CALCIUM BINDING TO PORCINE PANCREATIC PROPHOSPHOLIPASE A_2 STUDIED BY ^{43}Ca NMR

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1. Introduction

Pancreatic phospholipase A_2 (PLA_2) catalyzes the hydrolysis of fatty acid ester bonds at the 2-position of 1,2-diacyl *sn*-phospholipids [1]. The enzyme is secreted by the pancreas in an inactive form. In the duodenum the zymogen is activated by trypsin cleavage [1]. The enzyme is capable of degrading phospholipids in micellar form, while the proenzyme is only able to hydrolyze monomeric substrates [2,3]. It has been shown that Ca^{2+} are essential for the catalytic properties of both the enzyme and the zymogen. Ca^{2+} binding to both forms has been studied by a variety of physicochemical techniques, showing that Ca^{2+} binds in a 1:1 molar ratio with association constants in the range of $1-5 \times 10^3$ M^{-1} [4,5]. From X-ray crystallographic data the Ca^{2+} binding site has been found to be located in a cavity of the protein surface near Asp_{49} [6].

Here we report the results of ^{43}Ca NMR measurements of Ca^{2+} binding to prophospholipase A_2 ($PPLA_2$). It is shown that the method is useful in providing information about: (i) association constants; (ii) the exchange rate of Ca^{2+} (i.e., k_{off}); (iii) dynamics in the Ca^{2+} binding site; and (iv) the apparent pK value for the group(s) involved in Ca^{2+} binding.

2. Materials and methods

2.1. *Materials*

Porcine pancreatic phospholipase A_2 was purified from a fortified preparation obtained as a side fraction during insulin production. The chromatographic steps were performed essentially as in [7]. The prophospholipase obtained showed no activity in an egg yolk assay [7]. Protein concentrations were determined spectrophotometrically using the absorption coefficient $E^{1\,cm}_{1\%} = 12.3$ at 280 nm [7]. A 0.086 M $CaCl_2$ solution was prepared by dissolving $CaCO_3$ (60% isotropically enriched in ^{43}Ca, Oak Ridge Nat. Lab.) in 0.1 M HCl and the solution was neutralized to a final pH at 7.0.

2.2. *Methods and data treatment*

The ^{43}Ca NMR spectra were obtained at 17.16 MHz, using a homemade Fourier transform spectrometer [8]. The relaxation of ^{43}Ca can safely be assumed to be exclusively quadrupolar. For correlation times sufficiently short, such that $\omega\tau_c \lesssim 1$, the relaxation can be approximated by a single exponential, corresponding to a Lorentzian line in the Fourier transformed spectrum. When the observed signal is due to metal ions exchanging between two sites, the line shape is no longer Lorentzian, except for very slow and very fast exchange rates. When the intermediate exchange condition applies the full width at half height of the NMR signal, $\Delta\nu$, is not only influenced by the transverse relaxation rates in the two sites and their populations, but also the exchange rate (i.e., k_{off}), cf. the Swift-Connick equation ($x = 1$ indicates longitudinal and $x = 2$ transverse relaxation rates):

$$R_{x,obs} = (1 - p_b)R_{x,f} + p_b/(R^{-1}_{x,b} + k^{-1}_{off})$$

which is valid for p_b values $\ll 1$ [9]. When the Swift-Connick approximation is not fulfilled, a total band-shape analysis is needed to obtain values of k_{off} and $R_{2,b}$. The details of the band-shape analysis will be reported elsewhere. To visualize the agreement

between calculated and observed signals, the full width at half height ($\Delta\nu = R_{2,obs}/\pi$) will be used in the same way as when the Swift-Connick equation is valid.

3. Results

Fig.1 shows the line-width of the observed ^{43}Ca NMR signal as a function of the [Ca^{2+}] at a constant [protein]. The broadening of the ^{43}Ca signal is most likely due to Ca^{2+} exchanging with the catalytic site of $PPLA_2$. The data can be fitted to the model of a simple chemical equilibrium:

$$PPLA_2 + Ca^{2+} \overset{K_a}{\rightleftarrows} Ca^{2+} - PPLA_2 \qquad (1)$$

The details of the fitting procedure will be published elsewhere. The value obtained for the association constant K_a is $2.5 \pm 1 \times 10^3$ M^{-1} at 24°C.

The temperature dependence of the ^{43}Ca NMR line-width is shown in fig.2. By fitting the data we obtained: $k_{off} = 2.5 \pm 1 \times 10^3$ s^{-1} and the line-width of the bound ion $\Delta\nu_b = 5 \pm 0.6 \times 10^2$ Hz (corresponding to $R_{2,b} = 1.6 \pm 0.2 \times 10^3$ s^{-1}) at 24°C. Measurements of the longitudinal relaxation rate, $R_{1,obs}$, for a 1.7 mM $PPLA_2$ solution containing 10.0 mM Ca^{2+} (pH 7.0) results in a value of 79 ± 4 s^{-1}.

The pH dependence of the observed ^{43}Ca NMR line-width is shown in fig.3. The data can be fitted to the model of a single protonation step:

$$\Delta\nu = [\Delta\nu_i]/[1 + 10^{(pK_{a,i} - pH)}] + C \qquad (2)$$

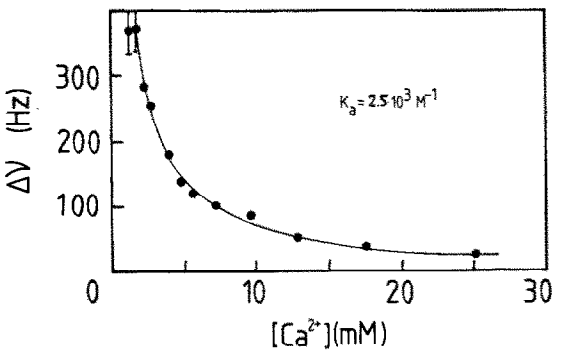

Fig.1. The line-width of the ^{43}Ca NMR signal as a function of the [Ca^{2+}] for a 2.0 mM $PPLA_2$ solution (pH 7.5). The spectra were recorded at 24°C. The solid line represents the theoretical curve calculated as described in the text.

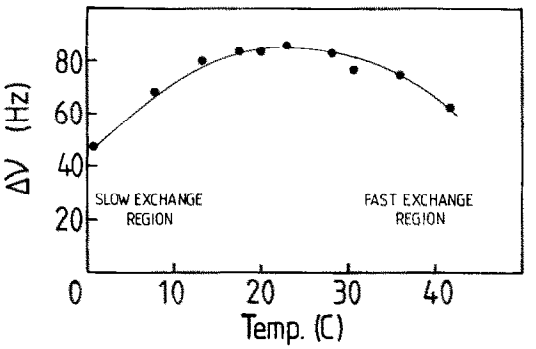

Fig.2. The temperature dependence of the ^{43}Ca NMR line-width for a 1.7 mM $PPLA_2$ solution (pH 7.4) containing 5.9 mM Ca^{2+}. The solid line is the theoretical curve drawn as described in the text.

In this equation $\Delta\nu_i$ is the step in the line-width associated with the apparent pK value $pK_{a,i}$. C is the contribution to the observed line-width that stays unaffected during the titration. In the fitting procedure we obtained the following parameters: $pK_{a,i} = 5.2$, $\Delta\nu_i = 91$ Hz and $C = 8$ Hz.

4. Discussion

Ca^{2+} binding to $PPLA_2$ has been studied using an equilibrium gel filtration technique [4] and UV difference spectrophotometry [4,5]. In these investigations, association constants in the range $2–4 \times 10^3$ M^{-1} were reported. The value of the association constant obtained from the ^{43}Ca concentration dependence, $K_a = 2.5 \pm 1 \times 10^3$ M^{-1}, is in good agreement with these results.

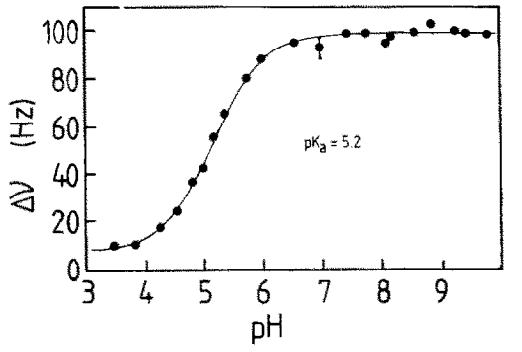

Fig.3. The pH dependence of the ^{43}Ca NMR line-width for a 1.7 mM $PPLA_2$ solution in the presence of 5.9 mM Ca^{2+}. The spectra were recorded at 23°C. The solid curve represents the model of a single protonation step with an app. pK of 5.2.

The value obtained for the rate of the dissociation of Ca^{2+} from $PPLA_2$ (i.e., $k_{off} = 2.5 \pm 1 \times 10^3\ s^{-1}$ at 24°C) may be compared with the corresponding figure for rabbit skeletal muscle troponin C (TnC). The off-rate for Ca^{2+} binding to the regulatory sites of TnC ($k_{off} = 1 \times 10^3\ s^{-1}$; T. A. et al., unpublished) is of the order of magnitude that one would expect if the on-rate were essentially diffusion controlled. (Taking the association constant of Ca^{2+} to the regulatory sites to be $3 \times 10^5\ M^{-1}$ [10], we obtain $k_{on} = 3 \times 10^8\ s^{-1}\ M^{-1}$.)

In contrast, the on-rate of Ca^{2+} binding to $PPLA_2$ must be at least two orders of magnitude slower than the on-rates to the regulatory sites of TnC in order to account for the relation between the association constant and the off-rate. This large difference reflects the different nature of the regulatory Ca^{2+} binding sites of TnC on the one hand and that of $PPLA_2$ on the other. The former sites seem to have a flexible structure, such that the Ca^{2+} binding amino acid residues may easily wrap around an incoming ion. The Ca^{2+} binding site of $PPLA_2$ seems to be more rigid or generally less accessible to an incoming Ca^{2+}.

The value of the longitudinal relaxation rate, $R_{1,b}$, of the Ca^{2+} at the $PPLA_2$ binding site may be estimated from the Swift-Connick equation (see above). By using the experimentally obtained values: $R_{1,obs} = 79 \pm 4\ s^{-1}$, $R_{1,f} = 0.71\ s^{-1}$, $p_b = 0.17$ and $k_{off} = 2.5 \pm 1 \times 10^3\ s^{-1}$ we may calculate $R_{1,b}$ to be $5.7 \pm 0.8 \times 10^2\ s^{-1}$.

To obtain an estimate of $R_{2,b}$ we have not followed a procedure similar to that described above for $R_{1,b}$ since chemical exchange effects distort the observed ^{43}Ca NMR signal from a Lorentzian line shape. Instead we have used the value obtained from fitting the variable temperature data: $R_{2,b} = 1.6 \pm 0.2 \times 10^3\ s^{-1}$. From the ratio $R_{2,b}/R_{1,b}$ it is possible to calculate the correlation time τ_c for the bound Ca^{2+}. Under conditions such that $\omega\tau_c \lesssim 1$ it may be shown that [11,12] $R_{2,b}/R_{1,b} = (0.3 + 0.5\,\tilde{J}_1 + 0.2\,\tilde{J}_2)/(0.2\,\tilde{J}_1 + 0.8\,\tilde{J}_2)$, where $\tilde{J}_1 = (1 + (\omega\tau_c)^2)^{-1}$ and $\tilde{J}_2 = (1 + (2\omega\tau_c)^2)^{-1}$. The quotient $R_{2,b}/R_{1,b} = 2.9 \pm 0.7$ corresponding to $\omega\tau_c = 1.3 \pm 0.3$ or $\tau_c = 12 \pm 3$ ns. This value is close to that expected for the rotational diffusion of the entire $PPLA_2$ molecule. Taking the average hydrodynamic radius to be 2.1 nm this gives $\tau_c = 8$ ns from the Debye-Stokes-Einstein equation [13]. This may be taken to indicate that the Ca^{2+} binding region has a relatively low internal mobility.

From the relaxation rate of the bound $^{43}Ca^{2+}$ ($R_{2,b} = 1.6 \pm 0.2 \times 10^3\ s^{-1}$) and the correlation time ($\tau_c = 12$ ns) we may calculate the quadrupolar coupling constant, χ, of the bound $^{43}Ca^{2+}$ from the expression [11,12] $R_{2,b} = (2\pi^2\chi^2/49)(0.3\tau_c + 0.5\,\tau_c\tilde{J}_1 + 0.2\,\tau_c\tilde{J}_2)$ to be 0.8 ± 0.1 MHz. This value, which is the first to be reported for a Ca^{2+} binding protein may be compared with the quadrupole coupling constants observed for $^{43}Ca^{2+}$ bound to EDTA (0.5 MHz) and EGTA (2.1 MHz) (T.D., unpublished).

The apparent pK value ($pK_a = 5.2$) from the pH dependence of the ^{43}Ca line-width probably reflects the ionization of Asp_{49}, the only charged amino acid residue that is directly involved in the Ca^{2+} binding [6]. Our value is in excellent agreement with the value in [14] ($pK_a = 5.2$) for the active enzyme.

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