

Metal Ion Binding to Parvalbumin. A Proton NMR Study

Enzio Ragg,^a Adrien Cavé^b and Torbjörn Drakenberg*

Department of Physical Chemistry 2, Chemical Center, University of Lund, S-221 00 Lund, Sweden ^aInstituto di Biochimica Generale, Via Celoria 2, I-20133 Milano, Italy and ^bC.C.I.P.E., rue de la Cordonille, BP 5055, F-34033 Montpellier-Cedex, France

Ragg, Enzio, Cave, Adrien and Drakenberg, Torbjörn. 1986. Metal Ion Binding to Parvalbumin. A Proton NMR Study. – Acta Chem. Scand. B 40: 6–14.

The ¹H NMR spectra of carp parvalbumin saturated with Ca²⁺, Cd²⁺, La³⁺ and Lu³⁺ were compared, using 2D ¹H NMR techniques as well as conventional ¹H NMR spectra. The Ca²⁺ and Cd²⁺ saturated parvalbumin (with both high affinity Ca²⁺-binding sites occupied) gave rise to very similar spectra. This shows that these two species have almost identical protein conformations. The ¹H NMR spectrum from the Ln³⁺ saturated parvalbumins deviated from the other two and it was therefore concluded that Cd²⁺ is a better probe for Ca²⁺ than Ln³⁺ in parvalbumin and probably also for related calcium binding proteins. The addition of excess of divalent metal ions, such as Mg²⁺ or Ca²⁺, causes small changes in the chemical shift of some methyl resonances. This is presumably caused by binding of these metal ions to a third site close to the CD site which is made up of the carboxylic groups from Glu 60 and Asp 61.

Parvalbumins are a class of small ($M = 11500$), acidic ($pI = 4-5$) and very heat-stable calcium binding proteins.^{1,2} They appear in abundance in fast skeletal muscle, especially in fish. The crystal structures of the calcium and terbium saturated carp parvalbumin P3 have been determined and refined to a resolution of 1.9 Å.^{3,4} It has been found that the protein contains 6 helices, labelled A to F starting from the N terminus. The calcium binding loops are flanked by helices C,D and E,F, respectively and are named the CD and EF sites.

The physiological function of the parvalbumins is presently not fully understood. However, evidence has been presented which suggests that they are involved in the relaxation of the fast muscle, probably by functioning as a calcium carrier.⁵⁻⁷

The calcium ion has a noble gas-like electronic configuration and it is therefore spectroscopically inert except for X-ray absorption³ and ⁴³Ca NMR.^{8,9} Because of the unattractive spectroscopic properties of calcium, various other metal

ions have been used as probes for the calcium-binding sites. The lanthanides substitute very well for calcium. In fact they bind even more strongly than the calcium ion.^{10,11} The lanthanides have therefore been used extensively as probes for metal ion binding to parvalbumins.^{4,10-16} Another probe which has also been used is the Cd²⁺ ion in ¹¹³Cd NMR studies.^{17,18} In all these studies it has been assumed that the used probe ion will induce similar, if not identical, conformational changes in the protein as the native ion calcium does.

It has been shown with X-ray crystallography that the terbium ion first replaces the calcium ion bound to the EF site in the crystal.⁴ Based on this observation it has been assumed that all lanthanides will replace calcium in the same order. We have shown in a preceding article, however, that this is not always the case.¹⁹ Furthermore, we have also presented evidence for the existence of a third metal ion binding site close to the CD site.

The proton NMR spectra of metal saturated parvalbumins show several well resolved resonances.²⁰ We therefore performed a 2D proton NMR study of parvalbumin P3 from carp sat-

*To whom correspondence should be addressed.

urated with various metal ions in order to verify whether or not it is justified to assume that the protein conformation is the same for parvalbumin saturated with Ca^{2+} , Cd^{2+} , La^{3+} or Lu^{3+} .

Experimental

All proton NMR experiments were performed on a Nicolet 360 WB spectrometer operating at 361.79 MHz for protons. Standard ^1H NMR spectra were recorded using a modified WEFT sequence.²¹ The 180° pulse was a soft 5 ms pulse centered at the HDO resonance. In the 2D COSY experiment²² the HDO resonance was saturated using the proton decoupler, which was gated off during acquisition.

Material. The calcium saturated protein (PaCa_2) was prepared as previously reported¹⁸. The lanthanide saturated protein (PaLa_2) was obtained by addition of La^{3+} to PaCa_2 . The cadmium-loaded protein (PaCd_2) was prepared by first adding an excess of $\text{Cd}(\text{ClO}_4)_2$ to PaCa_2 , which was then dialysed against water. This was repeated twice before the protein was lyophilized. The titration experiments were performed by adding small aliquots of stock solutions of the actual metal ion directly into the NMR tube. All experiments were performed at 40°C and at pH 6.5 if not stated otherwise.

Results and discussion

Replacement of Ca^{2+} by Cd^{2+} . The proton NMR spectrum of the Cd saturated parvalbumin of carp 4.25 (PaCd_2) is not very different from that of the native protein which is in the Ca^{2+} form (PaCa_2). The chemical shifts of the most characteristic resonances in the aromatic and aliphatic regions are given in Tables 1 and 2, respectively. The main difference in the aromatic region between the two species is a 0.04 ppm variation for the resonance labelled P_4 (para proton triplet of a phenylalanine labelled number 4) at 6.45 ppm in

Table 2. The ^1H NMR chemical shifts (ppm) of the high field shifted methyl resonances and the protons to which they are spin-coupled.

Ca_2		La_2		Cd_2	LuCa	Lu_2
Me	Me	Me	Me	Me	Me	Me
-0.41	1.54	-0.23	1.58	-0.43	-0.36	-0.36
0.12	0.78	0.08	0.77	0.12	0.12	0.10
0.14	1.37	0.10	1.42	0.17	0.16	0.11
0.26	1.36	0.29	1.37	0.24	0.28	0.30
0.26	1.78	0.29	1.81	0.26	0.28	0.30
0.31	1.55	0.32	1.41	0.31	0.31	0.30
0.31	1.63	0.32	1.61	0.31	0.31	0.30
0.40	1.13	0.48	1.19	0.38	0.43	0.42
0.44	1.54	0.50	1.54	0.44	0.44	0.44
0.52	1.79	0.52	1.83	0.52	0.53	0.52

Table 1. The ^1H chemical shift (ppm) for the aromatic protons in the calcium, cadmium and lanthanum saturated parvalbumin.

	<i>ortho</i>			<i>meta</i>			<i>para</i>		
	Ca	Cd	La	Ca	Cd	La	Ca	Cd	La
1	6.94	6.94	6.99	6.53	6.53	6.54	5.58	5.58	5.62
2	7.22	-	7.23	6.25	6.27	6.27	5.92	5.92	5.92
3	7.04	-	7.07	6.16	6.16	6.13	6.69	-	6.67
4	6.93	-	6.95	6.41	6.41	6.41	6.45	6.49	6.50
5	6.84	6.84	6.85	6.53	6.52	6.54	6.65	-	6.70
6	6.56	6.65	6.70	6.92	-	6.92	-	-	-
7	7.07	-	7.21	6.95	-	6.94	-	-	-
8	7.37	-	7.38	6.98	-	6.99	-	-	-
9	7.45	-	7.44	7.17	-	7.14	-	-	-
10	7.45	7.45	7.49	7.32	-	7.35	-	-	-

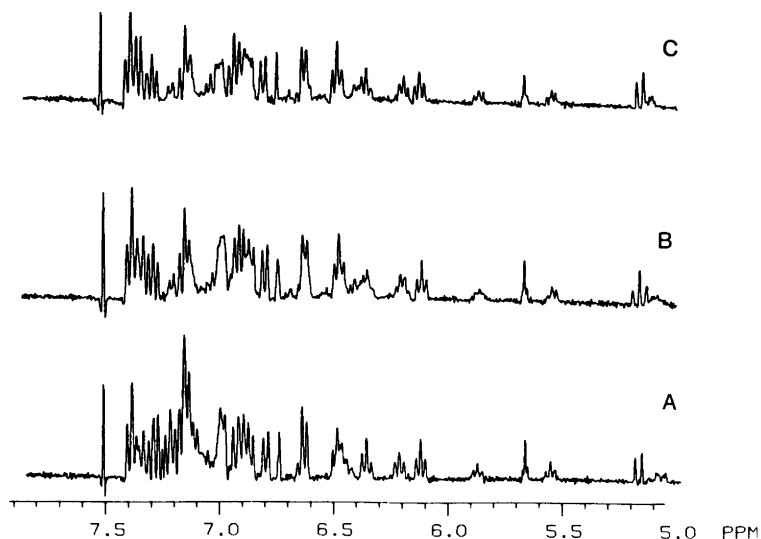


Fig. 1. The aromatic part of the ^1H NMR spectrum from metal saturated parvalbumin. A, PaCd_2 ; B, mixture of PaCd_2 , PaCa_2 and PaCaCd ; C, PaCa_2 . B and C have been obtained by adding Ca^{2+} -ions to A.

PaCa_2 and at 6.49 ppm in PaCd_2 . The main differences observable in the methyl region of the two species are 0.02 ppm variations for the methyl resonances at -0.41 ppm and at 0.40 ppm, which are shifted to -0.43 ppm and 0.38 ppm in the Cd form. The chemical shift variations of resonances at 0.14 ppm and 0.26 ppm in the PaCa_2 species observed at 0.17 ppm and 0.24 ppm in the PaCd_2 species may partly arise from the binding of cations at the secondary site of the protein, as will be described below.

The small amplitude of the chemical shift variations which occur in the proton NMR spectra when going from Cd-loaded to Ca-loaded parvalbumin are well evidenced in the titration of the PaCd_2 species by Ca^{2+} ions, as shown in Figs. 1 and 2. As the Cd^{2+} ions that are bound in the two high affinity sites of parvalbumin are progressively replaced by Ca^{2+} ions several resonances in the aliphatic as well as in the aromatic regions are affected. For instance the two doublets at about -0.4 ppm and $+0.4$ ppm appear transiently as

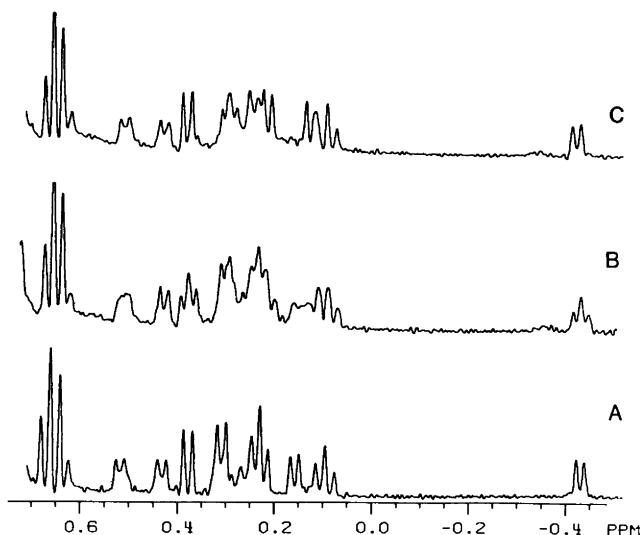


Fig. 2. The upfield shifted region of the ^1H NMR spectrum from metal saturated parvalbumin, 2 mM protein at pH 6.5. A, PaCd_2 ; B, mixture of PaCd_2 , PaCa_2 and PaCaCd ; C, PaCa_2 . B and C have been obtained by adding Ca^{2+} -ions to A.

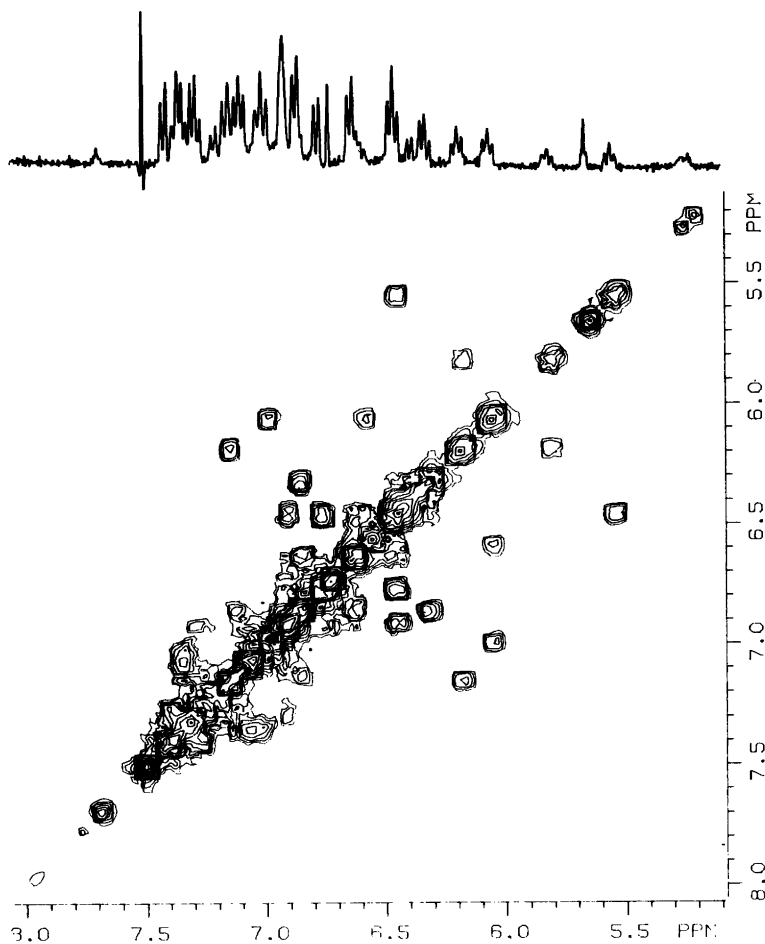
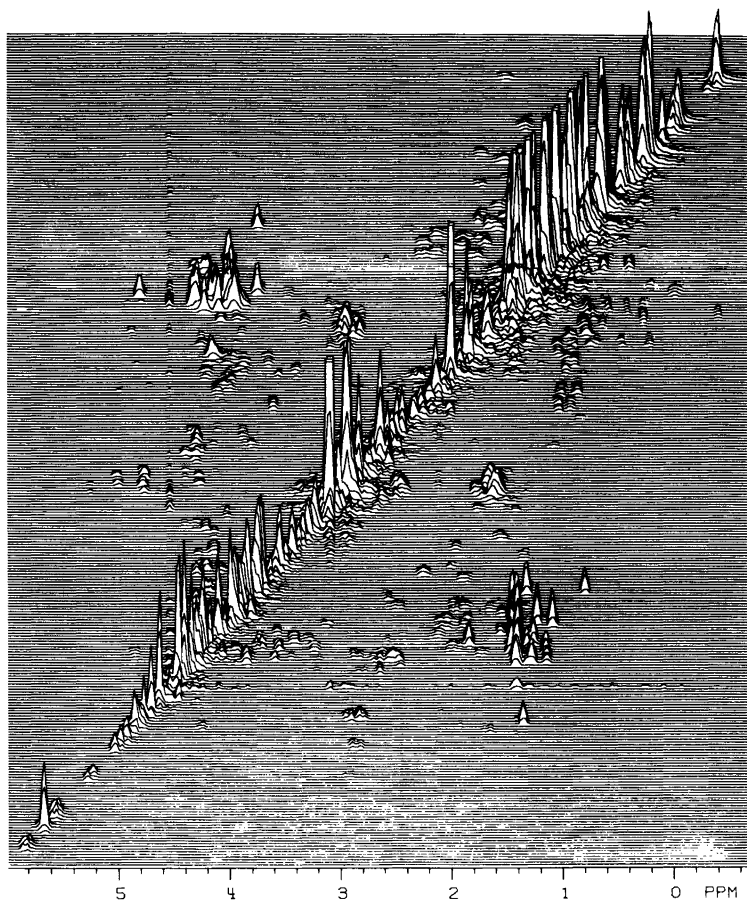


Fig. 3. Contour plot for the COSY ^1H 2D NMR data from PaLa₂, showing the aromatic part of the ^1H NMR spectrum. The protein concentration was 2 mM at pH 6.5 and 40°C. The data was treated with a double exponential multiplication in both dimensions to improve resolution.

well-resolved triplets and then reappear again at higher concentrations of Ca^{2+} ions as well-resolved doublets. Note also the triplet at 6.3 ppm, which appears transiently as a quartet. The shift of the triplet of the para proton of Phe4 at about 6.5 ppm is also well evidenced in this titration. A few resonances like the one at 0.55 ppm broaden transiently. However, most of the aliphatic and aromatic resonances remain completely unperturbed by the Ca/Cd exchange. It can be inferred from these results that the Cd^{2+} ion constitutes an excellent probe for the study of parvalbumins and probably also for other related calcium binding proteins with "EF hand" calcium binding sites.⁴ A shift of only 0.04 ppm is small in comparison with the variations induced by temperature changes²³ in these resonances and they are

also small in comparison to the ring current shifts. These originate from proximal aromatic rings of Phe residues, which are responsible for shifting many resonances of methyl groups and of aromatic protons more than 1 ppm to high field. These ring current shift effects are very sensitive to distance as well as to angular variations.²⁸ Structural changes involving the repositioning of the perturbing aromatic rings would therefore have been observed directly with proton NMR. Since the aromatic rings are essentially localized to the hydrophobic core of the protein it can be assumed that the core of the protein is not affected by the exchange of Ca^{2+} ions by Cd^{2+} ions. This is probably also true for the surface of the protein. However, the proton NMR results cannot be considered conclusive in this respect.

Fig. 4. Stacked plot for the COSY ^1H 2D NMR data from PaLa_2 , showing the high field half of the spectrum. Same conditions as for Fig. 3.



Replacement of Ca^{2+} by La^{3+} or Lu^{3+} . The lanthanides have been very popular as Ca probes due to their various optical and magnetic properties.²⁴ However, they differ from the Ca^{2+} ion by an additional ionic charge and by the ionic size, which decreases through the lanthanide series from La^{3+} (1.15Å) to Lu^{3+} (0.93Å). For comparison the ionic size of Ca^{2+} and Cd^{2+} is 0.99Å and 0.97Å respectively. It is therefore of interest to check by proton NMR if the substitution of Ca^{2+} ions bound in the high affinity sites of parvalbumin by the lanthanides is isomorphous. Many changes occur in the proton NMR spectrum of parvalbumin when La^{3+} ions are added to the sample. These changes are, however, not very extensive as can be seen from data in Tables 1 and 2. The 2D COSY spectra of PaLa_2 (Figs. 3 and 4) were compared to those of PaCa_2 and

PaCd_2 (not shown). The contour plot of the aromatic region of the PaLa_2 sample is shown in Fig. 3. The high-field resonances have nearly the same off-diagonal correlation peaks as in PaCa_2 and PaCd_2 and the resonances have not been shifted by more than 0.05 ppm. Only a few resonances have been assigned to the low field region of the aromatic part due to severe overlap of resonances. However, the 2D NMR spectra allowed us to observe that several of these low field resonances were shifted by as much as 0.1 ppm for PaLa_2 , as compared to PaCa_2 . If the aromatic protons shifted to high field are more representative of the hydrophobic core than the low field, resonances may be assigned to the aromatic residues that are located on the surface of the protein as is the case for Phe 57, whose aromatic ring is located between the primary sites CD and EF.

This would thus indicate that the lanthanum ion perturbs the structure of the surface more extensively than the interior of the protein.

The stacked plot of the 2D NMR spectrum for the aliphatic region of PaLa₂ is shown in Fig. 4. It contains the resonances for the 63 methyl groups that are present in the carp 4.25 parvalbumin. The methyl resonances of Leu, Ile and Val residues are readily distinguished from those of the Ala and Thr residues, the correlation peaks of which appear in quite distinct parts of the plot, around 2 ppm and 4 ppm, respectively. It is not possible to find all the 25 methyl correlation peaks from the 20 Ala and 5 Thr residues, however, among the correlation peaks of these residues one signal appear to be shifted to higher field than the other ones. A previous calculation²³ has established that the methyl group of Ala 14 should be shifted to higher field and the present signal could therefore be assigned to that residue. Similarly it is not possible to find all the correlation peaks belonging to the 5 Val, 9 Leu and 5 Ile residues. The chemical shift of the most upfield shifted resonance and of their connected resonances are reported in Table 2 for comparison with the corresponding resonances of PaCa₂ and PaCd₂. The resonance at -0.4 ppm is predicted to correspond to one of the methyl groups of Val 106 and the triplet resonance at 0.1-0.12 ppm probably correspond to the methyl group of either Ile 97 or Ile 58.²³

The comparison of the stacked plot for the aliphatic region of PaLa₂ with the one of PaCa₂ does not reveal any important changes. This confirms, as previously indicated from the study of the aromatic region, that the tertiary structure of the protein has been conserved, not as well however, as with Cd²⁺ ions. The variations remain small in comparison with the thermal dependencies of many of these resonances and, as previously discussed, they are also small compared to the shifts induced by the rings from nearby phenylalanines, exemplified with the resonance at -0.4 ppm which has been shifted by as much as 1.5 ppm. The variation of 0.1 ppm observed for this high field resonance attributed to Val 106²³ corresponds to a change in the distance of ca 3% between this proton and the aromatic ring that is causing the shift, assuming no change in the angles. We can thus conclude that for parvalbumin also lanthanum functions as a reasonable isomor-

phous replacement for calcium, (For a discussion of ring current shifts see Ref. 25).

The Lu³⁺ ion is an interesting probe ion for the study of parvalbumins since it has been shown that one calcium can be selectively replaced by one lutetium ion.²⁶ Similarly for PaCd₂ this selectivity has also been observed¹⁹ and it is assumed by analogy with the X-ray data^{3,4} that the

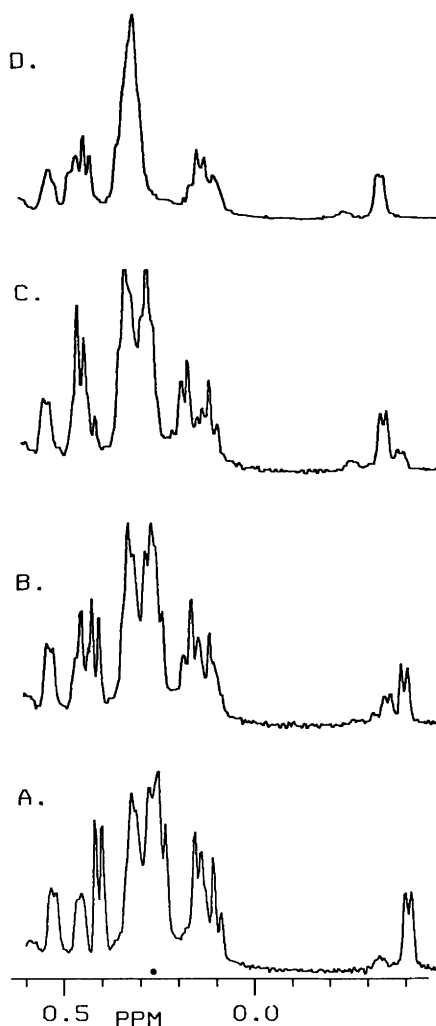


Fig. 5. ¹H NMR spectrum of parvalbumin showing the high field shifted region as a function of added Lu³⁺ ions. Protein concentration 2 mM at pH 6.5. A, no added Lu³⁺; B, 0.7 mM Lu³⁺; C, 1.3 mM Lu³⁺; D, 4 mM Lu³⁺.

EF site is selectively filled with the smaller lanthanides such as Yb^{3+} , Tb^{3+} and Lu^{3+} . The sequential substitution of the Ca^{2+} ions bound in the primary sites of the carp 4.25 parvalbumin has therefore been followed by proton NMR spectroscopy. Fig. 5 shows the ^1H NMR spectra of the carp 4.25 parvalbumin corresponding to the titration of the Ca-loaded protein by Lu^{3+} ions. With 1 equivalent of Lu^{3+} the intermediate

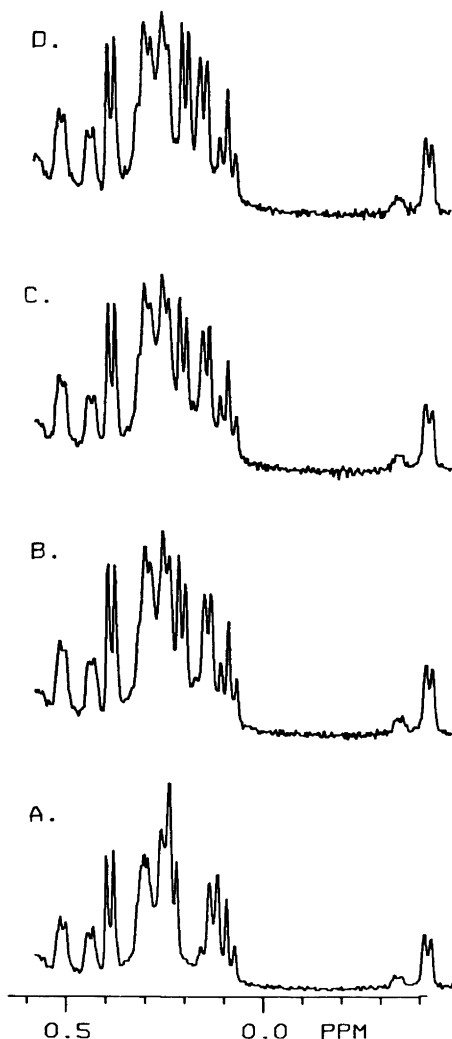


Fig. 6. ^1H NMR spectrum of parvalbumin showing the high field shifted region as a function of added Mg^{2+} ions. 2 mM PaCa_2 at pH 6.5. A, no added Mg^{2+} ; B, 2 mM Mg^{2+} ; C, 4 mM Mg^{2+} ; D, 20 mM Mg^{2+} .

species PaLuCa is produced and the corresponding spectrum is shown in Fig. 5. A distinct high field methyl resonance appears at about 0.05 ppm downfield from the corresponding one in the PaCa_2 species. Similarly the well-resolved doublet at about 0.4 ppm is slightly shifted to low field by about 0.04 ppm. When an excess of Lu^{3+} ions are added to produce the PaLu_2 species the high-field resonance at -0.4 ppm remains unchanged whereas the resonance at about 0.4 ppm is again shifted to higher field as in the PaLa_2 species. The highfield resonance at -0.4 ppm may therefore be considered as representative for the EF primary site (Val 106), whereas the resonance at about 0.4 ppm is representative for the CD site. This is discussed in detail by Corson *et al.*²⁶ The replacement of Ca^{2+} ions by Lu^{3+} is also isomorphous as for cadmium and lanthanum ions. The selective binding of one Lu^{3+} ion offers a possibility to gain information regarding the localization of protons with respect to EF and CD site.

The third metal ion binding site. ^{113}Cd NMR¹⁸ and ^{25}Mg NMR²⁷ studies have shown that an additional cationic site different from the two primary sites is located in the vicinity of one of these two high affinity sites. It is now apparent that the vicinal primary site should be the CD site as was proposed by Rhee *et al.*¹⁵ Proton relaxation enhancement studies using Mn^{2+} have also shown the presence of this additional cationic site, which binds Ca^{2+} and Mg^{2+} ions with the same affinity, whereas the two primary sites bind Ca^{2+} ions preferentially over Mg^{2+} ions.¹¹ The third site is highly hydrated and is therefore located at the surface of the protein.^{15,18} When Mg^{2+} ions are added to PaCa_2 no changes are observed in the aromatic part of the proton NMR spectrum and the Mg^{2+} ions are obviously not able to compete with Ca^{2+} ions for the occupation of the two high affinity sites. However, some significant changes do take place in the methyl region of the NMR spectrum: the two resonance at 0.14 and 0.26 ppm, are shifted by about 0.04 ppm to 0.18 and 0.22 ppm, respectively. The binding constant calculated from these chemical shift changes with variations in Mg^{2+} concentration are in agreement with the one previously calculated for the binding of Mg^{2+} ions to the secondary site, $K = 10^3 \text{ M}^{-1}$. Thus, in contrast to what was observed for the primary sites, the binding of a divalent ca-

tion to the secondary site of the protein does not lead to the appearance of any new resonances, but causes small gradual shifts of some of the resonances. This is related to the shorter life time of the cations bound to this site, which can be estimated to $2 \cdot 10^{-4}$ s for Mg^{2+} .

If, as suggested by Rhee *et al.*¹⁵, the binding of cations in the secondary site involves Asp 53, Glu 59, Asp 61 and Glu 62 then the two methyl resonances at 0.14 and 0.26 ppm, which are both coupled to a proton at 1.36 ppm, are likely to belong to the two methyl groups of Leu 63. These two resonances are predicted to be high field shifted by the ring current effects of Phe residues.²⁸ This secondary site is present in $PaCa_2$ as well as in $PaCd_2$ and the same chemical shift variations of the resonances at 0.14 and 0.26 ppm can be observed upon addition of Mg^{2+} ions or Ca^{2+} ions to the latter (Fig. 6). However, for the La or Lu-saturated parvalbumins this site is not detected in similar proton NMR experiments (data not shown). This is probably related to the additional ionic charge of the lanthanides and to the fact that in the hypothesis of Rhee *et al.*¹⁵ the primary CD site and the secondary site share three carboxylic groups, which could be more attracted by the trivalent cation bound to the CD site than by a divalent cation bound to the secondary site. However, as has been observed by fluorescence, a trivalent cation bound to the secondary site can compete with a trivalent ion bound to the CD site for the bridging carboxylic groups.¹³⁻¹⁵ According to the hypothesis of Ree *et al.*¹⁵ the difference between the parvalbumins of the β -phylogenetic lineage and the parvalbumins belonging to the α -lineage could be related to the exchange of Asp 61 in the β -lineage by a Glu 61 in the α -lineage. It is not immediately obvious that the presence of an extra CH_2 group in the Glu residue would change the binding of cations to the secondary site of parvalbumins as it is presently observed.¹⁸ However, as we observed in our ¹¹³Cd NMR study¹⁹ of the pike parvalbumin (one of the α -lineage proteins) there is also a secondary site in this protein, although it is much weaker. Consequently only trivalent cations do bind to this site in the α -lineage parvalbumins and not divalent cations such as Mg^{2+} or Ca^{2+} .

Conclusion

It has been shown that the structure of parvalbumin (Carp pI 4.25) is very similar, whether it is in the $PaCa_2$ or $PaCd_2$ form. Substitution with La^{3+} and Lu^{3+} causes larger perturbations in the spectra. However, the deviations from the structure with Ca^{2+} are expected to be quite small.

Binding of magnesium to the third site on parvalbumin, which is probably composed of the amino acid side-chains of Glu 60 and Asp 61, causes changes in the chemical shifts of two methyl group resonances, which have been tentatively assigned to Leu 63. Proton NMR spectroscopy appears to be a very sensitive method for detecting small structural changes in parvalbumins.

References

1. Pechère, J. F., Capony, J. P. and Demaille, J. G. *Syst. Zool.* 22 (1973) 533.
2. Kretsinger, R. H. *Annu. Rev. Biochem.* 45 (1976) 239.
3. Moewe, P. C. and Kretsinger, R. H. *J. Mol. Biol.* 91 (1975) 201.
4. Sodwadski, J., Cornick, G. and Kretsinger, R. H. *J. Mol. Biol.* 124 (1978) 123.
5. Heizman, C. H., Berchtold, M. W. and Rowler, R. M. *Proc. Natl. Acad. Sci. USA* 79 (1982) 7243.
6. Gerday, C. and Gillis, J. M. *J. Physiol.* 258 (1976) 96.
7. Haiech, J., Derancourt, J., Pechère, J. F. and Demaille, J. G. *Biochemistry* 18 (1979) 2752.
8. Andersson, T., Drakenberg, T., Forsén, S., Thulin, E. and Swärd, M. *J. Am. Chem. Soc.* 104 (1982) 576.
9. Forsén, S., Andersson, T., Drakenberg, T., Thulin, E. and Swärd, M. *Fed. Proc.* 41 (1982) 2981.
10. Lee, L. and Sykes, B. D. *Biochemistry* 20 (1981) 1156.
11. Cavé, A., Daures, M. F., Parello, F., Saint-Yves, A. and Sempere, R. *Biochimie* 61 (1979) 755.
12. Donato, H. and Martin, R. B. *Biochemistry* 13 (1974) 4575.
13. Nelson, D. J., Miller, T. L. and Martin, R. B. *Bioinorg. Chem.* 8 (1977) 229.
14. Horrocks, W. DeW., Jr. and Collier, W. E. *J. Am. Chem. Soc.* 103 (1981) 2856.
15. Rhee, M.-J., Sudnick, D. R., Arkle, V. K. and Horrocks, W. DeW., Jr. *Biochemistry* 20 (1981) 3328.

16. Cavé, A., Parello, J. and Saint-Yves, A. *Biochimie* 63 (1981) 457.
17. Drakenberg, T., Lindman, B., Cavé, A. and Parello, J. *FEBS Lett.* 92 (1978) 346.
18. Cavé, A., Saint-Yves, A., Parello, J., Swärd, M., Thulin, E. and Lindman, B. *Mol. Cell Biochem.* 44 (1982) 161.
19. Drakenberg, T., Swärd, M., Cavé, A. and Parello, J. *Biochem. J.* 227 (1985) 711.
20. Cavé, A., Pages, M., Morin, Ph. and Dobson, C. M. *Biochimie* 61 (1979) 607.
21. Patt, S. L. and Sykes, B. D. *J. Chem. Phys.* 56 (1972) 3182.
22. Bax, A. *Two-Dimensional Nuclear Magnetic Resonance in Liquids*, Delft University Press, Delft 1982.
23. Cavé, A. and Parello, J. In Balian, R. *et al.*, Eds., *Les Houches*, North Holland Publ. Co., Amsterdam 1981, Session 23R.
24. Reuben, J. *Naturwissenschaften* 62 (1975) 172.
25. Perkins, S. J. In Berliner, L. J. and Reuben, J., Eds., *Biological Magnetic Resonance*, Plenum Press, New York 1982.
26. Corson, D. C., Williams, T. C. and Syke, B. D. *Biochemistry* 22 (1983) 5882.
27. Cavé, A., Parello, J., Drakenberg, T., Thulin, E. and Lindman, B., *FEBS Lett.* 100 (1979) 148.
28. Parello, J., Cavé, A., Quigdomenech, C., Maury, C., Capony, J. P. and Pechère, J. F. *Biochimie* 56 (1974) 61.

Received March 8, 1985.