A ^{43}Ca NMR STUDY OF THE BINDING OF CALCIUM TO PARVALBUMINS

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1. Introduction

Association between ions and biological molecules (proteins, nucleic acids, sugars) is important in the structure and function of many biological systems. Many of the ions so involved (alkali, alkaline earth, halide ions) lack characteristic spectroscopic properties. However, NMR detectable isotopes can sometimes be used. The spin quantum numbers of these nuclei are usually greater than 1/2 so that the nuclear magnetic relaxation is governed mainly by quadrupolar effects which show important variations when an ion–macromolecule complex is formed, due to the local dynamics and the electrical environment of the bound ion.

The quadrupolar relaxation of alkali and halide nuclei has been extensively studied and several chemical and biological applications have been developed [1,2]. For biological systems, interest is mainly on Mg^{2+} and Ca^{2+} but because of the low inherent sensitivity of the NMR detectable isotopes there have been very few studies using these nuclei [3]. However, ^{25}Mg and ^{43}Ca NMR investigations of isotopically-enriched samples on Fourier transform spectrometers should be most significant for many biological problems.

We report here on the feasibility of using the resonance of calcium-43 in the study of parvalbumins. These calcium-binding proteins, present in the muscles of almost all vertebrates, have a low molecular weight (about 11 500), a high water solubility, an acidic isoelectric point, a high phenylalanine content and a strong affinity for calcium ions [4,5]. The tertiary structure of a parvalbumin component from carp muscle (characterized by its isoelectric point, pI 4.25; [6]) has been established by X-ray crystallography [7,8]. The two calcium ions are located at very specific sites in the molecule. In both sites, the immediate environment of the calcium ion is an octahedral arrangement of oxygen atoms in carboxyl, carbonyl and hydroxyl groups. However, the two sites differ in their exposure to the solvent: one site is occupied by ligand groups belonging exclusively to the protein, whereas the other contains a water molecule as an external ligand [9].

The arrangement of the first coordination sphere of the parvalbumin-bound Ca^{2+} ions is less symmetrical than that of the aquo-cation. A considerable change in the ^{43}Ca quadrupole relaxation can therefore be expected to accompany Ca^{2+} fixation and will affect the half-width and the relaxation times of the ^{43}Ca resonance of a calcium–parvalbumin solution. These will also be affected by the rate of exchange of the Ca^{2+} ions between the free and the protein-bound states.

In the present investigation, the half-width of the ^{43}Ca signal was measured for isotopically enriched ^{43}Ca solutions containing varying concentrations of carp parvalbumin, pI 4.25, under different conditions of temperature and pH. Some T_1 measurements were also made in parallel. This is the first demonstration of an effect of a biological macromolecule on the relaxation of $^{43}Ca^{2+}$.

2. Materials and methods

Parvalbumin, pI 4.25, from carp muscle (*Cyprinus carpio*) was isolated by the procedure in [6] and used as a lyophilized powder. The calcium content was

2.4 Ca^{2+} ions/parvalbumin molecule, as determined by atomic absorption spectroscopy (Perkin-Elmer 303 apparatus) in the presence of La^{3+} ions.

^{43}Ca was purchased from Oak Ridge (TN USA) as $^{43}CaCO_3$ (61.63% isotopic enrichment).

All chemicals were of analytical grade. Tris was either 'Trizma base' or 'Trizma HCl' from Sigma (St Louis, MO). Dithiothreitol (DTT) was from Calbiochem (San Diego, CA). Tris-buffer, 75 mM, pH 8.1 containing 15 mM DTT was prepared by dissolving the required quantities of Trizma HCl and Trizma. ^{43}Ca solutions were prepared by dissolving a carefully weighed quantity of $CaCO_3$ in Tris-buffer and the pH adjusted to the desired value using 1 M HCl or 1 M NaOH. Total Ca^{2+} conc. approx. 0.1 M. A stock solution containing 9 mM parvalbumin was prepared in Tris-buffer. High protein concentrations were obtained by adding the lyophilized powder directly.

All pH measurements were made at room temp. (about 20°C) using a Radiometer PHM 64 pH-meter. NMR experiments were performed with a Varian XL-100 spectrometer adapted for ^{43}Ca resonance in the Fourier transform mode by using a specially built probe operating at 6.73 MHz and equipped with a variable temperature device. Tubes of 12 mm diameter filled with 1.5–2.0 ml solution were used. Line-shape experiments, performed under non-spinning conditions, usually required 500 transients with an acquisition time of 1 s or more in order to achieve a reasonable signal-to-noise ratio. The half-width of the observed signal is denoted as $\Delta\nu_{1/2}$ (in Hz). The accuracy of the half-width measurements was estimated to be ± 0.6 Hz. T_1 measurements were carried out using the $(180°–\tau–90°)_n$ pulse sequence. In the absence of parvalbumin these showed that the half-width $\Delta\nu_{1/2}$ is due mainly to magnet inhomogeneity.

3. Results

3.1. *Effect of the calcium-to-protein ratio*

When parvalbumin was added progressively (up to 7×10^{-4} M) to a 0.1 M Ca^{2+} solution (Ca^{2+}/protein molar ratio about 150) no significant variation of $\Delta\nu_{1/2}$ was observed, at pH 7.2, even when raised to 58°C, a temperature within the stability range of the native structure [10,11]. When the protein concentration was raised to 2.5×10^{-3} M a slight increase in $\Delta\nu_{1/2}$ of about 1.5 Hz was observed at 58°C. This is certainly because the exchange between bound and free calcium ions is relatively slow on the NMR time scale. At this parvalbumin concentration the molar fraction of bound calcium ions does not exceed 5%, at which concentration of bound calcium, rapid exchange conditions would be characterized by a very significant signal broadening, taking into account the local dynamics and electric field gradients around the bound cation. It can be estimated that the correlation time would increase by a factor of about 10^2 if protein molecule reorientation causes relaxation. The contribution due to variations in electric field gradients is difficult to evaluate because precise information on the symmetry of the Ca^{2+}-ligand coordination is required.

3.2. *Effect of temperature*

The temperature was gradually increased using a 0.1 M Ca^{2+} solution containing 7×10^{-4} M parvalbumin. A significant broadening was observed above 65°C and this increased progressively on raising the temperature (fig.1). At 95°C the broadening was about 3 times that at 65°C. An independent experiment with a 0.085 M Ca^{2+} solution containing 2.5×10^{-3} M parvalbumin (Ca^{2+}/protein molar ratio about 35) showed a more pronounced effect. The observed dependence of $\Delta\nu_{1/2}$ on the parvalbumin concentration at high temperatures indicates that conditions allow rapid exchange of calcium ions between the free and the protein-bound states. This phenomenon is reversible since the half-width was practically re-established at its initial value when lowered to 55°C (fig.1).

Proton NMR studies have shown that the tertiary structure of carp parvalbumin, pI 4.25, is deeply perturbed when raised above 80°C [10,11]. The high-temperature state is less organized than the native globular structure defined at lower temperature. At least a part of the increase of the ^{43}Ca half-width in the 65–95°C range (fig.1) is therefore associated with a conformational transition in the protein. There are two possible interpretations of this result:

1. The mean life-time of calcium ions on the native protein diminishes so that rapid exchange conditions are approached as the temperature is increased (an estimation based on the relaxation rate at high

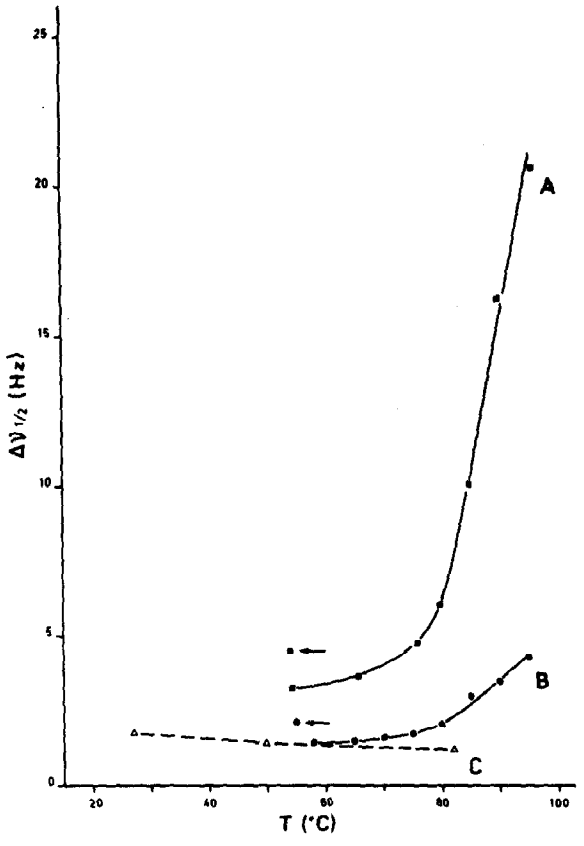

Fig.1. ^{43}Ca resonance experiment at variable temperature. Variation of $\Delta\nu_{1/2}$ at pH 7.7 (reversibility is indicated by an arrow). (A) Solution containing 0.085 M Ca^{2+}, 0.17 M Na^+, 0.044 M Tris, 0.009 M DTT and 2.4 × 10^{-3} M carp parvalbumin. (B) Solution containing 0.1 M Ca^{2+}, 0.1 M Na^+, 0.045 M Tris, 0.007 M DTT and 6.8 × 10^{-4} M carp parvalbumin. (C) Same solutions as (B) without added parvalbumin.

temperature gives a life-time for the Ca^{2+}-parvalbumin complex of the order of 10^{-3} s or longer).

2. The high-temperature conformation of parvalbumin is able to interact with calcium ions and the exchange between the free and the bound states is rapid on the NMR time scale.

Both processes may play a role during the melting transition. The fact that no sharpening of the ^{43}Ca signal is observed at very high temperature establishes the participation of the second process. It is known that parvalbumin loses its affinity for calcium when the temperature is raised [12]. This does not preclude parvalbumin retaining a more or less organized structure with some affinity for calcium ions at high temperatures. ORD measurements have shown that the structure of carp parvalbumin, pI 4.25, does not correspond to a characteristic random coil even at very high temperatures [13].

3.3. *Effect of pH*

This was investigated at 54°C in the pH range 7–12, using a calcium to protein ratio of about 35 (fig.2). Between pH 7 and pH 8.5 no effect on the

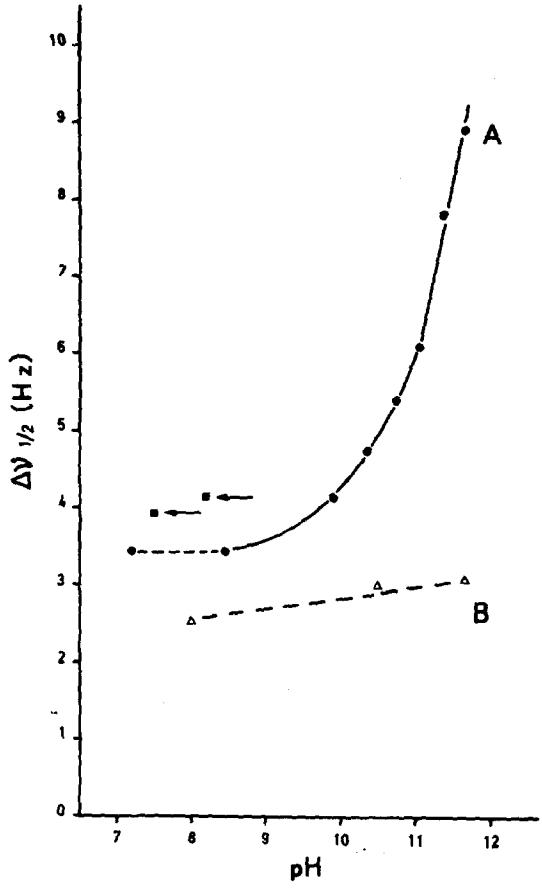

Fig.2. ^{43}Ca resonance experiment at variable pH. Variation of $\Delta\nu_{1/2}$ at 54°C. (A) To a solution containing 0.089 M Ca^{2+}, 0.089 M Na^+, 0.04 M Tris, 0.006 M DTT and 2.6 × 10^{-3} M carp parvalbumin, at pH 7.2, very small vol. 4 M NaOH were added successively up to pH 11.6. Reversibility was checked by adding small vol. 4 M HCl (indicated by an arrow). (B) Without protein containing 0.085 M Ca^{2+}, 0.1 M Na^+, 0.045 M Tris and 0.009 M DTT, at pH 2.2. Small vol. 4 M NaOH were added to increase pH up to 11.6 with a final Na^+ concentration of about 0.17 M.

^{43}Ca half-width was observed. Above pH 9, a broadening was readily observed and at pH 11.6 this was about 3 times larger than that at lower pH. The reversibility of the phenomenon was established by progressively adding HCl. The values of $\Delta\nu_{1/2}$ observed at pH 8.2 and 7.5 in samples which had first been brought to pH 11.6 were only slightly higher than those originally observed in the lower pH range. Effects of the changes in ionic strength on $\Delta\nu_{1/2}$ during the pH variations were not investigated.

High resolution ^{1}H NMR spectroscopy was used to establish independently whether the broadening observed when the pH was varied is due to a conformational change of the parvalbumin molecule. A reversible transition was observed (at 52°C) in the pH range 10–12. Figure 3 shows a comparison between the low-field region of the NMR spectra of carp parvalbumin at pH 6.8 and at pH 12.5. In the native state (neutral pH) the phenylalanine aromatic resonances are spread between 5.5 ppm and 7.5 ppm [11], characteristic of the presence of a highly-structured internal hydrophobic core [10]. At high pH the aromatic resonances appear between 6.5 ppm and 7.5 ppm, the protein still retaining some of the characteristics of the globular native structure. As

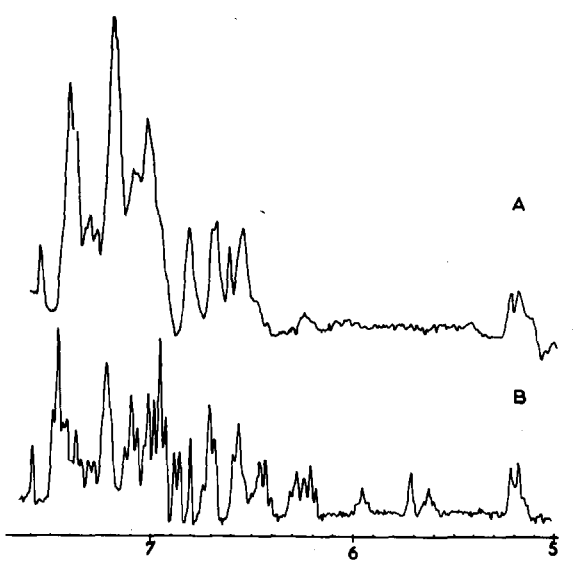

Fig.3. Low-field region of ^{1}H NMR spectra at 270 MHz of carp parvalbumin, pI 4.25, in D_2O at 52°C (protein conc. approx. 8×10^{-3} M; chemical shifts referred to internal DSS): (A) pH 12.5 (with added NaOH). (B) pH 6.8.

for the melting transition, the results in fig.2 suggest that parvalbumin is able to interact with calcium ions even at a very high pH with rapid exchange rates on the NMR time scale.

4. Discussion

The present ^{43}Ca experiments clearly show that calcium exchange in parvalbumin, pI 4.25, is slow on the NMR time scale under physiological conditions, with a rate of decomposition of the calcium–parvalbumin complex less than 10^3 s^{-1}. It is likely that these slow exchange characteristics are related to the very significant loss of water molecules on binding of calcium. Kinetic effects have recently been invoked to explain the role of calcium in parvalbumins during muscle contraction [14]. Rapid exchange is observed either at high temperatures or at high pH values. The parvalbumin molecule adopts defined conformational states different from the native state as inferred from ^{1}H NMR spectroscopy. The new conformations still interact with calcium ions but with rapid exchange rates on the NMR time scale.

The fact that the ^{43}Ca resonance in solutions of carp parvalbumin is unaffected in the pH range 7–9 deserves consideration. It has been reported that the Ca^{2+} binding capacity of a related parvalbumin (component, pI 4.47, from carp muscle) was strongly pH dependent within this range [15] and that about 6 calcium ions are liberated from the protein at pH 8.5. This large number of calcium ions would be consistent with the reported occurrence of secondary binding sites in muscular parvalbumins [16]. However this result is questionable. If secondary Ca^{2+} binding sites were to exist on parvalbumins, they would be on the protein surface where most of the Asp and Glu residues are located [7]. The present results with ^{43}Ca NMR were obtained using relatively high calcium concentrations (up to 150 Ca^{2+}/parvalbumin at which the secondary sites would be filled with Ca^{2+} ions. In such conditions, calcium exchange would be expected to be rapid on the NMR time scale and the half-width of the ^{43}Ca-resonance to be dependent on the concentration of bound calcium ions. This was not observed experimentally. Competitive studies between Ca^{2+} and Gd^{3+} also suggest that there are only 2 calcium sites on parvalbumins [17].

5. Conclusion

The present studies show that calcium-43 resonance is well suited for detection of changes in the binding of Ca^{2+} ions to proteins. With parvalbumins, the method is limited to conditions (high temperature and pH) where rapid exchange is observed on the NMR time scale. Calcium binding to parvalbumins is characterized by a very important loss of water molecules from the aquo-cation. Calcium binding with retention of water molecules (such as in calcium proteins with external binding, Ca^{2+}–nucleic acid interactions) might be characterized by higher complex decomposition rates, allowing a better determination of the Ca^{2+}–macromolecule interaction by ^{43}Ca resonance methods.

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