NON-EQUIVALENCE OF THE CD AND EF SITES OF MUSCULAR PARVALBUMINS. A ^{113}Cd NMR STUDY

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1. Introduction

The tertiary structure of parvalbumins is well documented by the determination of atomic coordinates for the component pI 4.25 from carp muscle by X-ray crystallography [1]. A significant feature of this structure is the presence of two calcium ions at sites named CD and EF respectively [2]. Six oxygen atoms are liganded to the central cation in an octahedral arrangement, with Ca · · · O distances ranging from 2.15–2.85 Å [1]. The first site contains solely protein ligands: four carboxyl groups, a peptide bond carbonyl group and a serine hydroxyl group. The site EF also contains four carboxyl groups and a carbonyl group but the sixth ligand is provided by a water molecule [1]. This suggests that the parvalbumin sites CD and EF will have different physico-chemical and functional properties.

Studies of the binding of Ca(II) by various techniques have shown that parvalbumins have two cationic sites with high affinity for calcium ions [3–5], but have not been able to distinguish between the two sites. It has also been shown that many other cations compete for the ionic sites of parvalbumins [6–9]. Specific binding of Na(I) [10] and Mg(II) [11] has been observed using ^{23}Na and ^{25}Mg NMR spectroscopy. Kinetic aspects of the binding of Ca(II) to parvalbumins have been investigated directly by ^{43}Ca NMR [12]. Although well adapted to characterization of cation binding, these NMR studies have not provided evidence of any non-equivalence of the two ionic sites.

As the chemical shifts of ^{113}Cd(II) are very sensitive to the chemical nature of the ligand groups involved in complex formation [13], it seemed likely that the CD and EF sites could be distinguished by ^{113}Cd NMR spectroscopy. Calcium and cadmium have very similar ionic radii and are likely to compete for the two sites. It has been shown that the ^{113}Cd NMR signal from cadmium bound to various proteins can be readily observed [14–16]. In the present work it is shown that the cadmium ions bound to the carp parvalbumin molecule give rise to two ^{113}Cd NMR signals with distinct chemical shifts. It is therefore possible to make competition studies, giving information for each site separately, by ^{113}Cd NMR spectroscopy. This is greatly facilitated by a most favourable signal-to-noise ratio as compared with previous reports on ^{113}Cd NMR of protein solutions.

2. Materials and methods

Carp parvalbumin, component pI 4.25 from the muscle of *Cyprinus carpio*, was used throughout this work. It was prepared according to the procedure of Pechère [17] and contained 2.4 calcium ions per molecule (atomic absorption spectrophotometry

[5]). The concentration was determined by amino acid analysis.

A solution of $^{113}Cd(II)$ was prepared by dissolving ^{113}CdO (from Oak Ridge, USA; isotopic enrichment: 96.3%) in 0.5 M H_2SO_4 and made up to a final volume of 4 ml containing 21 mM Cd(II), 0.23 M Na(I), 63 mM Tris–H_2SO_4, 0.5 mM dithiothreitol (DTT) at pH 6.9. To this solution lyophilized parvalbumin was gradually added to a final concentration of 10.4 mM. pH was varied using 1 M NaOH or 1 M H_2SO_4. Gd(III) was added from a solution containing 80 mM $GdCl_3$ and 100 mM Tris–HCl at pH ~ 6.

The ^{113}Cd NMR spectra were run at 30°C on a modified Varian XL-100-15 spectrometer operating on the FT mode at 22.2 MHz and using a normal 20 mm O.D. test tube in a laboratory made probe. The FT parameters used throughout this work were: spectral width 5000 Hz, acquisition time 0.5 s, weight function 0.02 s (16 Hz line-broadening), flip angle 70° (pulse width 70 μs) and 2000 transients. A 1 M $Cd(ClO_4)_2$ solution was used as an external reference (positive shifts to low field).

3. Results and discussion

Figure 1 shows a ^{113}Cd NMR spectrum of a cadmium–parvalbumin solution at pH 5.9 with a molar ratio Cd(II)/protein close to 2. The free cations appear as a single signal at +3.5 ppm while protein-bound $^{113}Cd(II)$ ions appear as two distinct signals of nearly equal intensity at –93.8 ppm (signal 1) and –97.5 ppm (signal 2). It is reasonable to assume that the two signals correspond to cadmium ions bound to the CD and EF sites of the parvalbumin molecule.

The observation of two distinct chemical shifts clearly shows that the two ionic sites of parvalbumin in solution have different structures. The small shift difference indicates however that the local environment for the cadmium nuclei in the two sites is similar. This is in agreement with observations on the solid state [1]. The displacement towards high-field of the protein-bound ^{113}Cd signals, relative to the free ^{113}Cd signal indicates that the cadmium ions are coordinated only to oxygen atoms. The carboxyl group is known to cause upfield shifts of the $^{113}Cd(II)$ NMR signal [18] and preliminary data indicate that the shift of $(RCOO)_2Cd$ complexes is about –35 ppm [19]. The most negative shift reported previously is that of 4 M $Cd(NO_3)_2$ in water, –65 ppm [18]. The shift values of about –100 ppm observed for the cadmium nuclei bound to the parvalbumin molecule, can be related to the presence of four carboxyl groups in the CD and EF sites. Ligands containing coordination

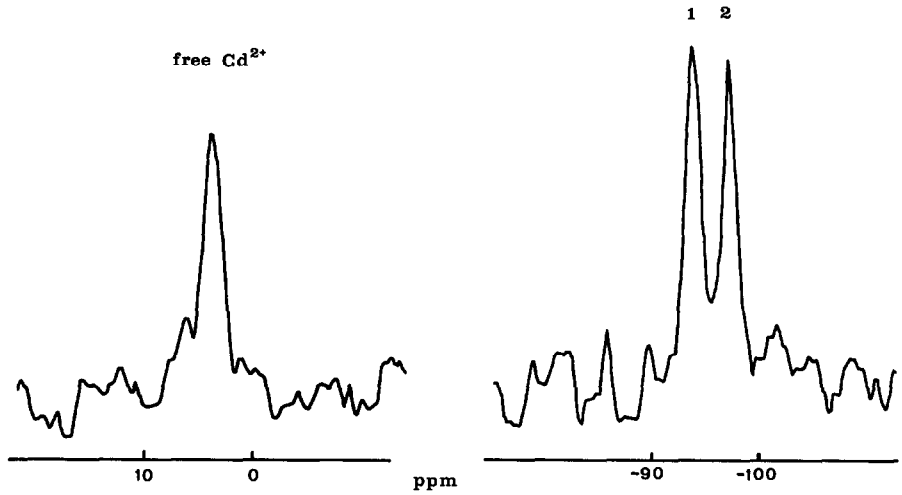

Fig.1. ^{113}Cd NMR spectrum of $^{113}Cd(II)$–parvalbumin at 30°C. Shifts relative to external 1 M $Cd(ClO_4)_2$. Conditions: 8.8 mM carp parvalbumin pI 4.25, approx. 21 mM Ca^{2+}, 17.3 mM Cd^{2+} (16.6 mM $^{113}Cd^{2+}$), 0.28 M Na^+, 5.3 mM K^+, 5.3 mM Cl^-, 53 mM Tris–sulphate, 0.45 mM DTT at pH 5.9. Volume 4.74 ml. Total acquisition time 17 min.

atoms other than oxygen are found to induce downfield shifts [13].

The fact that no broadening of signals 1 and 2 is observed under various conditions indicates that the exchange rate between free and bound cadmium ions is low on the NMR time scale (less than 10^2 s^{-1} for the decomposition rate). In this respect Cd(II) behaves similarly to Ca(II) as shown by ^{43}Ca NMR spectroscopy [12]. However, in the last case no chemical shift effects could be detected on binding to the protein. The only divalent cations in the parvalbumin solution were Cd(II) and Ca(II) in nearly equal amounts, the latter coming from the protein itself. The reported experiment in fig.1 therefore represents a study of the competition between these two ions monitored by ^{113}Cd NMR spectroscopy. About 30–40% of the total cadmium content remains free in solution, and the intensities of signals 1 and 2 are similar. This indicates that Cd(II) has a slightly greater affinity for parvalbumin than Ca(II), in agreement with competition experiments carried out with carp parvalbumin loaded with Gd(III) [9]. The ^{113}Cd NMR results show further that the binding constants of Ca(II) and Cd(II) are very similar for both CD and EF sites. Signals 1 and 2 will include contributions from the parvalbumin species, Pa(Cd, Ca) and/or Pa(Ca, Cd), since these are unlikely to be resolved in the ^{113}Cd NMR spectra from signals due to the fully-loaded parvalbumin, Pa(Cd, Cd).

The effect of pH upon the chemical shifts of signals 1 and 2 was investigated in the range 5.9–9.75. In contrast to the free cadmium signal, which is displaced by more than 50 ppm downfield as pH is varied from 6.9 to 8.2, signals 1 and 2 are relatively insensitive to pH variations from 5.9 to 9.3 (fig.2). This accords with the relative inaccessibility of the parvalbumin-bound cations as shown by X-ray crystallography [1]. However a slight dependence of the chemical shift is observed for signal 1 (about 1 ppm downfield from pH 6 to 9). This variation, while signal 2 remains unaffected within the error limits (± 0.2 ppm), suggests that signal 1 corresponds to the ^{113}Cd(II) ions bound to the site EF which is in contact with the external medium through a water molecule.

Above pH 9 the intensity of signals 1 and 2 is reduced, but no broadening is observed, and at pH 9.75 both disappear in the noise. In contrast the free cadmium signal is very broad at pH 8.5 and is no longer observed above pH 9.3. A plausible explanation for this broadening is the formation of complexes between Cd(II) and free amino groups on the exterior of the protein. It has, for example, been shown that the exchange between free cadmium and cadmium–glycine complexes is of the right magnitude to give extensive broadening [18,19]. The disappearance of signals 1 and 2 at pH values higher than those where the free cadmium signal vanishes also accords with a protecting effect due to complexation of cadmium at sites CD and EF. A similar situation has been

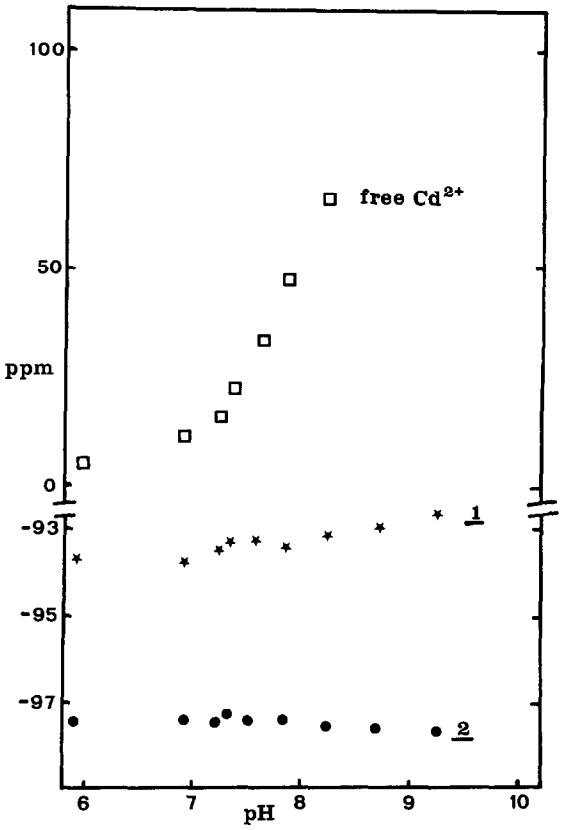

Fig.2. pH dependence at 30°C of the ^{113}Cd NMR chemical shifts. Initial solution (4.0 ml) containing 10.4 mM carp parvalbumin pI 4.25, approx. 25 mM Ca^{2+}, 21 mM Cd^{2+} (20.2 mM $^{113}Cd^{2+}$), 0.23 M Na^+, 63 mM Tris–sulphate, 0.5 mM DTT at pH 6.9. Aliquots of 1 M NaOH were added up to pH 9.75. Reversibility was checked by adding 1 M H_2SO_4 progressively to pH 5.9. Final volume 4.69 ml. Signal 1 ★, signal 2 ●, free Cd^{2+} □.

described for free and protein-bound gadolinium : in the latter case pH can be increased by about one additional unit before metal-hydroxide formation occurs [20].

The effect of chloride anions on the chemical shifts of ^{113}Cd signals was investigated by adding increasing amounts of KCl to the cadmium–parvalbumin solution at pH 5.9. It is known that chloride affects the chemical shift of $^{113}Cd(II)$ in water [21]. A variation in chemical shift from +4.0 ppm to +8.3 ppm is observed for the free cadmium signal when the KCl concentration is increased to a final value of 0.01 M. Under the same conditions there is no displacement of signals 1 and 2. This indicates again that the cadmium ions remain essentially inaccessible to the external medium when bound to the protein.

Competition experiments between Ca(II) and Gd(III) ions have shown that the two calcium ions found in native carp parvalbumin pI 4.25 can be replaced by two gadolinium ions [5]. Because of the difference in chemical shift between signals 1 and 2 observed with carp parvalbumin, ^{113}Cd NMR spectroscopy is a very suitable method for the study of ionic exchange in parvalbumins. When Cd(III) is added progressively to the cadmium–parvalbumin solution, signal 1 disappears first, then at higher metal concentration signal 2 vanishes. In both cases a progressive reduction of the signal intensities is observed without any significant broadening. Since Ca(II) in site EF of carp parvalbumin crystals can be selectively replaced by the lanthanide ion Tb(III) [6], it can be assumed that signal 1 corresponds to the $^{113}Cd(II)$ ions located in the EF site of the parvalbumin molecule in solution. This is in agreement with the pH dependence of signal 1. The data of fig.3 give an association constant ratio, K_{Gd}/K_{Cd}, for the EF site that is at least 8 times larger than for the CD site.

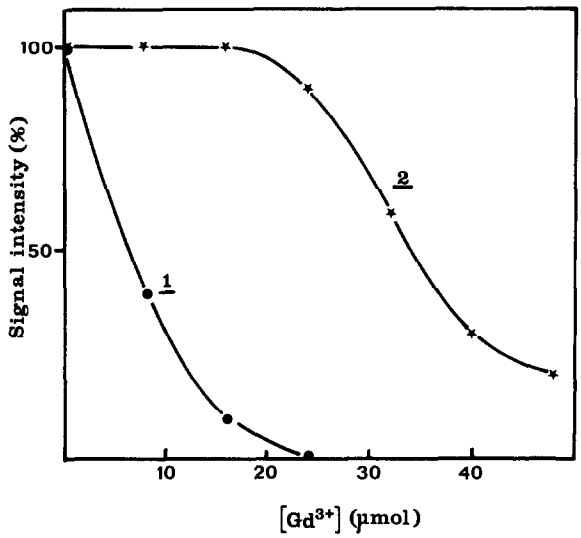

Fig.3. Dependence of the intensity of signals 1 and 2 from parvalbumin-bound ^{113}Cd ions as a function of added Gd^{3+}. Same conditions as in fig.1. Six aliquots of 100 µl of an 80 mM Gd^{3+} solution (section 2) were added successively. Signal 1 • and signal 2 ⋆.

4. Conclusion

The present work shows that ^{113}Cd NMR spectroscopy is particularly well adapted for characterization of the ionic sites CD and EF of parvalbumins in solution. This is due to the strong dependence of ^{113}Cd chemical shifts on the chemical nature of the ligands encountered in cadmium complexes. Sharp signals are observed for parvalbumin-bound cadmium ions since the decomposition rate of the cadmium–parvalbumin complex is very low on the NMR time scale.

Parvalbumins are acidic proteins and it can be expected that the carboxyl groups of the aspartic and glutamic residues, which are mainly located on the surface of the molecule [22], interact with cations. ^{113}Cd NMR signals, corresponding to secondary sites, were not seen and may be difficult to detect because of low affinity and unfavourable kinetic effects. Controlled competition experiments can be performed with the aim to prepare parvalbumins substituted with different cations on the two CD and EF sites. It has been considered that such hybrid structures may play a role in muscle activity through calcium–magnesium exchange [23]. The results of fig.3 are of particular interest as they suggest a method of preparing lanthanide-parvalbumins with a well-defined occupancy of the two sites. These molecular species would allow a careful analysis of the high-resolution ^{1}H NMR spectra [24,25] and thus the possibility of studying the conformational features of parvalbumins in solution.

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