Na^+ BINDING TO PARVALBUMINS STUDIED BY ^{23}Na NMR

J. PARELLO, P. REIMARSSON[+], E. THULIN[+] and B. LINDMAN[+]

Equipe de Recherche de Biophysique No. 140, CNRS, USTL, 34060 Montpellier, France and *[+]Divisions of Physical Chemistry 1 and 2, Chemical Center, P.O.B. 740, S-220 07 Lund, Sweden*

Received 2 February 1979

1. Introduction

Parvalbumins (Pa) are low molecular weight (~11 500) proteins found in the muscles of most vertebrates [1,2]. The strong interaction of parvalbumins with Ca^{2+} is well documented and evidence for a strong interaction with other divalent ions as well as with trivalent ions is accumulating rapidly [3–5]. In addition to divalent metal ions (Mg^{2+}, Ca^{2+}), muscles also contain high concentrations of monovalent metal ions, i.e., Na^+ and K^+. The interaction of these with parvalbumins might well be important either through a direct competition with other metal ions [6] or through an interaction influencing the protein conformation. It is well known that there is a strong coupling between parvalbumin conformation and the binding of Ca^{2+} [7,8]. Since ^{23}Na NMR is an extremely powerful method for the study of ion–macromolecule interactions [9–12] it was natural to extend our studies on metal ion binding to parvalbumins by ^{43}Ca [13], ^{113}Cd [14,15] and ^{25}Mg [25] NMR to include ^{23}Na NMR. In particular it seemed feasible to approach the following questions:

(1) Is there a significant interaction between Na^+ and parvalbumin?
(2) In the absence of Ca^{2+}, does Na^+ occupy the Ca^{2+} binding sites?

Parvalbumins with a calcium content lower than that of native parvalbumins (usually 2 Ca^{2+}/protein molecule) are usually prepared in the presence of a calcium chelating agent such as EDTA or EGTA [8]. However, it was observed that the removal of Ca^{2+} is accompanied by a binding of EGTA to the protein molecule [16] and this may influence the interaction of metal ions. A method for preparing metal-free parvalbumins or apoparvalbumins in the absence of EGTA was therefore developed.

2. Materials and methods

A crude parvalbumin extract was prepared from the white muscle of hake *(Merluccius merluccius)* by the procedure in [17]. The main component, pI 4.36, was isolated by DEAE-cellulose chromatography according to [18]. Its purity was checked by agarose gel electrophoresis [19].

Ca^{2+} free parvalbumin: Hake apoparvalbumin was prepared by treating the native Ca^{2+}-loaded parvalbumin with an excess of $DyCl_3$ in 50 mM Na-cacodylate buffer at pH 6.4. Removal of unbound Ca^{2+} and Dy^{3+} was carried out by dialyzing against distilled water. The pH was adjusted to 10.5 with NaOH. The hydroxide precipitate was removed by centrifugation. After lowering to pH 7 by addition of HCl, the Dy^{3+} content was below 0.5 ions/protein molecule (determined by ultraviolet absorption) and the Ca^{2+} content was 0.2 ions/protein molecule (determined by atomic absorption spectroscopy).

^{23}Na NMR spectra were obtained using a modified Varian XL-100 spectrometer operating in the Fourier transform mode at 26.46 MHz. Linewidths were measured at halfheight of the signals. The experimental temperature was 28°C. The results are presented as excess linewidths ($\Delta\nu_{ex}$), i.e., the difference of the experimental line width and that of a protein-free solution. In the present case with rapid exchange conditions (verified by variable temperature studies), $\Delta\nu_{ex}$ is given by:

$$\Delta\nu_{ex} = \Sigma p_i \Delta\nu_i$$

where p_i is the fraction of Na^+ at site i on the protein, characterized by the intrinsic line width $\Delta\nu_i$. Since ^{23}Na relaxation is strongly enhanced on binding to a macromolecule, $\Delta\nu_{ex}$ is a sensitive measure of the extent of binding (principles detailed in [20]).

3. Results and discussion

Studies of the ^{23}Na NMR line width as a function of the concentration of added $Pa(Ca_2)$ gave only a very slight line broadening, demonstrating that for the fully Ca^{2+}-loaded protein there is no appreciable Na^+ binding. Since this could be due to common binding sites of Na^+ and Ca^{2+} and a very much higher Ca^{2+} affinity, studies were performed with parvalbumins where Ca^{2+} had been fully or partly removed. Studies at 0.1 M Na^+ and pH 7.4 gave an excess ^{23}Na linewidth of <9 Hz for both $Pa(Ca_2)$ and the Ca^{2+}-free apoparvalbumin when protein was increased to 3.8 mM. No significant difference between the two proteins was detected. In fig.1, a study of the effect of Ca^{2+} content up to very high concentrations is presented for a lower Na^+ concentration. (At 10–100 Ca^{2+}/protein the ^{23}Na linewidth was found to be independent of Ca^{2+} content.) Again a small line-broadening is found and, furthermore, it is demonstrated that at <2 Ca^{2+}/protein molecule does not cause an enhanced Na^+ binding. The variations observed at 0–2 Ca^{2+}/protein molecule, which are relatively minor, can certainly be ascribed to conformational changes associated with Ca^{2+} binding. These conformational changes would affect the binding of Na^+ at low affinity sites on the exterior of the protein. The presence of a distinct minimum suggests the presence of a significant amount of $Pa(Ca_1)$.

Since Ca^{2+} addition up to very high concentrations

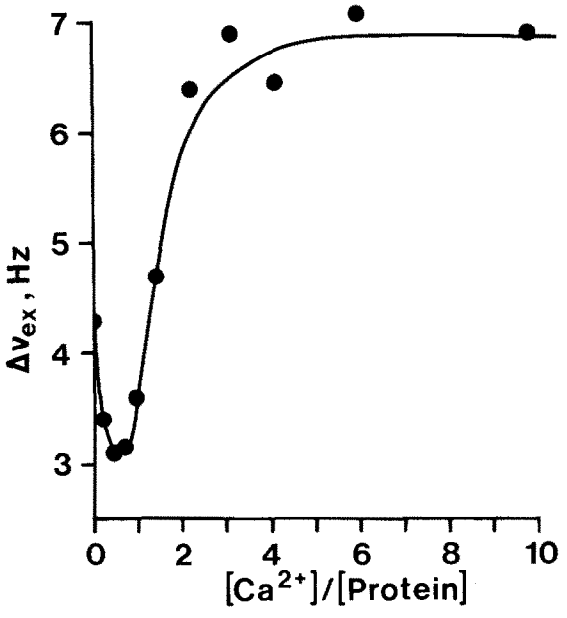

Fig.1. ^{23}Na resonance experiment at variable Ca^{2+} concentration by adding $CaCl_2$ to a 1.57 mM hake apoparvalbumin solution in 10 mM Tris–HCl, at pH 7.4 and containing 30 mM NaCl. Variation of $\Delta\nu_{ex}$ as a function of $[Ca^{2+}]$/[protein].

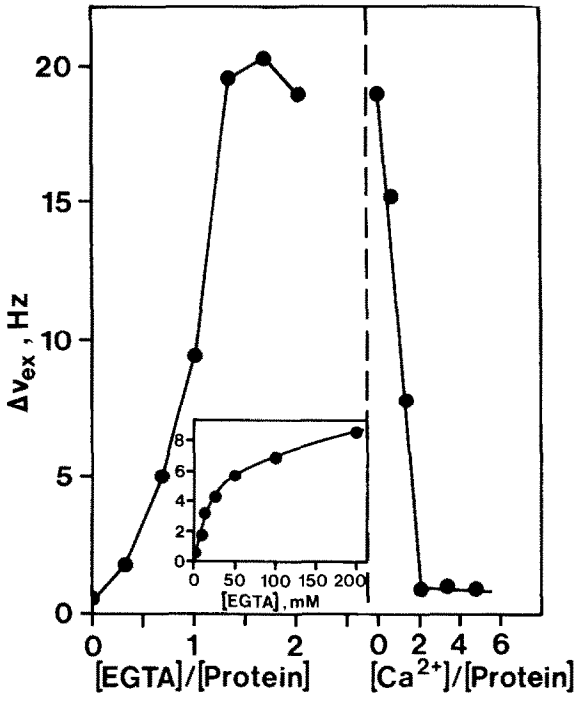

Fig.2. ^{23}Na resonance experiment with hake apoparvalbumin. Left figure: Effect of EGTA concentration on the halfwidth of the ^{23}Na signal of a 30 mM NaCl solution containing 0.37 mM apoparvalbumin in 10 mM Tris–HCl, at pH 7.4. Variation of $\Delta\nu_{ex}$ as a function of [EGTA]/[protein]. Right figure: Variation of $\Delta\nu_{ex}$ as a function of $[Ca^{2+}]$/[protein]. $CaCl_2$ was added to the EGTA–apoparvalbumin solution. Insert. Variation of $\Delta\nu_{ex}$ with the EGTA concentration of a 30 mM NaCl solution in Tris–HCl, at pH 7.4.

has no effect on the ^{23}Na relaxation, it can be established that the Na^+ binding sites have no appreciable affinity for Ca^{2+}. In fig.2, it is shown that addition of EGTA has an extremely strong influence on Na^+ binding and also that this additional Na^+ binding is in strong competition with Ca^{2+}. Thus apoparvalbumin itself only has a very small influence on ^{23}Na relaxation, but this is not the case in the presence of EGTA. When the EGTA content increases above 1.5 equivalents, $\Delta\nu_{ex}$ remains nearly constant. This suggests that EGTA binds to the apoparvalbumin molecule with a relatively high affinity. (The present results do not, on the other hand, give direct information on the binding of EGTA in the presence of Ca^{2+}.) Under the same experimental conditions, free EGTA contributes negligibly to the ^{23}Na relaxation (see insert in fig.2).

Addition of 2 Ca^{2+} equivalents re-establishes the ^{23}Na relaxation at its initial value (fig.2). It is likely that the added Ca^{2+} first interact with the EGTA molecules before filling the high-affinity Ca^{2+} sites CD and EF of the parvalbumin molecule. (Alternatively, but less likely, there is a dissociation of EGTA from the protein on Ca^{2+} addition. A distinction between possibilities should be feasible from direct studies of EGTA binding.) At higher Ca^{2+} concentrations, $\Delta\nu_{ex}$ remains practically constant. This is in agreement with the observation that the influence of the Ca^{2+} concentration is small as may be inferred from the results of fig.1. A titration was also performed up to 0.5 EGTA equivalents/apoparvalbumin. The addition of ~0.5 equivalents Ca^{2+} was then sufficient to eliminate the EGTA-induced ^{23}Na relaxation. This supports the conclusion that Ca^{2+} interacts preferentially with EGTA while interaction with the CD and EF sites of the protein is of lower affinity under the present conditions.

The binding of EGTA to a Ca^{2+}-free parvalbumin is also supported by independent experiments where removal of Ca^{2+} from a $Pa(Ca_2)$ molecule (from carp muscle) was carried out by an excess of EGTA at pH 9. It was observed that a certain amount of EGTA (~2 molecules/protein) is eluted with the protein during gel filtration [16].

In conclusion, our ^{23}Na NMR studies demonstrate that there is only weak Na^+ binding to parvalbumin even at reduced Ca^{2+} contents and that there is no Ca^{2+}-Na^+ competitive binding. In the presence of EGTA, on the other hand, Na^+ binding is dramatically enhanced and for this additional Na^+ binding there is a strong competition with Ca^{2+}.

Our conclusions are in complete contradiction to those in [6,21]. In our opinion this is due to the presence of some EGTA, added to remove Ca^{2+}, in their solutions.

Acknowledgements

We are grateful to Hans Gustavsson for a preliminary study of Na^+–parvalbumin interactions (in 1973), to Adrien Cavé for discussion and to David Turner for linguistic criticism. This work is contribution no. 7 from Equipe de Recherche de Biophysique and was partly supported by the Délégation Générale à la Recherche Scientifique et Technique (Mécanismes de reconnaissance a l'échelle moléculaire). The Centre National de la Recherche Scientifique and the Swedish Natural Science Research Council have provided travel and project grants.

References

[1] Goodman, M. and Pechère, J.-F. (1977) J. Molec. Evol. 9, 131–158.
[2] Coffee, C. F., Bradshaw, R. A. and Kretsinger, R. H. (1973) Adv. Exp. Med. Biol. 48, 211–233.
[3] Moews, P. C. and Kretsinger, R. H. (1975) J. Mol. Biol. 91, 229–232.
[4] Nelson, S. J., Miller, T. L. and Martin, R. B. (1977) Bioinorg. Chem. 7, 325–334.
[5] Daurès, M.-F. (1977) Thesis, USTL, Montpellier.
[6] Grandjean, J., Laszlo, P. and Gerday, C. (1977) FEBS Lett. 81, 376–380.
[7] Parello, J., Cavé, A., Puigdomènech, P., Maury, C., Capony, J.-P. and Pechère, J.-F. (1974) Biochimie 56, 61–76.
[8] Donato, H., jr and Martin, R. B. (1974) Biochemistry 13, 4575–4579.
[9] Laszlo, P. (1978) Angew. Chemie 90, 271–283.
[10] Forsén, S. and Lindman, B. (1978) Chem. Brit. 14, 29–35.
[11] Gustavsson, H., Lindman, B. and Bull, T. (1978) J. Am. Chem. Soc. 100, 4655–4661.
[12] Gustavsson, H., Siegel, G., Lindman, B. and Fransson, L-Å. (1978) FEBS Lett. 86, 127–130.
[13] Parello, J., Lilja, H., Cavé, A. and Lindman, B. (1978) FEBS Lett. 87, 191–195.

[14] Drakenberg, T., Lindman, B., Cavé, A. and Parello, J. (1978) FEBS Lett. 92, 346–350.
[15] Cavé, A., Parello, J., Drakenberg, T., Thulin, E. and Lindman, B. (1979) FEBS Lett. 100, 148–152.
[16] Cavé, A. (1979) personal communication.
[17] Strehler, E. E., Eppenberger, H. M. and Heizmann, C. W. (1977) FEBS Lett. 78, 127–133.
[18] Pechère, J.-F., Demaille, J. and Capony, J.-P. (1971) Biochim. Biophys. Acta 236, 391–408.
[19] Johansson, B. G. (1972) Scand. J. Clin. Lab. Invest. 29, suppl. 124, 7–19.
[20] Lindman, B. and Forsén, S. (1976) Chlorine, Bromine and Iodine NMR, Physico-Chemical and Biological Applications, ch. 8, Springer Verlag, Heidelberg.
[21] Grandjean, J. and Laszlo, P. (1978) in: Protons and Ions Involved in Fast Dynamic Phenomena, pp. 373–380, Elsevier, Amsterdam, New York.