Mg^{2+} BINDING TO PARVALBUMINS STUDIED BY ^{25}Mg AND ^{113}Cd NMR SPECTROSCOPY

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1. Introduction

Parvalbumins (Pa) are low molecular weight (~11 500) proteins found in the muscles of most vertebrates. They have a strong affinity for Ca^{2+} [1,2]. It has been established by X-ray crystallographic studies of parvalbumin component pI 4.25 from carp muscle that 2 Ca^{2+} occupy 2 sites within the protein molecule. These sites are called CD and EF [3] and they certainly correspond to the high-affinity Ca^{2+} sites (K_d ~10^{-7} M) which were observed in solution [4]. The CD and EF sites are non-equivalent as shown by ^{113}Cd NMR spectroscopy studies of a Cd^{2+}-loaded carp parvalbumin: two ^{113}Cd signals with different chemical shifts are observed in the NMR spectrum [5].

Conformational studies of parvalbumins in solution have revealed that the tertiary structure of these globular proteins is dependent on the Ca^{2+} content [6–8]. ^1H NMR spectroscopy clearly demonstrated that the highly compact structure of the 2 Ca^{2+} form is lost after removal of Ca^{2+} [6]. The Ca^{2+}-free form corresponds to a less compact structure which is converted back to the initial one on addition of the missing Ca^{2+} [9].

Two different forms of the same parvalbumin molecule have been suggested to occur during the activation–relaxation cycle of the muscle [10]. These forms might be related to the occurrence of different parvalbumin conformations depending on the Ca^{2+} concentration in the sarcoplasm. The interaction of Mg^{2+} might also play a role in the conformational transition of parvalbumins because of the relatively high concentration of Mg^{2+} in the muscle, i.e., 2–6 mM [11] which should be compared to 0.05–1 mM protein [12].

The interaction of Ca^{2+} with parvalbumins was recently studied by ^{43}Ca NMR spectroscopy on the basis of quadrupolar relaxation effects which occur upon binding of Ca^{2+} to the protein [14]. In a similar way the interaction of Mg^{2+} with parvalbumins can be studied by NMR of the quadrupolar nucleus ^{25}Mg. The association between Mg^{2+} and biological molecules rarely has been investigated using ^{25}Mg NMR spectroscopy (reviewed [15]). We report here on the feasibility of using the resonance of ^{25}Mg in the study of ion binding of parvalbumins.

In this investigation, the halfwidth of the ^{25}Mg signal was measured for solutions containing varying concentrations of parvalbumin under different conditions of temperature and content of Ca^{2+} and Mg^{2+}. Parallel experiments by ^{113}Cd NMR spectroscopy were also performed using a Ca^{2+}-loaded parvalbumin. The chemical shifts of the CD and EF ^{113}Cd signals were investigated as a function of the Mg^{2+} concentration. The combination of the results obtained by ^{25}Mg and ^{113}Cd NMR spectroscopy established the existence of Mg^{2+} specific sites which differ from the CD and EF Ca^{2+}-sites of parvalbumins.

2. Materials and methods

Parvalbumins from carp muscle (*Cyprinus carpio*) and from hake muscle (*Merluccius merluccius*) were isolated by the procedure in [26] and used as lyophilized powders. The purity of the protein was checked by agarose gel electrophoresis [17]. The Ca^{2+} content was ~2.4 Ca^{2+}/parvalbumin molecule as determined by atomic absorption spectroscopy (Perkin-Elmer 303) in the presence of La^{3+}. Ca^{2+}-free

parvalbumin or apoparvalbumin was obtained by gadolinium hydroxide precipitation, at pH 10.5, from a Gd^{3+}-loaded parvalbumin. Samples with about 0.4 Gd^{3+} equivalents were obtained.

^{25}Mg was purchased from Oak Ridge (Tennessee) as ^{25}MgO (97.9% isotopic enrichment). All chemicals were of analytical grade. Tris-buffer (75 mM, pH 8.1) was prepared by dissolving the required quantities of Trizma HCl and Trizma from Sigma (St Louis). A ^{25}Mg stock solution was prepared by dissolving a carefully weighed quantity of ^{25}MgO in a small volume of 4 M HCl whereafter the pH was adjusted to 7.5 by adding Tris-buffer and 1 M NaOH at ~0.1 M final Mg^{2+} conc. Protein concentrations were obtained directly from the amount of added lyophilized powder.

All pH measurements were made at room temperature (~20°C). NMR experiments were performed with a Varian XL-100 spectrometer adapted for ^{25}Mg resonance in the Fourier transform mode by using a specially built probe operating at 6. 12 MHz and equipped with a variable temperature device. Tubes of 12 mm diam. filled with 1.5–2.0 ml solution were used under non-spinning conditions. The FT parameters used throughout this work were: spectral width 4000 Hz, acquisition time 0.15 s, pulse-width 35 μs and 2000–4000 transients depending on the linewidth. The halfwidth of the observed signals is denoted as $\Delta\nu_{1/2}$ (in Hz). The accuracy of the halfwidth measurements was estimated to be ± 0.6 Hz. A 1 M $MgCl_2$ solution in 2 M HCl was used as a ^{25}Mg reference (natural abundance). The excess linewidth, $\Delta\nu_{ex}$, is the difference between the observed linewidth and that of a corresponding protein-free solution.

^{113}Cd NMR spectra were obtained as in [5].

3. Results

3.1. *^{25}Mg NMR studies with Ca^{2+}-loaded parvalbumin, $Pa(Ca_2)$*

When $Pa(Ca_2)$ is added to a 0.5 M Mg^{2+} solution, the ^{25}Mg resonance is progressively broadened (fig.1A). This indicates that an interaction between Mg^{2+} and parvalbumin occurs and that the chemical exchange of Mg^{2+} is relatively fast on the NMR time scale. A study of the dependence of the halfwidth

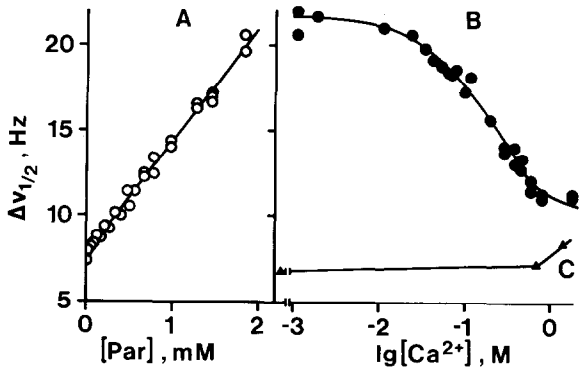

Fig.1. ^{25}Mg NMR study of Mg^{2+} affinity for carp parvalbumin at 28°C. (A) $Pa(Ca_2)$ progressively added to a solution containing 0.5 M Mg^{2+} (^{25}Mg natural abundance) and 0.043 M Tris, at pH 7.0. (B) Ca^{2+} is progressively added to a solution containing 0.1 M Mg^{2+} (^{25}Mg enriched sample), 0.58 mM $Pa(Ca_2)$ and 0.07 M Tris, at pH 7.1. (C) Addition of Ca^{2+} to a 1 M Mg^{2+} (natural abundance) solution.

of the ^{25}Mg NMR signal on temperature shows that $\Delta\nu_{1/2}$ goes through a maximum in the range 35–40°C (fig.2), indicating that fast exchange conditions prevail above 40–50°C.

When Ca^{2+} is added to a 0.1 M Mg^{2+} solution containing 0.57 mM $Pa(Ca_2)$, a decrease in the ^{25}Mg linewidth is observed around 0.1 M Ca^{2+}, indicating that

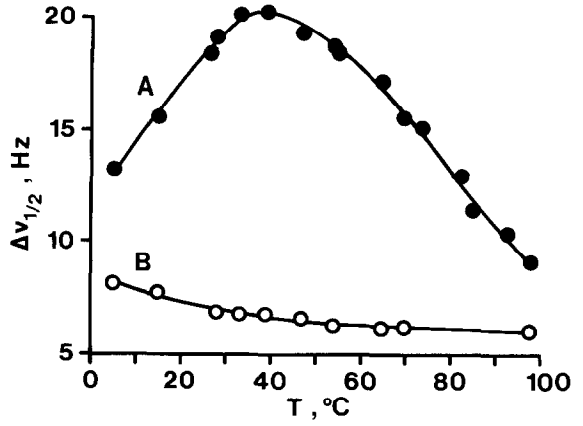

Fig.2. ^{25}Mg NMR study at variable temperature. (A) Solution containing 97 mM Mg^{2+} (^{25}Mg enriched sample), 0.2 M Na^+, 50 mM Tris, 9 mM DTT and 0.57 mM carp $Pa(Ca_2)$ at pH 7.5. (B) 1 M $MgCl_2$ (natural abundance) in acidic solution (~2 M HCl).

Ca^{2+} and Mg^{2+} are competing for parvalbumin sites with similar affinities, $K_{Mg}/K_{Ca} \simeq 2$ (fig.1B). This similarity is unexpected on the basis of studies [18] on the competition of these ions with Gd^{3+} which showed that if Mg^{2+} binding occurs to the CD and EF sites the affinity constant would be lower than that of Ca^{2+} by 2–3 orders of magnitude. Thus the effect of parvalbumins on the ^{25}Mg NMR linewidth should correspond to Mg^{2+} binding at secondary sites.

3.2. *^{113}Cd NMR studies with a Cd^{2+}-loaded parvalbumin, $Pa(Cd_2)$*

It has been established that the CD and EF sites can be occupied by Cd^{2+}, which give rise to two characteristic ^{113}Cd signals with distinct chemical shifts [5] and that Ca^{2+} and Cd^{2+} have similar affinities ($K_{Cd}/K_{Ca} = 2$–4) [19]. When Mg^{2+} is added to $Pa(Cd_2)$, the chemical shift of the ^{113}Cd signal from the CD site remains unaffected whereas the EF signal is markedly shifted upfield following a saturation curve (fig.3). A similar result is obtained when Ca^{2+} is added to $Pa(Cd_2)$: only the EF signal is upfield shifted by Ca^{2+} addition (not shown). When Mg^{2+} is added to $Pa(Cd)_2$ neither of the two signals disappears even at high Mg^{2+}/protein ratios (up to 130). In contrast, the intensity of both signals decreases rapidly when Ca^{2+} is added. Under conditions similar to those in fig.3, < 25% of the initial intensity is measured when Ca^{2+}/protein = 4. This again indicates that the affinity constants of Ca^{2+} and Mg^{2+} for the CD and EF sites must differ by at least two orders of magnitude. Another significant point is that there is a specific dependence of the chemical shift of the EF ^{113}Cd signal on the concentration of divalent ions such as Mg^{2+}, Ca^{2+} and Cd^{2+}. This has been observed for several parvalbumins. It is likely that this effect is due to the binding of these divalent cations to a secondary site of parvalbumin, located near the EF primary site or conformationally related to this site. A lower limit (10^2 M^{-1}) for the affinity constant of Mg^{2+} for this site can be estimated. This is an apparent binding constant because of the unavoidable presence of other divalent cations (Ca^{2+}, Cd^{2+}).

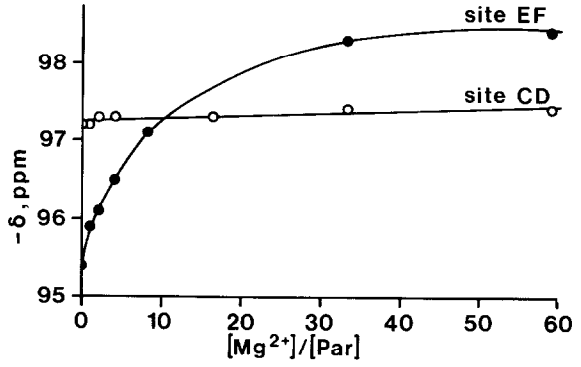

Fig.3. Effect of Mg^{2+} concentration on the chemical shifts of the 2 $^{113}Cd^{2+}$ bound in the CD and EF sites of carp parvalbumin (see [5]). The solution contains 18.6 mM Cd^{2+} (96.3% ^{113}Cd enriched sample), 53 mM Tris–sulphate, at pH 7.0, and 4.8 mM carp $Pa(Ca_2)$. The temperature is 28°C.

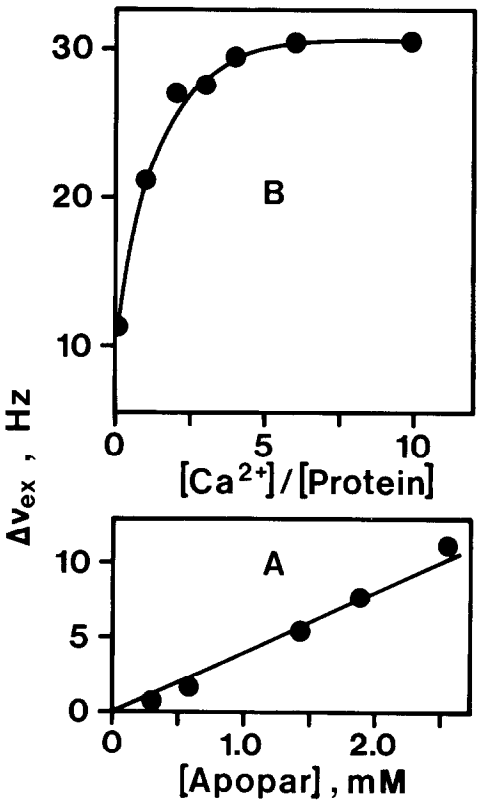

Fig.4. ^{25}Mg NMR study of Mg^{2+} affinity for hake apoparvalbumin (Pa(o)). (A) Dependence of $\Delta\nu_{ex}$ upon protein concentration for a solution containing 99 mM Mg^{2+} (^{25}Mg enriched sample), ~0.13 M Na^{2+}, 0.05 M cacodylate at pH 6.5 and 28°C. (B) Dependence of ^{25}Mg halfwidth $\Delta\nu_{ex}$ on Ca^{2+} concentration.

3.3. *^{25}Mg NMR studies with Ca^{2+}-free parvalbumin, Pa(o)*

It is known that the affinity of parvalbumins for Ca^{2+} is dependent on the integrity of the whole protein structure. Localized chemical modifications, such as substitution of Arg 75, affect the binding of Ca^{2+} [20]. It is well established that the compact tertiary structure of parvalbumin is dependent on the Ca^{2+} content and that Pa(o) corresponds to a more relaxed tertiary structure [6–8, 19]. It was therefore interesting to investigate the interaction of Mg^{2+} with Pa(o). Figure 4A shows the dependence of the ^{25}Mg linewidth on the concentration of hake Pa(o). As in fig.1, a broadening of the NMR signal is observed with a nearly linear dependence of $\Delta\nu_{1/2}$ on protein concentration. This clearly indicates that apoparvalbumin interacts with Mg^{2+}. When Ca^{2+} is added to a Mg^{2+}-saturated apoparvalbumin, the ^{25}Mg linewidth is further increased and a saturation value is reached beyond 5 equivalents of Ca^{2+} (fig.4B). Because of the high affinity of the CD and EF sites for Ca^{2+}, the addition of Ca^{2+} yields Pa(Ca_2). The observed increase in $\Delta\nu_{ex}$ is to be related to a change in the Mg^{2+} binding characteristics (structural, thermodynamic or kinetic) of the secondary site as a consequence of structural changes of the protein molecule when Ca^{2+} enter the CD and EF sites. Preliminary results show that the dependence of the ^{25}Mg linewidth on temperature is markedly different for Pa(o) and Pa(Ca_2).

4. Discussion

The results described clearly demonstrate that ^{25}Mg NMR spectroscopy is well suited for monitoring cation binding to proteins. The important question in the case of parvalbumin is to establish the nature of the Mg^{2+} binding sites. In these respects, the various competition experiments with both ^{25}Mg and ^{113}Cd NMR are especially informative. The situation is complex, however, with several interdependent sites, and furthermore, it should be noted that ^{25}Mg and ^{113}Cd NMR do not necessarily monitor the same Mg^{2+} site.

The main part of the ^{25}Mg relaxation enhancement due to parvalbumin is affected in competition with Ca^{2+}, with about equal binding affinity of the two ions. Because Ca^{2+} binding to CD and EF sites is much stronger than Mg^{2+} binding, the present results demonstrate the presence of a secondary cation (Mg^{2+}, Ca^{2+}) binding site. The ^{113}Cd NMR chemical shifts and signal intensities in combination show that there is Mg^{2+} binding to parvalbumin but that this binding does not displace Cd^{2+} bound at either the CD or the EF sites. The chemical shift change of the EF ^{113}Cd signal on Mg^{2+} binding, as well as on Ca^{2+} and Cd^{2+} binding, suggests that there is a secondary cation binding site close to the EF site. This probably corresponds to the 'third site' described [21] where evidence is presented for a site close to the EF site from fluorescence experiments using a Tb^{3+}-loaded parvalbumin.

Both the ^{25}Mg and ^{113}Cd NMR results show that in the presence of other divalent ions like Ca^{2+} and Cd^{2+} there is no binding of Mg^{2+} to the CD and EF sites. This is in agreement with the much lower affinity of Mg^{2+}, as inferred from, e.g., competition studies with Gd^{3+} observing the water proton relaxation [18]. An important question to answer is if there is Mg^{2+} binding to the CD and EF sites in the absence of other divalent ions. Adding Ca^{2+} to an apoparvalbumin solution containing Mg^{2+} leads to an increase in ^{25}Mg relaxation rate rather than a decrease as would be expected with an appreciable contribution to relaxation from the CD and EF sites. Variable temperature studies indicate that even at rather high temperatures (60°C) there is no additional relaxation contribution that can be ascribed to the CD and EF sites. These results suggest that there is (under our experimental conditions) either no Mg^{2+} binding to the high affinity sites or that there is such a binding but occurring under unfavourable exchange conditions. Under conditions similar to those used in the present study, the binding of Ca^{2+} to the high affinity sites was not detected in ^{43}Ca NMR because the dissociation rate of the Pa(Ca_2) complex over a wide range of temperature is too slow [14]. While the present study does not give definitive results on Mg^{2+} binding to the CD and EF sites there are alternative possibilities which can be utilized, i.e., performing studies of the type presented in fig.4B with different Mg^{2+} concentrations and doing competition experiments using ^{45}Ca tracer diffusion. From the results of competition experiments between Ca^{2+}, Mg^{2+}, Cd^{2+} and Gd^{3+} it can be inferred that the binding constant of Mg^{2+} for the CD and EF sites is at least 2 orders of magnitude less than that

of Ca^{2+} or Cd^{2+} [19]. It has been reported that Mg^{2+} and Ca^{2+} are competing from the same sites on a parvalbumin molecule immobilized on a polyacrylamide matrix and that the affinity of the protein for Ca^{2+} is 3.5 orders of magnitude higher than for Mg^{2+} [13]. It must be noted though that in this and other studies on parvalbumins, which find evidence for Mg^{2+} binding to CD and EF sites, soluble chelators such as EGTA or EDTA have been used to remove Ca^{2+} from the native protein. It has been shown that EGTA is able to bind to apoparvalbumin and thereby to interact strongly with different cations such as Ca^{2+} and Na^{+} [22]. In view of this we make presently no attempt to correlate our data, obtained without using a soluble chelator, with literature data on cation binding to parvalbumins.

It may be questioned if the occurrence of a Ca–Mg secondary site is relevant to the function of parvalbumins. Studies by ^{113}Cd NMR show that the dependence of the chemical shift of the two cadmium signals differs according to the origin of the protein. For instance, components pI 5.0 and pI 4.2 from pike muscle, which belong to different phylogenetic groups, differ markedly in their ^{113}Cd NMR behaviour at Mg^{2+} addition [23].

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References

[1] Goodman, M. and Pechère, J.-F. (1977) J. Mol. Evol. 9, 131–158.
[2] Coffee, C. J., Bradshaw, R. A. and Kretsinger, R. H. (1973) Adv. Exp. Med. Biol. 48, 211–233.
[3] Kretsinger, R. H. and Nockolds, C. E. (1973) J. Biol. Chem. 248, 3313–3326.
[4] Benzonana, G., Capony, J.-P. and Pechère, J.-F. (1972) Biochim. Biophys. Acta 278, 110–116.
[5] Drakenberg, T., Lindman, B., Cavé, A. and Parello, J. (1978) FEBS Lett. 92, 346–350.
[6] Parello, J., Cavé, A., Puigdomènech, P., Maury, C., Capony, J.-P. and Pechère, J.-P. (1974) Biochimie 56, 61–76.
[7] Donato, H. and Martin, R. B. (1974) Biochemistry 13, 4575–4579.
[8] Nelson, D. J., Opella, S. J. and Jardetzky, O. (1976) Biochemistry 15, 5552–5560.
[9] Cavé, A., Morin, Ph. and Dobson, C. M. (1979) submitted.
[10] Pechère, J.-F., Derancourt, J. and Haiech, J. (1977) FEBS Lett. 75, 111–114.
[11] Brinley, F. J., Scarpa, F. and Tiffert, J. F. (1977) J. Physiol. 266, 545.
[12] Baron, G., Demaille, J. and Dutruge, E. (1975) FEBS Lett. 56, 156–160.
[13] Lehky, P., Comte, M., Fisher, E. H. and Stein, E. A. (1977) Anal. Biochem. 82, 158–169.
[14] Parello, J., Lilja, H., Cavé, A. and Lindman, B. (1978) FEBS Lett. 87, 191–195.
[15] Lindman, B. and Forsén, S. (1979) in: NMR and the Periodic Table (Harris, R. and Mann, B. eds) ch. 7, Academic Press, New York.
[16] Pechère, J.-P., Demaille, J. and Capony, J.-P. (1971) Biochim. Biophys. Acta 236, 391–408.
[17] Johansson, B. G. (1972) Scand. J. Clin. Lab. Invest. 29, suppl. 124, 7–19.
[18] Daurès, M.-F. (1977) Thesis, USTL Montpellier.
[19] Giulioli, C., Cavé, A. and Parello, J. (1979) in preparation.
[20] Coffee, C. J. and Solano, C. (1976) Biochim. Biophys. Acta 453, 67–80.
[21] Nelson, D. J., Miller, T. L. and Martin, R. B. (1977) Bioinorganic chemistry 7, 325–334.
[22] Parello, J., Reimarsson, P., Thulin, E. and Lindman, B. (1979) FEBS Lett. 100, 153–156.
[23] Thulin, E., Lindman, B., Cavé, A. and Parello, J. (1979) in preparation.