SODIUM COMPLEXATION BY THE CALCIUM BINDING SITE OF PARVALBUMIN

J. GRANDJEAN and P. LÁSZLÓ
Laboratoire de chimie organique physique

and

C. GERDAY
Laboratoire de biochimie musculaire, Institut de Chimie Université de Liège, Sart-Tilman, par 4000 Liège, Belgium

Received 5 July 1977

1. Introduction

Parvalbumins are a class of muscular proteins with calcium binding properties, distributed in all classes of vertebrates [1]. The amino acid sequence is known for a number of parvalbumins. The tertiary structure has been established by X-ray crystallography for carp parvalbumin B [2]: it shows two calcium sites; each of these has an octahedral arrangement of oxygen atoms from carboxyl, carbonyl and hydroxyl groups coordinating the Ca^{2+} cations. The calcium binding sites are the same in parvalbumin III from pike, with mol. wt 11 780 [3], chosen for this study.

^{23}Na nuclear magnetic resonance is establishing itself as an extremely powerful method for studying the interactions between cations and organic or biological molecules [4]. A major impetus for our study is the finding that half-decalcified parvalbumin (PAB-1) of pike or carp will return reversibly to the native form (PAB-2) when the missing calcium is restored to it [5,6]. Furthermore, internal mobility remains similar in PAB-1 and PAB-2 [7]. Therefore, we set upon an exploration of the vacant calcium binding site in PAB-1, using the sodium cation as our probe.

We do find indeed that Na^+ will bind to PAB-1; it affixes itself to the calcium-binding site, albeit much more weakly than calcium, by some five orders of magnitude. However, and rather surprisingly, the sodium cation has a residence time on the protein long with respect to the PAB-1 reorientational correlation time of 4 ns. Thus, the off and on rate constants k_- and k_+ for complex formation between Na^+ and PAB-1 can be bracketed with upper and lower limits. Finally, competition experiments display the sequence:

$$Na^+ < K^+ \ll Mg^{2+} < Ca^{2+} \leqslant Mn^{2+}$$

of complexing abilities.

2. Materials and methods

Parvalbumin III of pike is obtained and decalcified according to published procedures [5]. Calcium content of the purified parvalbumins is determined by atomic absorption spectrometry (Perkin Elmer model 103). While the protein concentration is 1.7 mM, the sodium concentration is varied between 10^{-2} and 2.10^{-1} M by dissolving $NaHCO_3$ and eventually NaCl to maintain pH constant at 8.0 ± 0.2. Likewise, solutions of other salts (chlorides in all cases) are made from compounds of the best commercial grade. Concentrations are determined by titration and/or weighting. No calcium contamination from the NMR tubes could be detected.

Nuclear magnetic resonance parameters are obtained with the Bruker HFX-90 instrument, at 23.81 MHz, and with the Cameca 250 instrument, at 62.86 MHz. The longitudinal relaxation times T_1 are determined using the $(180^\circ-\tau-90^\circ-T)_n$ pulse

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sequence [8]. The transverse relaxation times T_2 are obtained from the linewidths at half-height: $T_2^{-1} = \pi.\Delta\nu_{1/2}$. It was checked that all band shapes are pure Lorentzian. Viscosities are determined using Desreux-Bischoff viscosimeters [9] for the aqueous solutions, made of H_2O–D_2O mixtures (approximately 1:1) in order to allow for the deuterium lock.

3. Results

The sodium linewidth $\Delta\nu_{1/2}$ increases greatly with the [PAB-1]/[Na^+] ratio. By contrast, $\Delta\nu_{1/2}$ is nearly invariant in the presence of the native protein PAB-2. Addition of Ca^{2+} ions to the PAB-1 + Na^+ system displaces sodium ions: the sodium linewidth becomes that for free sodium ions, ca. 10 Hz.

The ^{23}Na linewidth does not vary significantly between pH 7.5 and 9. It decreases slightly below pH 7.5. Precipitation occurs at the iso-electric point, of pH $\simeq$ 5.0 for pike parvalbumin III. These observations are consistent with involvement of carboxyl groups ($pK_a < 4$) in the cation-binding site.

All of the other experiments are performed at pH 8.0 ± 0.2. Fast sodium exchange occurs between the free form in the aqueous solution and the bound state on the protein, as shown by the reduction of the linewidth $\Delta\nu_{1/2}$ as the temperature increases [10]. We shall thus assume existence of Na^+ in two connected compartments: free and strongly-bound; substraction from the observed linewidth $\Delta\nu_{1/2}^{obs}$ with PAB-1 of the linewidth $\Delta\nu_{1/2}^{0}$ observed with the native parvalbumin PAB-2 at the same protein and salt concentrations eliminates weak binding sites on the protein from consideration.

Under our experimental conditions, viz., a large excess of Na^+ with respect to the protein, the appropriate expression for the 1:1 PAB-1 sodium complex is [11,12]:

$$[Na^+]_t = [PAB\text{-}1]_t \cdot \frac{\Delta\nu_{1/2}^{B}}{\Delta} - \frac{1}{K_f} \qquad (1)$$

where $\Delta = \Delta\nu_{1/2}^{obs} - \Delta\nu_{1/2}^{0}$, $[Na^+]_t$ and $[PAB\text{-}1]_t$ are total concentrations, $\Delta\nu_{1/2}^{B}$ is the linewidth in the pure complex, and K_f is the stability constant for this complex.

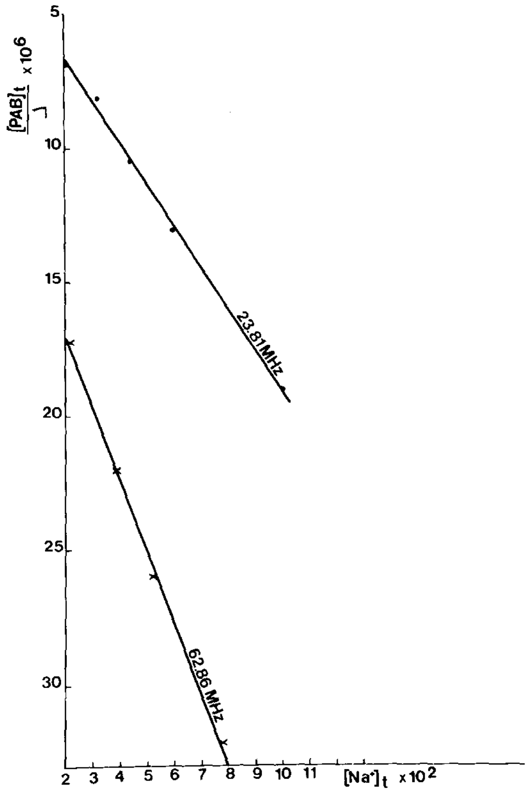

Fig.1. Fit of eq. (1) to the experimental points.

The experimental results conform nicely to eq. (1): in all cases, straight lines are obtained when plotting $[Na^+]_t$ as a function of $[PAB\text{-}1]_t.\Delta^{-1}$, with correlation coefficients greater than 0.995 (N = 4–5) (fig.1).

The apparent equilibrium constant $K_f = 30 \pm 10$ (3σ) M^{-1}. The linewidth characteristic of the pure complex and normalized to unit viscosity is:

$$1/\pi T_2^B \equiv \Delta\nu_{1/2}^{B}$$

$$= 6000 \pm 900\ (3\sigma)\ \text{Hz.cP}^{-1}\ \text{(at 23.81 MHz)}$$

$$1/\pi T_2^B \equiv \Delta\nu_{1/2}^{B}$$

$$= 3800 \pm 550\ (3\sigma)\ \text{Hz.cP}^{-1}\ \text{(at 62.86 MHz)}$$

The chemical shift difference between the free and the bound states is negligible. Then, the limiting linewidths $1/\pi T_2^{\mathrm{B}}$ are described by the following equations, derived by Bull for a spin 3/2 nucleus [13,14]:

$$1/T_2^{\mathrm{B}} = 0.6/T_2'^{\mathrm{B}} + 0.4/T_2''^{\mathrm{B}} \qquad (2)$$

where

$$1/T_2'^{\mathrm{B}} = \frac{\pi^2}{5} \cdot \left(\frac{e^2qQ}{h}\right)^2 \cdot \left[\tau_{\mathrm{c}} + \frac{\tau_{\mathrm{c}}}{1+\omega^2\tau_{\mathrm{c}}^2}\right]$$

$$1/T_2''^{\mathrm{B}} = \frac{\pi^2}{5} \left(\frac{e^2qQ}{h}\right)^2 \left[\frac{\tau_{\mathrm{c}}}{1+4\omega^2\tau_{\mathrm{c}}^2} + \frac{\tau_{\mathrm{c}}}{1+\omega^2\tau_{\mathrm{c}}^2}\right]$$

The term e^2qQ/h is the quadrupolar constant, τ_{c} is a correlation time and ω is the Larmor frequency.

Determination of the ratio at two different frequencies of $\Delta\nu_{1/2}^{\mathrm{B}}$ may yield the magnitude of the correlation time τ_{c}: the experimental value of 1.58 ± 0.4 is consistent (fig.2) with a correlation time τ_{c} of either 3.4 ± 1.0 ns or 7.0 ± 2.0 ns.

A choice between these two values can be made from the independent determination of the $\Delta(T_1^{-1})/\Delta(T_2^{-1})$ ratio [10]. At 62.86 MHz, the observed value of 0.22 yields τ_{c} 4.8 ± 0.5 ns. Taking the average between these two values (3.4 and 4.8), τ_{c} 4 ± 1 ns.

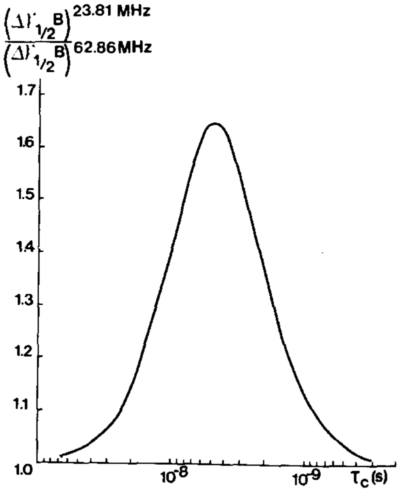

Fig.2. Plot of the limiting linewidth ratio, at two different examining frequencies, against the ^{23}Na correlation time τ_{c}; the experimental value of 1.58 is close to the maximum of this curve.

4. Discussion

The value for τ_{c} 4 ± 1 ns compares very well with that derived from viscosities under Debye-Stokes-Einstein assumptions: τ_{R} 3.5 ± 0.2 ns. The mean Stokes radius inferred from these values for the globular protein is 14.9 Å: the published crystallographic dimensions of ca. 16 Å [2] come close. Taking into account the ten-fold difference in protein concentration, our value of τ_{R} agrees with those of ∿ 12 ns obtained for more viscous samples both from ^{13}C longitudinal relaxation times [7] and from depolarized light scattering experiments [15]. Reorientation of the protein is isotropic [7], a feature highly favorable to interpretation of nmr relaxation times.

Another important consequence of the agreement between τ_{c} and τ_{R} values is the small magnitude of the exchange contribution to the linewidth : the sodium cation resides on the macromolecule for a time τ_{M} long with respect to τ_{c} 4 ns.

From eq. (2) and the above results, one may write:

$$T_2'^{\mathrm{B}}, T_2''^{\mathrm{B}} \gg \tau_{\mathrm{M}} \gg \tau_{\mathrm{c}}, \tau_{\mathrm{R}}$$

Then, with respect to the equilibrium:

$$\mathrm{Na}^+ + \text{PAB-1} \underset{k_-}{\overset{k_+}{\rightleftarrows}} \text{complex}$$

the following limits obtain:

$$2 \times 10^4 < k_- < 2.5 \times 10^8\ \mathrm{s}^{-1}$$

$$6 \times 10^5 < k_+ < 7 \times 10^9\ \mathrm{s}^{-1}$$

Using competition experiments, we have obtained the following sequence of complexing abilities (K_{f} values):

$$\mathrm{Na}^+ < \mathrm{K}^+ \ll \mathrm{Mg}^{2+} < \mathrm{Ca}^{2+} \leqslant \mathrm{Mn}^{2+}$$

as already reported for troponin C [16] and the myosin light chains [17]. The ionic radii are very similar for Na$^+$, Ca^{2+} and Mn^{2+} [18]. The ratio $K_{\mathrm{f}}(\mathrm{K}^+)/K_{\mathrm{f}}(\mathrm{Na}^+)$ is close to 3, and the ratio $K_{\mathrm{f}}(\mathrm{Ca}^{2+})/$

$K_f(Na^+)$ is close to 10^5 [19]. The quadrupolar coupling constant (e^2qQ/h) derived from $\Delta\nu^B_{1/2}$ and the correlation time τ_R has a value 1.3 ± 0.1 MHz.

5. Conclusions

Putting together all these results, we are led to the following inferences:

(i) Whereas Ca^{2+} is bound together with one water molecule [20], Na^+ is bound with two to four water molecules; the stronger K^+-binding comes about from easier water release by K^+ than by Na^+ in the forward step.

(ii) The quadrupolar coupling constant is close to the value of 1.2 MHz appropriate for $(Na^+)_4\ H_2O$ chelated by two carboxyl groups at van der Waals contact [21]; the K_f value of 30 M^{-1} comes close to that appropriate for coordination of Na^+ by bidentate or tridentate ligands [21,22].

(iii) The sodium ion is rigidly bound, a feature more likely to be displayed by a relatively small protein [23,24] and which may be conversely complementary to the high mobility observed for the phenylalanines in the molecule [25].

(iv) The much stronger calcium binding originates predominantly in a greatly decreased value of the off rate k_-, in accord again with the notion of a highly rigid cation-binding site.

(v) This is the first reported example of sodium cation binding to a biological macromolecule having such a long duration. The recent statement by Reuben [26]: 'Because of short lifetimes of the macromolecular complexes of sodium, the relaxation rate of ^{23}Na cannot be used in studies of macromolecular dynamics in solution' is unduly pessimistic. All of this work shows the contrary to be true, at least for this example of a calcium binding protein.

Acknowledgements

We wish to thank Professor G. Hamoir (University of Liège) for his continued interest in this study, Professor A. Gaudemer and Dr C. Merienne (University of Orsay) for use of the Cameca Spectrometer, the CNRS (Paris) for award of ATP Internationale 1976, and Fonds de la Recherche Fondamentale Collective (Brussels) for a grant towards purchase of the Bruker Spectrometer. We acknowledge with gratitude the expertise of Dr C. Detellier.

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