a}}/K_b^{\text{c}}$ is twice as large for the CD site as for the EF site, whereas $K_b^{\text{u}}/K_b^{\text{c}}$ is 8 times as high for the EF site as for the CD site. The whole variation lies in the CD site, whose relative binding constant increases 18-fold from Lu^{3+} to La^{3+} , resulting in a slight preference of La^{3+} for the CD site and a strong preference of Lu^{3+} for the EF site.

To our knowledge there is, before this present work, no information available on the lanthanide ion- Ca^{2+} or lanthanide ion- Cd^{2+} ion competition for a parvalbumin belonging to the α -phylogenetic lineage. Our results, however, show quite convincingly that the two proteins studied behave very similarly with regard to the metal-ion competition. La^{3+} shows a slight preference for the CD site, and Lu^{3+} prefers the EF site strongly. There is, however, a difference in how well Lu^{3+} competes with Cd^{2+} for the EF site, as can be seen from Figs. 2 and 4. The ratio $K^{\text{Lu}}/K^{\text{Cd}}$ is larger for the pike protein (≥ 1) than for the carp protein (< 1).

The third site

When the Ln^{3+} - Ca^{2+} competitive binding to parvalbumin is monitored by, for example, Tb^{3+} fluorescence, the change in the fluorescence is found to go through a maximum for less than 2 equiv. of Tb^{3+} per parvalbumin molecule, and decreases at higher Tb^{3+} concentration. This quenching has been explained as caused by binding of Tb^{3+} ions to a third site close to one of the primary sites (Rhee *et al.*, 1981). In X-ray-crystallographic studies there was no sign of such a site (Sowadski *et al.*, 1978). Also, in ^{113}Cd -n.m.r. studies effects due to metal-ion binding to a third site were observed (Cavé *et al.*, 1979b, 1982). However, these effects were observed only for the β -

phylogenetic-lineage proteins. In the latter studies it was observed that univalent cations caused a shift of the ^{113}Cd resonances from one of the sites to lower field, whereas bivalent cations caused a shift to higher field (Cavé *et al.*, 1982). The ^{113}Cd -n.m.r. signal from the same site is also broadened by the addition of paramagnetic ions such as Mn^{2+} . From this broadening the distance between the third site and a Cd^{2+} ion in the CD site was estimated to be less than 0.5 nm. Likewise, the distance between the third site and the EF site was estimated to be more than 1.0 nm (Cavé *et al.*, 1982).

Various effects are observed on the ^{113}Cd resonance from one of the sites of the carp pI4.25 protein when lanthanide ions are added to the Cd^{2+} -loaded protein: broadening with Gd^{3+} ions, upfield shift with the diamagnetic La^{3+} and Lu^{3+} , as well as with Yb^{3+} , and downfield shift with Tb^{3+} . The broadening induced by Gd^{3+} and the different chemical shifts caused by Tb^{3+} as compared with Yb^{3+} are related to the paramagnetic properties of these ions, which act as relaxation or shift probes on the neighbouring $^{113}\text{Cd}^{2+}$ ions when they are bound to the third, secondary, site. The initial shift effect induced by the addition of the various lanthanide ions is always the same, and is most probably caused by the binding of the released Cd^{2+} ions to the third site, since an addition of excess Cd^{2+} results in similar shifts.

In our earlier ^{113}Cd -n.m.r. studies (Cavé *et al.*, 1982) we used the fact that Mg^{2+} selectively causes a high-field shift for the ^{113}Cd resonance of the CD site to assign the resonances for several parvalbumins belonging to the β -phylogenetic lineage (note that the assignments have been reversed). However, the ^{113}Cd -n.m.r. spectra of parvalbumins belonging to the α -lineage were found to be insensitive to addition of Mg^{2+} or other bivalent or univalent cations, and no assignments could be made. We have now observed that the lanthanide ions will cause shift and/or line broadening of the ^{113}Cd resonance from one of the primary sites for the α -lineage parvalbumin from pike. Thus, in contrast with uni- and bi-valent cations, the trivalent lanthanide ions do affect the chemical shift of the high-field-shifted ^{113}Cd resonance of pike parvalbumin in a fashion similar to that observed for the low-field ^{113}Cd resonance of carp parvalbumin. On the basis of an inspection of the three-dimensional structure of the carp pI4.25 parvalbumin Rhee *et al.* (1981) proposed that the third binding site is situated close to the CD site. This suggestion is confirmed by our ^{113}Cd -n.m.r. results. The only difference between the α -lineage and β -lineage parvalbumins in the amino acid residues suggested to comprise the third site is the

substitution of Asp-61 in the β -lineage proteins for Glu-61 in α -lineage proteins.

The Tb³⁺-fluorescence titration of the pike parvalbumin, as shown in Fig. 6, displays a quenching effect similar to the one observed for the carp parvalbumin. For the pike protein the quenching disappears at lower protein and Tb³⁺ concentrations. This was not observed in similar studies of the carp protein. This different behaviour for the two proteins is in agreement with the proposal of a weaker third site in the pike protein as compared with the carp protein. Curve A in Fig. 6 can be used to estimate the binding constant of Tb³⁺ to this site in the pike parvalbumin to be approx. 100M^{-1} . The binding constant for the binding of this cation to the third site in the carp protein can be estimated to be $>10^4\text{M}^{-1}$.

We therefore propose that the α -lineage parvalbumins also have a secondary site, assumed to be close to the CD site. However, this site is much weaker in the α -lineage proteins than in the β -lineage proteins and apparently only binds trivalent cations to a noticeable amount under our experimental conditions. This would explain why this site is not observed as an Mg²⁺ site in ²⁵Mg-n.m.r. or ¹¹³Cd-n.m.r. experiments (Cavé *et al.*, 1979b, 1982). Nevertheless, this weak third site may be useful to assign the ¹¹³Cd resonances in the parvalbumins from the α -lineage.

Intersite interaction

We have already noted above that there is an interaction between the metal ion bound to the CD site and the one bound to the third site, evidenced in ¹¹³Cd n.m.r. as well as in Tb³⁺ fluorescence. This interaction is probably relayed via one or two bridging carboxy groups and does not necessarily alter the structure of the protein. There is also in some examples a noticeable interaction between the two primary sites, which can be observed with ¹¹³Cd n.m.r. As can be seen in Fig. 2, there are during the Lu³⁺ titration transiently more than two ¹¹³Cd resonances from Cd²⁺ bound to the protein. The same is true for the La³⁺ competition experiments for both the carp protein and the pike protein. The new line(s) appearing is most easily explained as being due to the species PaCdLn (where Ln represents lanthanide). This therefore shows that the Cd²⁺ bound to one of the primary sites will sense whether there is a Cd²⁺ or a lanthanide ion in the other primary site. Since this effect is observed for the diamagnetic ions La³⁺ and Lu³⁺, as well as for the paramagnetic lanthanide ions, we conclude that there is a, maybe slight, change in one of the Ca²⁺-binding sites

when Ca²⁺ or Cd²⁺ in the other is replaced by a lanthanide ion. Similar and more dramatic intersite interactions have also been observed for the pair of Ca²⁺ binding sites in the intestinal Ca²⁺-binding protein (Vogel *et al.*, 1985).

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