plasmin(ogen) form of the complex and that our results constitute the first characterization of the Glu-plasminogen–streptokinase complex. Our results also explain the literature discrepancy on the value of the sedimentation coefficient. The value of s = 10 S reported by Ling et al. (1966) and Davies et al. (1961) was on a Lys-plasmin–streptokinase complex with an available active site, while the s value near 5 S reported by De Renzo et al. (1963) and Summaria et al. (1970) was on the Lys-plasmin–streptokinase complex in which the active site was blocked.

Registry No. Plasminogen, 9001-91-6.

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Calcium Ion Binding to Pancreatic Phospholipase A_2 and Its Zymogen: A ^{43}Ca NMR Study†

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ABSTRACT: Calcium ion binding to phospholipase A_2 and its zymogen has been studied by ^{43}Ca NMR. The temperature dependence of the band shape of the calcium-43 NMR signal has been used to calculate the calcium ion exchange rate. The on-rate was calculated to be 5×10^6 M^{-1} s^{-1}, which is 2 orders of magnitude less than the diffusion limit of the hydrated Ca^{2+} ion in water. The ^{43}Ca quadrupole coupling constant for calcium ions bound to phospholipase, χ = 1.4 MHz, is significantly larger than those found for EF-hand proteins, indicating a less symmetric site. For prophospholipase A_2, we found χ = 0.8 MHz, indicating a calcium binding site, which is somewhat more symmetric than the EF-hand sites. The dependence of the ^{43}Ca NMR band shape on the calcium ion concentration showed that there are two cation binding sites on the phospholipase A_2 molecule: $K_1 = 4 \times 10^3$ M^{-1} and K_2 = 20 M^{-1}. The strong site was found to be affected by a pK_a = 6.5 and the weak site by pK_a = 4.5.

Phospholipase A_2 (PLA_2)[1] specifically catalyses the hydrolysis of the 2-acyl linkage of all phospholipids (van Deenen & de Haas, 1964: de Haas et al., 1968). The enzyme has been isolated from several different sources including snake venom (Wells & Hanahan, 1969; Joubert & van der Walt, 1975) and bee venom (Shipolini et al., 1971), as well as from mammalian pancreas (de Haas et al., 1968; Dutilh et al., 1975). Indications of the presence of a similar intracellular activity in other mammalian tissues have been obtained (Brockerhoff & Jensen, 1974). Phospholipase is secreted as a zymogen by the pancreas. The amino acid sequences of bovine, porcine and equine pancreatic phospholipases A_2 as well as that of the zymogen have been determined (Puyk et al., 1977; Fleer et al., 1978; Evenberg et al., 1977). The zymogens are converted to the active enzyme via a specific tryptic cleavage of the Arg–Ala bond, which removes an N-terminal heptapeptide from the proenzyme (Abita et al., 1972).

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[1] Abbreviations: NMR, nuclear magnetic resonance; PLA_2, phospholipase A_2; $PPLA_2$, prophospholipase A_2; BAEE, N^{α}-benzoyl-L-arginine ethyl ester; DPPC, diphenylcarbamyl chloride; Tris, tris(hydroxymethyl)aminomethane.

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Recently, the crystal structure refined to 1.7-Å resolution of the bovine pancreatic phospholipase A_2 has been reported (Dijkstra et al., 1981). The calcium ion, which is an obligatory requirement for enzymatic activity, is located in the active site and is coordinated by one carboxylate group (Asp-49), three backbone carbonyl groups (Tyr-28, Gly-30 and Gly-32) and two water molecules. Dijkstra et al. (1981) did not find any indication for a second weaker binding site for calcium ions, which had been reported by Slotboom et al., (1978) and Donné-Op den Kelder et al., (1983). The pH dependence of the calcium ion binding has been studied (Pieterson et al., 1974). These authors suggested that a histidine (His-48) and a carboxylate (Asp-49) are involved in the binding of the cation.

Volwert et al., (1974) have shown that *p*-bromophenacyl bromide specifically reacts with His-48 in phospholipase and thereby inactivates the enzyme. Similarly, Slotboom et al., (1978) showed that complete inactivation of the enzyme occurs upon binding of 1 mol of 1-bromo-2-octanone/mol of phospholipase. They also showed that the strong Ca^{2+} binding site was absent in the modified protein.

Here we want to present our ^{43}Ca NMR studies dealing with the binding of calcium ions to phospholipase A_2 and its zymogen. Some preliminary results have been reported earlier (Andersson et al., 1981a). Even though the NMR properties of the ^{43}Ca nucleus are unfavorable (Neurohr et al., 1983), ^{43}Ca NMR is a valuable tool in studies of Ca^{2+} binding to proteins (Vogel et al., 1983). For example, information regarding the metal ion exchange rates can be obtained (Drakenberg et al., 1983), and furthermore, the quadrupole coupling constant determined for the protein bound $^{43}Ca^{2+}$ ion provides information regarding the symmetry of the metal ion binding site.

Materials and Methods

Prophospholipase A_2 was prepared from porcine pancreatic powder that was dissolved in distilled water and extensively dialyzed against distilled water overnight. The dialyzate was subsequently subjected to the purification procedure described by Nieuwenhuizen et al., (1974). The fractions containing prophospholipase A_2 were pooled, dialyzed against 0.02 M NH_4HCO_3, and lyophilized.

Activation of Prophospholipase A_2. Prophospholipase A_2 was converted into the active form by treatment with trypsingel. This gel was synthesized by immobilization of trypsin on Sephadex G-25 according to Axén & Ernbach (1971) and had a specific activity of 300 millunits/mg of gel with BAEE as substrate. A 100-mg aliquot of trypsingel was allowed to swell for 1 h in 1 mL of 50 mM Tris-HCl buffer, pH 8. A total of 200 mg of prophospholipase A_2 was dissolved in 15 mL of the same buffer and transferred to a test tube with a screw cap, containing the swollen trypsingel. The test tube was rotated in order to obtain good mixing. Every half hour, aliquots (100 μL) were withdrawn and analyed for phospholipase A_2 activity with the egg yolk assay (Nieuwenhuizen et al., 1974). After 2 h, the specific activity of the enzyme was 1000 units/mg, and the reaction was considered to be complete. The test tube was centrifuged and the supernatant lyophilized. The powder was redissolved in 0.02 M NH_4HCO_3 and chromatographed on a G-25 Sephadex column. The void fraction, containing pure phospholipase A_2, was lyophilized and stored at 4 °C until further use.

Modification of Phospholipase by p-Bromophenacyl Bromide. The enzyme was reacted with *p*-bromophenacyl bromide as described by Volwerk et al., (1974). The inactivation of the enzyme was followed by the egg yolk assay. After inactivation, the enzyme solution was dialyzed against 0.02 M NH_4HCO_3 and lyophilized.

Pancreatic powder was obtained from Novo research laboratories, Bagsvaerd, Denmark, trypsin (DPCC treated) was from Sigma, and *p*-bromophenacyl bromide was from Serva. Sephadex was from Pharmacia, and CM-cellulose was from Whatman.

The protein concentration was determined spectrophotometrically with an absorption coefficient $E_{1\%} = 12.3$ at 280 nm. An aqueous 0.115 M $CaCl_2$ solution was prepared by dissolving $CaCO_3$ (60% isotopically enriched in ^{43}Ca, Oak Ridge National Laboratory) in 0.1 M HCl, which was subsequently neutralized by a final pH of 7.0 by addition of NaOH. In the NMR measurements, no buffers were used. The pH of the protein solutions was adjusted by addition of dilute HCl or NaOH.

The ^{43}Ca NMR spectra were recorded at 17.16 MHz on a home-built spectrometer as described elsewhere (Drakenberg et al., 1983). Depending on the Ca^{2+} concentration, 10^5–10^6 spectra were accumulated with a 90° pulse (50-μs pulse length) and a total time between the pulses of 30–100 ms. In order to have a negligible effect from probe ringing, a delay of 300 μs had to be introduced between the end of the pulse and the beginning of data accumulation.

Band-Shape Calculations. The band shapes of the ^{43}Ca NMR signal in a system where the Ca^{2+} ions exchange between a protein site and free (solvated) Ca^{2+} ions depend on the exchange rate, k_{ex}, the binding constant, K_B, and the relaxation rate of the ions bound to the protein, R_2. We have previously shown how to extract these parameters from the temperature and calcium-concentration dependencies of the band shape of the ^{43}Ca NMR signal (Drakenberg et al., 1983). This method has been used here with the modification that now ca. 20 curves at various temperatures and calcium concentrations have been used in one single calculation, instead of two separate calculations for the temperature dependence and the calcium-concentration dependence. The shape of the ^{43}Ca NMR signals has been characterized by the width of the signal at 10 different heights. These observed data points (ca. 200) are then compared with calculated ones in iterative calculations in order to obtain the best agreement between the observed and calculated band shapes. The error square sum (ESS) is used as a measure for the fit.

For both PLA_2 and $PPLA_2$, only one complete set of spectra (temperature, calcium, and pH dependencies) were recorded; however, the reproducibility was checked several times with different protein preparations and was always found to be better than ±10% as measured from the line width at half-height of the signal. In all calculation reported here, the entropy of activation, ΔS^*, for the metal exchange was assumed to be 0. Nonzero values did not improve the overall agreement between observed and calculated band shapes. This means that $k_{off} = (kT/h)e^{-\Delta H^*/(RT)}$.

Results

Figure 1, upper trace, shows a ^{43}Ca NMR spectrum from a sample containing 1.2 mM $^{43}Ca^{2+}$ and 0.74 mM PLA_2, and the lower trace shows the spectrum of free (solvated) calcium ions. The resonance in the upper trace is broadened due to the interaction between the calcium ions and the protein. Since the ^{43}Ca nucleus has a quadrupole moment, the width of the signal (the relaxation rate) will be very sensitive to the correlation time of the Ca^{2+} ions bound to the protein, and in the slow exchange regime, one would expect to observe two ^{43}Ca NMR signals, one narrow signal due to free ions and one broad due to protein bound ions. This has been observed, for ex-

Table I: Binding Constants (K_1 and K_2), Quadrupole Constants (χ), Ca^{2+} Ion Residence Time in the Binding Site (τ_{B1}), and Ca^{2+} Ion On-Rate (k_{on1}) to PLA_2 and $PPLA_2$ As Obtained in the Computer Fitting of ^{43}Ca NMR Data

protein	calculation no.	K_1 (M^{-1})	χ_1 (MHz)	K_2 (M^{-1})	χ_2 (MHz)	τ_{B1} (ms)	k_{on1} (M^{-1} s^{-1})	ESS[a]
PPLA2	1	8603	0.78			0.31	2.9×10^7	21.5
PPLA2	2	14235	0.76	2.5	0.79	0.31	4.6×10^7	19.9
PLA2	3	240	1.79			0.12	2×10^6	38.9
PLA2	4	2000[b]	1.27			0.20	1×10^7	81.2
PLA2	5	4608	1.37	15	2.33	1.0	4.6×10^6	10.9
PLA2	6	2000[b]	1.40	14	2.31	0.8	2.5×10^6	12.4
PLA2	7	10000[b]	1.36	19	2.15	1.2	8.3×10^6	12.0
PLA2	8	4798	1.37	30[b]	1.78	1.1	4.4×10^6	12.8
PLA2	9	4720	1.36	40[b]	1.61	1.2	3.9×10^6	14.8
PLA2	10	7.5×10^9	0.84[b]	32	1.95	1.2	6×10^{12}	54.5

[a] ESS, error square sum. The ESS obtained when each line was treated separately as lorentzian lines was 14.7 for $PPLA_2$ and 6.2 for PLA_2. [b] Not iterated in the computer fitting procedure.

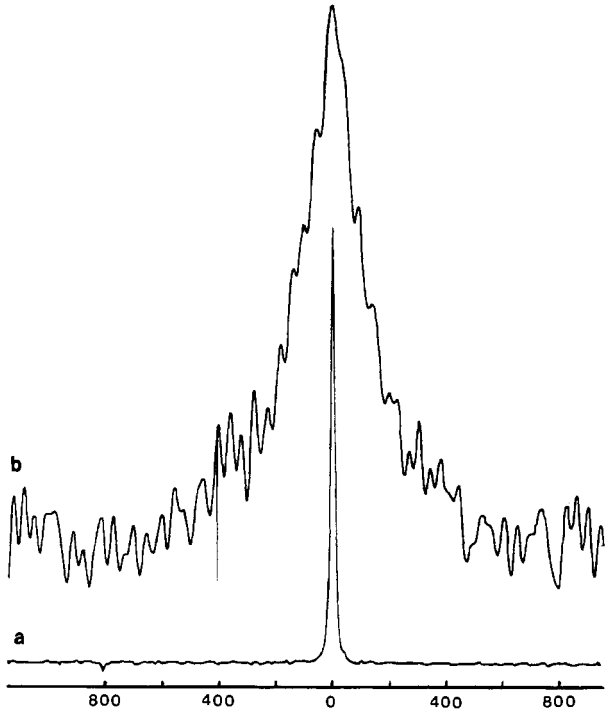

FIGURE 1: (a) ^{43}Ca NMR spectrum of free Ca^{2+} ions. (b) ^{43}Ca NMR spectrum of a solution containing 0.74 mM phospholipase A_2 and 1.20 mM $^{43}Ca^{2+}$ at pH 7.1 and 23 °C.

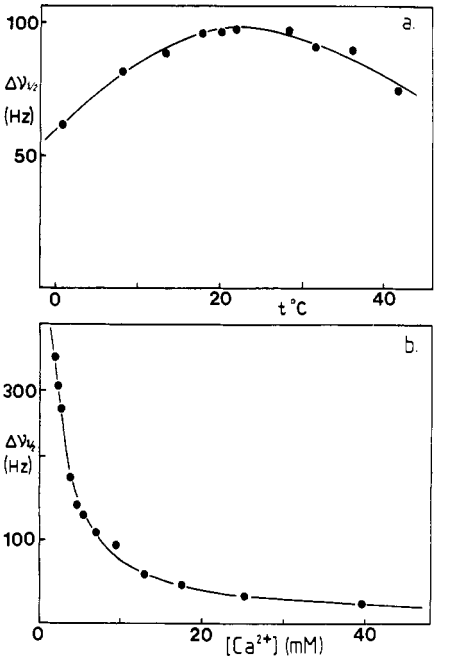

FIGURE 2: Line width of the ^{43}Ca NMR signal (a) as a function of temperature for a sample containing 1.7 mM $PPLA_2$ and 5.9 mM Ca^{2+} at pH 7.4 and (b) as a function of calcium concentration for a sample containing 2 mM $PPLA_2$ at 23 °C and pH 7.5. The solid curves are calculated line widths from the values given in Table I, calculation 1.

ample, for parvalbumin, which binds Ca^{2+} ions very strongly (Forsén et al., 1982; Drakenberg & Forsén, 1983). In contrast, for PLA_2 and $PPLA_2$ we have only observed one broad signal at all temperatures and Ca^{2+} to protein ratios.

Figure 2a shows experimental and calculated line widths for the ^{43}Ca NMR signal as a function of temperature for a sample containing 1.7 mM $PPLA_2$ and 5.9 mM $^{43}Ca^{2+}$. Figure 2b shows the dependence of the ^{43}Ca NMR line width on total calcium concentration. When a calculation was performed with these data and by assuming one single binding site, the parameters given in Table I (calculation 1) were obtained. In a second calculation, we assumed that there are two calcium binding sites on the protein. This did not result in a fit to the experimental data that was significantly better than that with the assumption of a single binding site. The values given in Table I differ slightly from the values reported in a preliminary report (Andersson et al., 1981a). This difference stems from the fact that in the present work we have treated the data from the concentration and temperature dependence in one single iterative calculation.

Figure 3 shows the observed ^{43}Ca NMR line width as a function of temperature and calcium concentration in a solution containng PLA_2. An attempt to simulate these dependences caused by a single binding site for Ca^{2+} ions on PLA_2 resulted in the line widths shown by the dashed curves in Figure 3, and the parameters are given in Table I, calculation 3, from 18 different ^{43}Ca NMR spectra. In a second iteration, we assumed that there are two Ca^{2+} ion binding sites, of which one is weak and has fast chemical exchange at all temperatures. This resulted in the line widths shown by the solid curves in Figure 3 and a binding constant for the strong site of 4×10^3 M^{-1}, in reasonable agreement with other reports (Pieterson et al., 1974; Slotboom et al., 1978).

In Table I are also included the results from several other calculations for PLA_2. A variation of the binding constant for the strong site (K_1) from 2×10^3 to 1×10^4 M^{-1} (calculations 5–7) shows that K_1 cannot be obtained with good accuracy in this type of experiment—it is obviously too strong. The constant can, however, with some confidence by assumed to fall in the range 2×10^3–1×10^4 M^{-1}, which is in good agreement with other reports (Pieterson et al., 1974; Slotboom et al., 1978). Calculations 5, 8, and 9 show the effect of a variation of the binding constant for the weak calcium site,

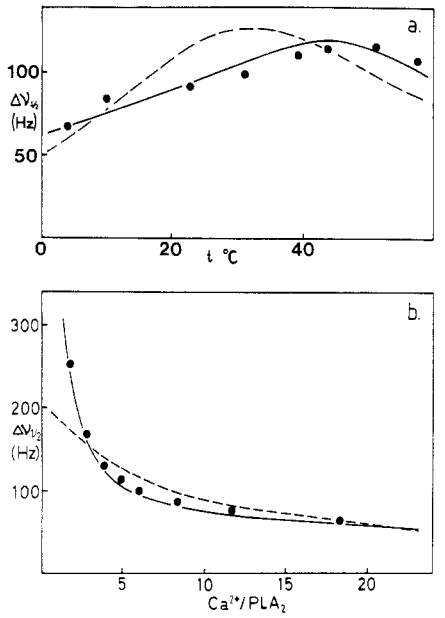

FIGURE 3: Line width of the ^{43}Ca NMR signal (a) as a function of temperature for a sample containing 0.74 mM PLA_2 and 4.4 mM Ca^{2+} at pH 7.2 and (b) as a function of Ca^{2+} to PLA_2 ratio for a sample containing 0.74 mM PLA_2 at 23 °C and pH 7.1. The dashed curves are calculated line widths from the values given in Table I, calculation 3, and the solid curves are from calculation 5.

K_2. The best fit is obtained with a binding constant to the weak site of 15–30 M^{-1}.

As we have demonstrated in our preliminary paper on $PPLA_2$ (Andersson et al., 1981a), the variation in the ^{43}Ca NMR line width with pH can be accurately explained with a single apparent pK_a value of 5.2. However, when the binding constant for Ca^{2+} ions to the protein is known, eq 1 can be used to obtain the "true" pK_a value, which is of course a value of more direct interest:

$$\Delta\nu_e^{obsd} = \Delta\nu \frac{K[E]}{1 + K[E]} \quad (1)$$

where

$$[E] = [E_{tot}]/(1 + K[Ca^{2+}] + 10^{pK_a - pH})$$

and

$$[Ca^{2+}] = [Ca_{tot}]/(1 + K[E])$$

Equation 1 is most easily solved by a computer to obtain self-consistent values for [E] and $[Ca^{2+}]$, which are then used to calculate $\Delta\nu_e^{obsd}$ as a function of pH. With a binding constant of 2×10^3 M^{-1}, this calculation resulted in a pK_a value of 6.5 for the Ca^{2+} ion binding to $PPLA_2$. The agreement between calculated and observed line widths is very similar to the one shown previously (Andersson et al., 1981a) and is therefore not reproduced here.

Figure 4 shows the pH dependence of the ^{43}Ca NMR line width for two different $[Ca^{2+}]$ to $[PLA_2]$ ratios. Using eq 1 and a binding constant of 4×10^3 M^{-1} for a single binding site gave the results shown by the dashed curves in Figure 4. For the low ratio of $[Ca^{2+}]$ to $[PLA_2]$, the agreement between calculated and observed line widths is almost as good as that for the $PPLA_2$ case; however, for the higher ratio (10) the agreement is less satisfactory, and the experimental line widths follow a curve indicative of a Hill coefficient less than 1. However, since the concentration dependence of the ^{43}Ca NMR line width has shown that there is also a second weaker binding site for calcium on PLA_2, we have used eq 2, which

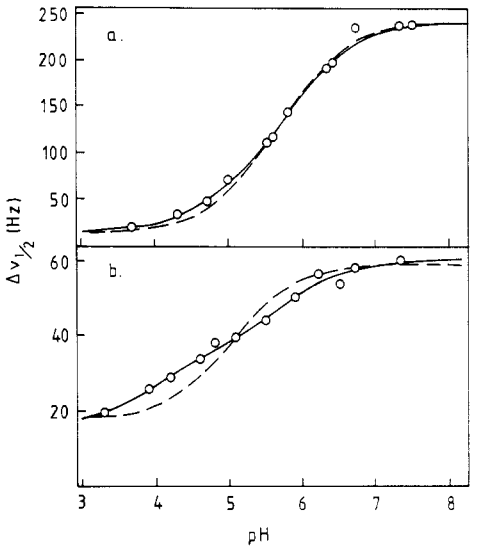

FIGURE 4: Dependence of the ^{43}Ca NMR line width on pH at 23 °C: (a) 0.98 mM phospholipase A_2 and 1.1 mM $^{43}Ca^{2+}$; (b) 1.1 mM phospholipase A_2 and 11.5 mM $^{43}Ca^{2+}$. The solid lines are calculated on the basis of eq 2, and the dashed curves are obtained by using eq 1.

is valid for the case with two independent binding sites with different pK_a values:

$$\Delta\nu_e^{obsd} = \Delta\nu_1 \frac{[CaE] + [CaECa] + [CaEH]}{[Ca_{tot}]} + \Delta\nu_2 \frac{[ECa] + [CaECa] + [HECa]}{[Ca_{tot}]} \quad (2)$$

where

$$[CaE] = K_1[Ca][E]$$

$$[CaECa] = K_1K_2[Ca]^2[E]$$

$$[CaEH] = K_1[Ca][E] \times 10^{pK_2 - pH}$$

$$[ECa] = K_2[Ca][E]$$

$$[HECa] = K_2[Ca][E] \times 10^{pK_1 - pH}$$

and

$$[E] = [E_{tot}]/(1 + K_1[Ca] + K_1K_2[Ca]^2 + K_1[Ca] \times 10^{pK_2 - pH} + K_2[Ca] + K_2[Ca] \times 10^{pK_1 - pH} + 10^{pK_1 - pH} + 10^{pK_2 - pH} + 10^{pK_1 + pK_2 - 2pH})$$

$$[Ca] = [Ca_{tot}]/[1 + K_1[E] + 2K_1K_2[Ca] \times [E] + K_1[E] \times 10^{pK_2 - pH} + K_2[E](1 + 10^{pK_1 - pH})]$$

Similarly to eq 1, eq 2 has to be solved for self-consistency in [E] and $[Ca^{2+}]$. Using eq 2 in an interative calculation to find the best agreement between experimental and calculated line widths resulted in a pK_a value for the strong site of 6.5 and for the weak site of 4.5. The calculated line widths are shown as the solid curves in Figure 4. This calculation used here is in fact similar to the one valid for the competition between two types of ligands, e.g., different metal ions for the same binding site [cf. the discussion on multiple binding of ligands by Klotz & Hunston [1979)].

We have also used ^{43}Ca NMR to study Ca^{2+} binding to the *p*-bromophenacyl bromide modified phospholipase A_2. There is only a moderate effect on the ^{43}Ca NMR line width caused by the addition of the *p*-bromphenacyl bromide modified protein to a $^{43}Ca^{2+}$ solution. The temperature dependence also showed a very small effect (data not shown). These results indicate that the lack of broadening of the ^{43}Ca NMR signal is not caused by slow exchange of calcium ions but finds its

origin in a drastic reduction of the binding constant for the strong site.

Discussion

The Ca^{2+} binding site in the refined (1.7-Å) crystal structure of bovine pancreatic phospholipase A_2 was found to be made up of three backbone carbonyl functions (Tyr-28, Gly-30, and Gly-32), both oxygens from the carboylate group of Asp-49, and two water molecules (Dijkstra et al., 1981). This site is thus very different from the calcium binding site in regulatory calcium binding proteins with known crystal structure; i.e., parvalbumin (Moews & Kretsinger, 1975) has two calcium binding sites containing four carboxylate groups each, and intestinal calcium binding protein (Szebengi et al., 1981) has one site with three carboxylate groups. In the latter two proteins, as well as in trypsin (Fehlhammer et al., 1777) and staphylococcal nuclease (Cotton et al., 1979), the calcium ion is thought to be octahedrally liganded to six oxygens. Also, for the regulatory calcium binding proteins, octacoordination has been suggested (Ellis, 1983; Kretsinger, 1980). A difference in the types of oxygen ligands and the geometry of the calcium binding site could a priori be expected to have an influence on the ^{43}Ca NMR spectra. We have previously shown for some EF-hand proteins (Andersson et al., 1982a; Forsén et al., 1982) that they have ^{43}Ca quadrupole coupling constants (χ) of ca. 1 MHz. From Table I, we can see that the ^{43}Ca quadrupole coupling constant for the strong site in PLA_2 is ca. 1.4 MHz and for the weak site χ is even larger, though not very well defined, 1.8–2.3 MHz.

In our preliminary paper (Andersson et al., 1981a), we reported that the quadrupole coupling constant, χ, for ^{43}Ca bound to $PPLA_2$ was only 0.8 MHz. In the light of the appreciably larger value of χ calculated here for PLA_2, we have reevaluated our previous $PPLA_2$ data once more by using the one-step calculation as indicated above. There is, however, no way in which we can account for a quadrupole coupling constant significantly larger than 0.8 MHz. Therefore, we conclude that the symmetry of the site in $PPLA_2$ differs significantly from that in PLA_2, with the $PPLA_2$ site being the more symmetric of the two. An alternative explanation could be that the Ca^{2+} site in $PPLA_2$ has a higher degree of flexibility than that in PLA_2. This would reduce the effective correlation time, which we have assumed to be the same in our calculations.

Another very interesting feature of the Ca^{2+} binding to PLA_2 and $PPLa_2$ is observed in the rates of metal ion exchange. For troponin C and calmodulin, the on-rate for the calcium binding is found to be essentially limited by diffusion of the calcion ion (Andersson et al., 1981b, 1982b). We have calculated (Table I) that the on-rate for Ca^{2+} to PLA_2 is about 2 orders of magnitude slower than what is found for troponin C and calmodulin. This difference may be related to the different biological roles for the proteins. In troponin C and calmodulin, calcium binding has a regulatory function, and both proteins have to respond quickly to variations in the Ca^{2+} concentration, whereas for PLA_2 and other extracellular enzymes, which are not subject to fluctuations in the Ca^{2+} concentration, there is no apparent need for such a fast metal exchange rate.

Aguiar et al., (1979) have shown, by using proton NMR, that the apparent pK_a for His-48 in bovine $PPLA_2$ is 6.2 in the apoprotein and that it decreases to 5.4 upon calcium addition. This is in good agreement with our observation that the calcium binding is sensitive to an apparent pK_a of 5.2 (Andersson et al., 1981a). It is of considerable interest that also others have found that the protonation of His-48 affects

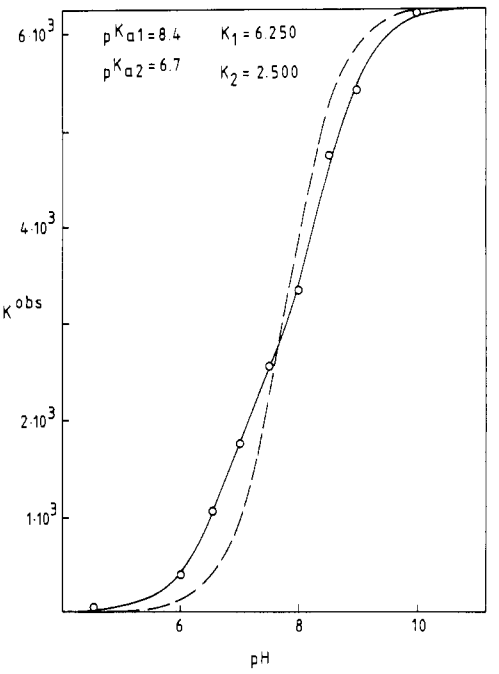

FIGURE 5: pH dependence of the apparent binding constant for calcium binding to phospholipase A_2 (Pieterson et al., 1974). The dashed curve is calculated by means of eq 3 (pK_a = (7.6), and the solid curve is calculated from eq 4 (pK_{a1} = 8.4, pK_{a2} = 6.7).

the binding of calcium even though it is not a ligand to the calcium ion (Dijkstra et al., 1981; Donné-Op den Kelder et al., 1983). Also our calculation of the "true" pK_a value of the group affecting the strong calcium binding with eq 1, pK_a = 6.5, agrees well with the value given by Aguiar et al. for the apoprotein, which is reassuring. Similarly, Aguiar et al. have reported the pK_a values for PLA_2 to be 6.5 and 5.7 for the apo and Ca forms, respectively. We have also found a value of 6.5 for the "true" pK_a, and the midpoint of the curve in Figure 4a gives an apparent pK_a of 5.5. Again, there is good agreement. In contrast, Pieterson et al. (1974) have argued that in addition to the histidine (pK_a = 6.5) an acidic group is affecting the binding of calcium. This cannot be verified with our ^{43}Ca NMR data, and the intent of the following discussion is to show that they may have interpreted their data erroneously. They argue that the large increase in the dissociation constant in the acidic pH region is caused by the protonation of one or more carboxylic groups. However, as can be seen from eq 3, which is valid for the case where the

$$K^{obsd} = K/(1 + 10^{pKa-pH}) \quad (3)$$

binding constant depends on only one site of protonation, the observed binding constant, K^{obsd}, should follow a sigmoid-shaped curve with its midpoint at pK_a. The equation also shows that K^{obsd} will decrease with a factor of 10 for the decrease in pH by 1 unit as long as pH < pK_a. Therefore, the variation in the binding, or dissociation, constant with pH below the highest pK_a value cannot be directly used to deduce a lower pK_a value. The dashed curve in Figure 5, which is calculated from eq 3, does not fit very well to the experimental data. The deviation is of the type that will result from a Hill coefficient less than 1. The fit is not much improved by using eq 4, which is valid for two ionizable groups, and by assuming

$$K^{obsd} = K_1/(1 + 10^{pK_{a1}-pH} + 10^{pK_{a1}+pK_{a2}-2pH}) + K_2/(1 + 10^{pK_{a2}-pH} + 10^{pH-pK_{a1}}) \quad (4)$$

an acidic pK_a (4–5). A good agreement between observed and calculated K^{obsd} values could only be obtained with the following constants: $K_1 = 6.3 \times 10^3$ M^{-1}, $K_2 = 2.5 \times 10^3$ M^{-1},

pK_{a1} = 8.4, and pK_{a2} = 6.7. Admittedly, this may be an overinterpretation of the data in Figure 5; however, the main point was to show that the experimental evidence for a pK_a value in the acidic region as discussed by Pieterson et al., (1974) could be interpreted differently.

The lower pK_a value (6.7) agrees very well with the one we have obtained from our ^{43}Ca NMR measurements on PLA_2 and $PPLA_2$ and with the value reported by Aguiar et al., (1979) for His. The higher value (8.4) on the other hand has no counterpart in our measurements, which do not extend above pH 7.6 for PLA_2 (pH 9.1 for $PPLA_2$). van Dam-Mieras et al., (1976) have, however, reported a conformational change of PLA_2 due to the protonation of the terminal α-NH_3 group with a pK_a = 8.4. It seems therefore possible that this transition may also affect the calcium binding constant. The calcium-43 NMR data are thus in good agreement with our analysis of the Ca binding data of Pieterson et al., (1974).

There is an agreement that the strong calcium binding site is sensitive to a $pK_a \approx 6.5$. With regard to the weak calcium binding site, there seem to be some disagreement. Donné-Op den Kelder et al., (1983) have used the proton titration technique to identify the groups involved in the calcium binding to the weak site in PLA_2. By comparing measurements in very high calcium concentrations ($[Ca^{2+}]$ = 250 mM) with those at low calcium concentrations, they have concluded that the weak site depends on a group with a pK_a = 6. In high calcium solutions, this apparent pK_a is reduced to 4.5. We have found that our ^{43}Ca NMR data from the pH titration can only be explained by assuming that the weak calcium binding depends on a group with a pK_a = 4.5. We can, however, not exclude the possibility that also a second group with a $pK_a \approx 6$ is involved since the effect on the ^{43}Ca NMR signal would be masked by the pH dependence due to the strong calcium binding site in the pH region 5–7.

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Registry No. Phospholipase A_2, 9001-84-7; prophospholipase A_2, 37350-21-3; calcium, 7440-70-2; calcium-43, 14333-06-3.

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