Cation Binding to Parvalbumin Studied by ^{113}Cd and ^{23}Na NMR. Peak Assignment of Rabbit (p*I* 5.5) Parvalbumin

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Cation binding to three apoparvalbumins was studied by means of ^{113}Cd NMR. The 3 parvalbumins that were investigated were carp p*I* 4.25, rabbit p*I* 5.5 and pike p*I* 5.0. The results showed that Cd^{2+} ions bind to the EF and CD sites of carp apoparvalbumin p*I* 4.25 with about the same affinity. For rabbit (p*I* 5.5) apoparvalbumin, Cd^{2+} binds preferentially to the EF site, while for pike (p*I* 5.0) apoparvalbumin, it was the CD site that exhibited somewhat higher affinity for Cd^{2+}. The effect of Mn^{2+} on the ^{113}Cd signals of rabbit parvalbumin was used to assign the ^{113}Cd NMR signals to the EF and CD sites. The Mn^{2+} paramagnetic effect on rabbit and pike parvalbumins differed from that obtained for carp parvalbumin. This is in agreement with the assumption that the β-lineage parvalbumins possess a third external site of higher affinity than the α-lineage parvalbumins. Furthermore, ^{23}Na NMR was used to study Na^+-Mg^{2+} competition in the native carp (p*I* 4.25) parvalbumin. The results showed that Na^+ and Mg^{2+} compete for the same site, the third external site.

Parvalbumins constitute a family of Ca^{2+} binding proteins found in rather high concentrations in the sarcoplasm of vertebrate skeletal muscles. The physiological function of parvalbumins is not fully understood but one suggestion is that it acts as a buffer for Ca^{2+} in the relaxation process of the muscle.[1-3]

The crystalline structure of 1 parvalbumin, component p*I* = 4.25 from carp, has been determined by X-ray diffraction.[4] Its X-ray crystallographic structure shows that the polypeptide chain is arranged in 6 helical fragments, denoted A to F. The 2 calcium binding sites are formed in the loops between the C and D and the E and F helices and are therefore termed the CD and the EF sites. The ligands in the sites are oxygen atoms in carboxyl, carbonyl and hydroxyl groups, which are octahedrally arranged around the metal ion.[4] In the study of cation binding to calcium binding proteins, ^{113}Cd NMR has been shown to be of particular importance.[5-9] The 2 calcium ions in the native protein can be readily replaced by $^{113}Cd^{2+}$ which possesses suitable magnetic properties for NMR studies. The 2 ions have about the same ionic radius ($r_{Cd^{2+}} = 0.97$ Å, $r_{Ca^{2+}} = 0.99$ Å) and both ions bind preferentially to oxygen atoms. ^1H NMR spectra of carp parvalbumin saturated with either Ca^{2+} or Cd^{2+} have been observed to be very similar.[10]

The chemical shift of the ^{113}Cd nucleus is very sensitive to the number and type of its ligands; the chemical shift range extends over about 800 ppm.[11] The strong binding of Cd^{2+} ions to parvalbumins together with the very sensitive chemical shift of the nuclei make a direct observation of $^{113}Cd^{2+}$ ions bound to the CD and EF sites possible.

The parvalbumins have been classified, on the basis of their primary structure, into α- and β-parvalbumins.[12] Differences in cation binding properties between the α- and the β-proteins have been observed using ^{113}Cd NMR.[5,6]

In this work, the binding of Cd^{2+} ions to 3 apoparvalbumins was studied using ^{113}Cd NMR. One

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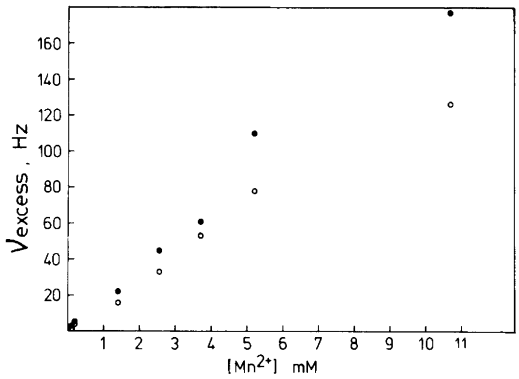

Fig. 1. Excess broadening of the ^{113}Cd NMR signals from 2.1 mM rabbit (p*I* 5.5) parvalbumin as a function of Mn^{2+} concentration. The signal which is somewhat more broadened than the other was assigned to the CD site-bound $^{113}Cd^{2+}$. This signal is the low-field signal in the spectrum. CD (●) and EF (○).

of the proteins, carp p*I* 4.25, belongs to the β-lineage in the phylogenetic tree as described by Pechere;[12] the other two proteins, rabbit p*I* 5.5 and pike p*I* 5.0, belong to the α lineage. ^{23}Na NMR has also been employed to study Na^+-Mg^{2+} binding and competition to the native carp (p*I* 4.25) parvalbumin.

Experimental

Rabbit parvalbumin was prepared, with slight modification, according to Lehky *et al.*,[13] pike parvalbumin was prepared by Eva Thulin, Department of Physical Chemistry 2, Chemical Center, Lund, Sweden and carp parvalbumin was supplied by Professor J. Parello, Montpellier, France. Purity of the proteins was checked by agarose gel electrophoresis. Apoparvalbumin, that is Ca^{2+}-free protein, was prepared by passing an aqueous solution of the protein through a column of Chelex-100. The lyophilized apoprotein was then dissolved in appropriate volumes of water or buffer solution at the time of use.

A 0.1 M ^{113}Cd solution was prepared by dissolving CdO (96.4% isotopically enriched in ^{113}Cd, Oak Ridge, National Laboratory, Oak Ridge, TN) in $HClO_4$. Adjustment of pH was made with Tris to a final pH value of 6.4. Final concentration of $HClO_4$ and Tris was 0.25 M and 0.125 M respectively.

Protein concentrations were determined either by weighing or by UV absorption measurements according to Cavé *et al.*[14]

All NMR spectra were obtained on a home-made Fourier transform spectrometer equipped with an Oxford Instruments 6T wide-bore magnet[15] using the same experimental conditions as reported elsewhere[6]. The ^{113}Cd NMR chemical shifts are given relative to the shift of an aqueous 0.1 M $Cd(ClO_4)_2$ solution with high-field shifts given negative values.

Results and Discussion

*The effect of Mn^{2+} on the ^{113}Cd NMR signals of rabbit (p*I *5.5) parvalbumin.* Fig. 1 shows the line broadening of the ^{113}Cd NMR signals of rabbit (p*I* 5.5) parvalbumin caused by addition of Mn^{2+}. One of the signals is slightly but significantly more affected than the other. This is in agreement with what has been observed for other parvalbumins belonging to the α-lineage in the phylogenetic tree.[6] Consequently, this result was used to assign the ^{113}Cd NMR signals of rabbit parvalbumin to the CD and EF sites.

A dramatic effect of the added paramagnetic ion Mn^{2+} on the ^{113}Cd spectra from the Cd^{2+}-loaded carp p*I* 4.25 parvalbumin has been observed by Cavé *et al.* [6]. Only 1 of the ^{113}Cd NMR signals was observed to broaden upon increasing Mn^{2+} ion concentration. This effect was attri-

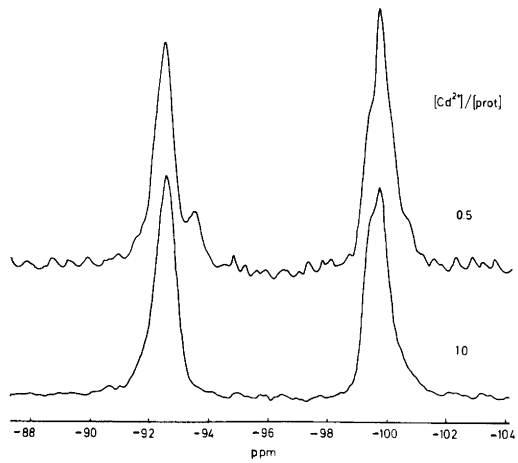

Fig. 2. ^{113}Cd NMR for carp (p*I* 4.25) parvalbumin at 2 different [Cd^{2+}] to [protein] ratios. The low-field signal in the spectrum corresponds to the CD site.[7]

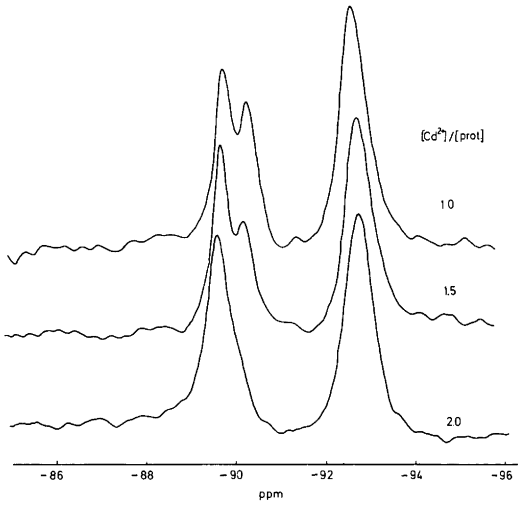

Fig. 3. ^{113}Cd NMR spectra for pike p*I* 5.0 parvalbumin at 3 different $[Cd^{2+}]$ to [protein] ratios. The high-field signal corresponds to CD.[7]

buted to the binding of Mn^{2+} to the third external site close to the CD high-affinity binding site. The effect of Mn^{2+} on $^{113}Cd^{2+}$-loaded pike (p*I* 5.0) parvalbumin was somewhat different.[7] For the pike protein, both ^{113}Cd signals were broadened in a similar way as for the rabbit (p*I* 5.5) parvalbumin.

Drakenberg *et al.*[7] showed from lanthanide-$^{113}Cd^{2+}$ competition experiments on pike that there also exists a third external site in this α-lineage protein. The site was suggested to consist of 2 carboxylate groups from Glu 60 and Asp 61 in β-lineage proteins and from Glu 60 and Glu 61 in α-lineage proteins. However, for the pike protein, it is weaker than for the β-lineage protein of carp (p*I* 4.25). Based on these results, we can thus conclude that the ^{113}Cd signal from rabbit (p*I* 5.5) parvalbumin that is slightly more broadened upon addition of Mn^{2+} ion corresponds to the CD site. This means that the low-field-shifted ^{113}Cd NMR signal from rabbit parvalbumin corresponds to the CD site. Consequently, the ^{113}Cd NMR peak assignments of rabbit parvalbumin (α) and carp parvalbumin (β) are the same, while for pike parvalbumin, it is the reverse, showing that it is, as yet, not possible to make the assignment of the ^{113}Cd NMR resonances based on shifts only.

Addition of $^{113}Cd^{2+}$ to apoparvalbumins. Fig. 2 shows the spectral appearance as $^{113}Cd^{2+}$ ions were added to the carp p*I* 4.25 apoprotein. The 2 signals that correspond to the EF and CD site-bound Cd^{2+} ions increased in intensity simultaneously. The high-field signal, i.e. EF (Cd^{2+}), showed only slightly higher affinity for Cd^{2+} ions. The assignment of the signals to the EF and CD sites are according to Drakenberg *et al.*[7] At low $[Cd^{2+}]$ to [protein] ratio (0.5), it was possible to distinguish 2 slightly shifted signals in addition to the 2 main resonances. These signals of lower intensity probably arose from protein molecules where only 1 of the sites was occupied by Cd^{2+}.

Fig. 3 shows ^{113}Cd spectra for pike p*I* 5.0 at different $[Cd^{2+}]$ to [protein] ratios. The peak assignments for the fully $^{113}Cd^{2+}$-loaded pike parvalbumin were the reverse of that for carp p*I* 4.25. The 2 signals around −90 ppm were probably due to Cd^{2+} ions in the EF site. The intensity of the signal at −90.5 ppm decreased as Cd^{2+} content increased and the CD site became more populated. Therefore, it is likely that the −90.5 ppm reso-

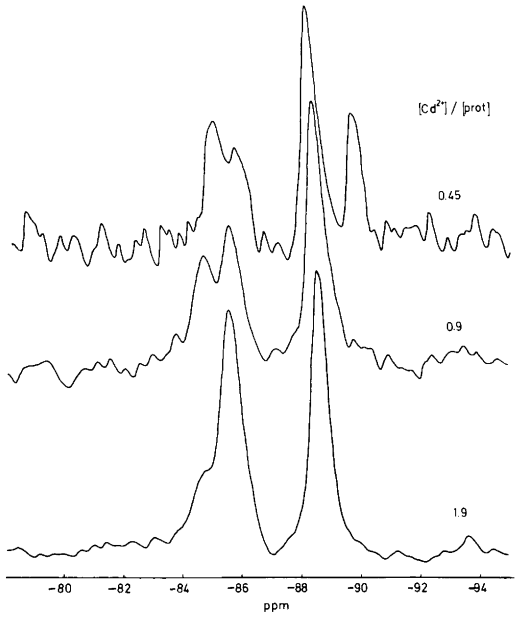

Fig. 4. ^{113}Cd NMR spectra for rabbit (p*I* 5.5) parvalbumin at 3 different $[Cd^{2+}]$ to [protein] ratios. The peak assignments are based upon results from Mn^{2+} line-broadening effects in Figure 1. consequently, the signal at −89 ppm corresponds to the EF site.

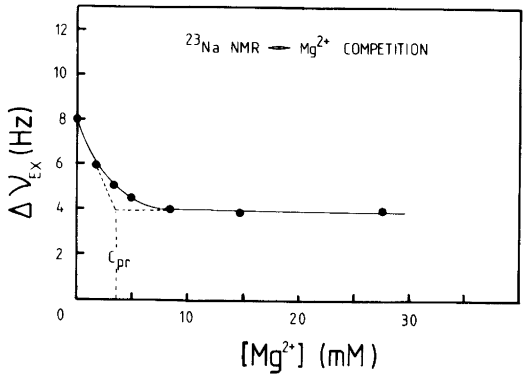

Fig. 5. Excess ^{23}Na linewidth for a 5 mM Na^+ solution, containing 3.8 mM carp (p*I* 4.25) parvalbumin as a function of Mg^{2+} concentration. The protein concentration is marked Cpr.

nance corresponds to EF–Cd^{2+} when there is no Cd^{2+} in the CD site. The signal at −89.5 ppm corresponds to EF–Cd^{+2} when the CD site is filled.

Fig. 4 shows the ^{113}Cd spectral appearances as Cd^{+2} ions are added to the apoparvalbumin of rabbit (p*I* 5.5). The Cd^{+2} ions showed a slight preference for the CD site. At [Cd^{+2}]/[protein]= 0.45, 4 signals appeared, which, from the left to right, probably correspond to CD–Cd^{+2} (EF–O), CD–Cd^{+2} (EF–Cd^{+2}), EF–Cd^{+2} (CD–Cd^{+2}) and EF–Cd^{+2} (CD–O).

The signal at −90 ppm disappeared at [Cd^{+2}]/[protein] = 0.9 depicting the somewhat higher affinity for Cd^{+2} at the EF site. The signals that correspond to the CD site (−84.5 and −85.5 ppm) showed the same behavior as the EF site signals for the pike (p*I* 5.0) parvalbumin. It is not surprising that the ^{113}Cd NMR shift for the Cd^{+2} ion in 1 site is dependent on whether or not there is a Cd^{+2} in the other site. Competition experiments using lanthanide ions has shown the existence of intersite interactions.[7] However, time-dependent spectral appearances for pike parvalbumin (p*I* 5.0) with [Cd^{+2}]/[protein] ≃ 1 have been observed.[20] This time dependence implies that the apoprotein regains its more structured native conformation quite slowly as Cd^{+2} is added. This suggests that the extra ^{113}Cd resonances observed in this work do not reflect the equilibrium population of the various species.

Na^+–Mg^{2+} competition observed from ^{23}Na NMR. Fig. 5 shows the excess ^{23}Na linewidth for a solution containing calcium-loaded carp (p*I* 4.25) parvalbumin as a function of Mg^{2+} concentration. The observed line broadening effect on the ^{23}Na NMR signal in the protein solution shows that Na^+ ions bind to the protein. Furthermore, the decrease in linewidth upon increasing Mg^{2+} concentration also shows that Na^+ and Mg^{2+} compete for the same site. It has been observed from ^{113}Cd NMR that Mg^{2+} does not compete with Cd^{+2} for the high-affinity sites.[6] It can thus be concluded that Mg^{2+} and Na^+ compete for the third external site. Earlier reports suggested that Na^+ binds with comparatively strong affinity for 1 of the high-affinity sites in a half-decalcified parvalbumin.[16,17] In another report, no significant binding of Na^+ to parvalbumin was observed and the results of Ref. 17 and 18 were attributed to a Na^+–EGTA complex interacting with the protein.[19] The result in Fig. 5 shows that Na^+ binds to parvalbumin which contains Ca^{2+} in the 2 high-affinity sites and that Mg^{2+} competes with Na^+ for this site.

Concluding remarks

Important differences in ion binding properties between α-and β-lineage proteins have been demonstrated.[6,7] From the work presented here, it is clear that the differences still prevail even though the proteins have been deionized and, in this form, are presumably in a less structured state. In the carp parvalbumin (p*I* 4.25), the EF and CD sites bind Cd^{2+} with about the same affinity. In the pike (p*I* 5.0) and the rabbit (p*I* 5.5) parvalbumins, 1 of the sites binds Cd^{2+} slightly stronger or faster. For pike parvalbumin, the EF site is preferentially filled, while for rabbit parvalbumin, it is the CD site that is filled first.

The line broadening effect of the paramagnetic ion Mn^{2+} on ^{113}Cd NMR signals of rabbit (p*I* 5.5) parvalbumin was used to assign the signals to the EF and CD sites. Furthermore, results from ^{23}Na NMR showed that Na^+ and Mg^{2+} compete for the same site, the third external site, in a Ca^{2+}-loaded carp (p*I* 4.25) parvalbumin.

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