Localization of the Second Calcium Ion Binding Site in Porcine and Equine Phospholipase A_2†

Gabriëlle M. Donné-Op den Kelder, Gerard H. de Haas,* and Maarten R. Egmond

ABSTRACT: At alkaline pH porcine pancreatic phospholipase A_2 is known to bind two Ca^{2+} ions per protein molecule. One Ca^{2+} ion is strongly bound to the active site and is essential for enzyme activity. A second Ca^{2+} ion binds more weakly to the protein and improves the affinity of the enzyme for lipid–water interfaces severalfold at high pH values. A group having a pK around 6 controls enzyme binding to lipid–water interfaces in the absence of Ca^{2+}. By use of proton titration techniques this group is now identified to be a carboxylate having an abnormally high pK. Its pK shifts to a value around 4.5 in the presence of high Ca^{2+} concentrations, suggesting that the carboxylate is involved in binding the second Ca^{2+} ion. The carboxylate was identified to be Glu^{71} by comparing proton titration experiments on porcine pancreatic phospholipase A_2 and an isoenzyme. The isoenzyme differs by only four residues from the most abundant enzyme, lacking the carboxylate at position 71 (Asn for Glu). The isoenzyme also appeared to be devoid of an abnormal carboxylate. Identification of Glu^{71} as the abnormal carboxylate in the porcine enzyme was substantiated by comparison with enzymes from other sources. Kinetic experiments on the various phospholipases finally demonstrated that enzyme species containing Glu^{71} bind a second Ca^{2+} ion to the low-affinity site, whereas enzymes lacking Glu^{71} also lack this second site. These experiments confirm the suggestion that Glu^{71} is one of the ligands for Ca^{2+} in the low-affinity site.

Phospholipase A_2 (EC 3.1.1.4) specifically catalyzes the hydrolysis of the 2-acyl ester linkage in 3-*sn*-phosphoglycerides. The pancreatic enzymes have an absolute requirement for Ca^{2+} ions which bind to the active site residue Asp^{49} (Fleer et al., 1981; Dijkstra et al., 1981). It was shown that binding of Ca^{2+} to the catalytic site of porcine PLA[1] perturbs the active site residue His^{48} and some tyrosine residues (Pieterson et al., 1974a; Volwerk et al., 1974; Aguiar et al., 1979; Verheij et al., 1980).

Apart from the active site there is another Ca^{2+} binding site in porcine PLA which has a lower affinity for Ca^{2+} than the former. It has been demonstrated that Ca^{2+} binding to the low-affinity site improves the affinity of the enzyme for organized lipid–water interfaces at alkaline pH (Pieterson, 1973; van Dam-Mieras et al., 1975). As confirmed by the X-ray diffraction studies on bovine PLA (Dijkstra et al., 1981), pancreatic PLA contains a region, distinct from its active site, that preferentially interacts with organized lipid–water interfaces (Verger & de Haas, 1973). This region, called the interface recognition site (IRS), includes several hydrophobic residues, among which is the only tryptophan in the protein (Trp^3). Also the NH_3^+ group of L-Ala^1 is part of the IRS (Slotboom & de Haas, 1975). In bovine PLA the NH_3^+ group of L-Ala^1 was found to be locked inside the protein, close to Gln^4, the carbonyl oxygen of Asn^{71}, and a buried H_2O molecule, which is in turn hydrogen bonded to Asp^{99}. The structural and functional importance of the N-terminal Ala^1 was demonstrated by specific modification of Ala^1 (Slotboom et al., 1978) and by recent monolayer studies (Pattus et al., 1979a,b).

Kinetic (Pieterson, 1973) and equilibrium dialysis experiments (Slotboom et al., 1978) revealed that binding of Ca^{2+} to the second site in porcine PLA is controlled by a group with a pK around 6–7. An exact value of 6.3 was derived from the ^{13}C NMR titration curve of ^{13}C enriched porcine [L-(3-^{13}C)Ala^1]AMPA (Jansen, 1979). ^{13}C NMR titration curves of equine and bovine [L-(3-^{13}C)Ala^1]AMPA (Jansen, 1979) showed that in the titration curve of the former enzyme a group with a pK of 6.3 is present while it is absent in bovine PLA. These experiments possibly suggest that bovine PLA lacks a low-affinity Ca^{2+} binding site.

It is the purpose of this study by means of the newly developed automated proton titration system (Donné-Op den Kelder et al., 1982) to investigate the nature of the unknown Ca–ligand in porcine PLA. For these studies, PLA's from several sources, modified enzymes, and isoenzymes were used. It was possible to identify the ligand for the second Ca^{2+} binding site in porcine phospholipase A_2.

Experimental Procedures

Materials and Methods. The pancreatic pro- and isoprophospholipases A_2 from pig, horse, ox, and human were purified from pancreatic tissue and converted into phospholipases A_2 by limited proteolysis as described by Nieuwenhuizen et al. (1974), van Wezel & de Haas (1975), Evenberg et al. (1977), Fleer et al. (1978), and Grataroli et al. (1981, 1982), respectively. ε-Amidinated prophospholipase A_2 (AMPREC) and phospholipase A_2 (AMPA) were prepared as described previously (Slotboom & de Haas, 1975). [1-(2-Oxooctyl)-His^{48}]PLA was prepared according to the procedure as described by Verheij et al. (1980). 1,2-Dioctanoyl-*sn*-glycero-3-phosphocholine was prepared as described by Cubero Robles & van den Berg (1969).

Protein concentrations were calculated from the absorbance at 280 nm by using $E^{1\%}_{1cm}$ values of 12.3 for bovine and equine PLA and a value of 11.6 for their precursors. For native and modified porcine PLA (and iso-PLA) a value of 13.0 was used

† From the Laboratory of Biochemistry, State University of Utrecht, Transitorium III, Padualaan 8, NL-3584 CH Utrecht, The Netherlands. *Received October 22, 1982.* This study was partly carried out under the auspices of The Netherlands Foundation of Chemical Research (SON) and with financial aid from The Netherlands Organization for the Advancement of Pure Research (ZWO).

[1] Abbreviations: PLA, phospholipase A_2; PREC, zymogen of PLA; AMPA, ε-amidinated PLA; AMPREC, zymogen of AMPA; [Oct-His^{48}]PLA, [1-(2-oxooctyl)-His^{48}]PLA; iso-PLA, isoenzyme of PLA; iso-AMPA, isoenzyme of AMPA; pGlu, pyroglutamic acid; C_{18}PN, *n*-octadecylphosphocholine; di-C_8-PC, 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine; di-C_{10}-PC, 1,2-didecanoyl-*sn*-glycero-3-phosphocholine; IIP, pH of isoionic solution; RAM, random access memory; BCD, binary coded decimal; IRS, interface recognition site; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

0006-2960/83/0422-2470$01.50/0

and a value of 12.3 for their precursors. All calculations were based on a molecular weight of 13 950 for porcine PLA (and iso-PLA), 13 750 for bovine PLA, and 13 900 for equine PLA, respectively, as can be derived from the amino acid compositions (Puijk et al., 1977, 1979; Fleer et al., 1978; Evenberg et al., 1977). For human PLA a value of 13.0 was used for $E^{1\%}_{1cm}$ and a value of 13 900 for its molecular weight. For the determination of the molecular weights of the zymogens of the above-mentioned enzymes, 700 (molecular weight of additional heptapeptide) was added to the molecular weight of the corresponding native enzymes.

Proton Titration Procedure. The enzymes were dissolved in distilled, degassed water and subsequently deionized by passage through a mixed-bed ion-exchange column in a closed system. Recycling was performed until a stable conductivity of about 1–2 $\mu\Omega^{-1}$ was reached. The protein solution collected from the column generally contained about 0.2% protein.

The pH meter equipped with a combined calomel electrode was calibrated with standard 0.05 M phthalate (Radiometer S1316) and 0.05 M phosphate (S1326) buffers. The isoionic pH (IIP) was measured for a fresh protein solution (5 mL). The solution was brought to the required ionic strength by adding solid KCl (Merck, pro analysi). When the titration behavior of the Ca^{2+}-bound protein was studied, solid $CaCl_2$ (Merck, pro analysi) was added to the required amount. The stirred solution was kept at 25.0 ± 0.2 °C and was held continuously under a nitrogen atmosphere. Generally, titrations were carried out with CO_2-free 0.0643 M/L NaOH (Titrisol, Merck) starting at pH 3 and titrating until pH 10–11. Usually 400 μL of titrant was added with step sizes of addition of 1 μL. The experiments were repeated at least 2 times, and data were averaged and stored for further analyses.

Proton Titration System. An automatic titration system has been developed and was described extensively elsewhere (Donné-Op den Kelder et al., 1982). A Radiometer titration system comprising a PHM 64 research pH meter, a GK 2321C combined electrode, an ABU 80 Autoburette, and a TTA 60 titration assembly was used. A Rockwell R6500 type microprocessor (48 kilobytes RAM) was used to read the BCD output of the digital pH meter and to control the titrator as follows: (a) wait for an initially preset time to allow the system to reach equilibrium; (b) read pH values at fixed time intervals (~1 s) until they become constant within operator-defined limits; (c) repeat the sequence from step b until the required data are collected. After the experiment the data are stored on floppy disk.

Procedure of Analysis. The analyses of the titration curves were performed according to the method developed by Linderstrøm-Lang (1924) and described by Tanford (1962) by using the relation

$$pH = pK_{int}^{(i)} + \log [\alpha_i/(1 - \alpha_i)] - 0.868w\bar{Z}_H \quad (1)$$

where α_i is the average degree of dissociation of a titratable group i and $K_{int}^{(i)}$ the intrinsic dissociation constant for that group (i.e., K_i in the absence of long-rang electrostatic interactions). $\bar{Z}_H$ is the average net proton charge of the protein and w the electrostatic interaction factor depending on size and shape of the molecule and on the type of solvent (dielectric constant, ionic strength). In all calculations w was assumed to be constant over the whole titration range. When a divalent metal ion binds to the protein, eq 1 is no longer valid and $\bar{Z}_H$ has to be replaced by $\bar{Z}_H + 2$. For $\bar{Z}_H$ we can write the relation

$$\bar{Z}_H = Z_{max} - \sum n_i\alpha_i \quad (2)$$

where Z_{max} is the maximum positive proton charge given by

$$Z_{max} = n_{His} + n_{\alpha\text{-amino}} + n_{Lys} + n_{Arg} \quad (3)$$

Table I: Partial Amino Acid Composition and Electrostatic Interaction Factor w (at 100 mM KCl) for Porcine, Bovine, and Equine Phospholipases A_2[a]

nature of groups	phospholipases A_2 from: pig PLA	pig iso-PLA	pig PREC	pig iso-PREC	ox PLA	ox PREC	horse PLA
α-COOH	1	1	1	1	1	1	1
aspartic acid	9	10	9	10	9	9	7[b]
glutamic acid	6	5	7	6	5	5	7
histidine	3	2	3	2	2	2	1
α-amino	1	1			1		1
lysine	9	9	9	9	11	11	8
tyrosine	8	8	8	8	7	7	7
arginine	4	4	5	5	2	3	4
pGlu			1	1		1	
w[c] × 10^3	87.4	87.4	86.5	86.5	87.8	86.7	87.5

[a] Puijk et al., 1977; Puijk et al., 1979; Fleer et al., 1978; Evenberg et al., 1977. [b] Corrected value determined from previous proton-titration experiments (Donné-Op den Kelder et al., 1982). [c] Calculation of w was based on the molecular weights as mentioned in the text (Materials and Methods) and the corresponding partial specific volumes as determined from the amino acid compositions ($\nu_p \simeq 0.696$–0.706).

For the calculation of the number of protons dissociated from the protein ($\bar{n}_H$) relation 4 is used:

$$\bar{n}_H = (m_t - m_w)/m_p \quad (4)$$

where m_t is the total amount of added titrant, m_w the fraction of m_t which does not interact with the protein, and m_p the total amount of protein. $\bar{n}_H$ Can simply be converted to $\bar{Z}_H$ when the IIP (pH at zero net proton charge) is known.

The partial amino acid composition and calculated electrostatic interaction factor w (at 100 mM KCl) for the several studied proteins are given in Table I.

Numerical Differentiation. In differential titration curves $-dpH/d\bar{Z}_H$, the numerical derivative of the titration curve, is plotted vs. $\bar{Z}_H$. With very small increments in the amount of added acid or base, $-dpH/d\bar{Z}_H$ approaches the reciprocal buffer capacity (de Bruin & van Os, 1968):

$$-dpH/d\bar{Z}_H = 1/[2.303\sum n_i\alpha_i(1 - \alpha_i)] + 0.868w \quad (5)$$

with n_i the number of groups titrating in class i. This function becomes very large at these values of $\bar{Z}_H$ where every α_i is either 1 or 0. Differential titration curves usually show two peaks, one near pH 6 (Max 1) and a second near pH 8–9 (Max 2). In case of phospholipase A_2 generally a well-defined Max 2 can be observed while Max 1 is only poorly resolved (Donné-Op den Kelder et al., 1982). The position of Max 2 on the $\bar{Z}_H$ axis (Z_{II}) is therefore very suitable for group counting. Z_{II} for native PLA (iso-PLA) is given by

$$Z_{II} = n_{Lys} + n_{Arg} - n_{COOH} + (1 - \alpha_{NH_2}) \quad (6)$$

assuming that carboxyl and histidyl groups have an α of 1 and the lysines and tyrosines an α of 0, while the single α-NH_3^+ group is titrating in the pH region where Max 2 is located. The last term of eq 6 vanishes when pGlu is present as the N-terminal residue. Usually Z_{II} values can be determined experimentally from the titration curve if the reference point for the ordinate is the point of zero proton charge (i.e., if the IIP has been determined). However, when substances other than H^+ ions (e.g., Ca^{2+}) bind to the protein, the IIP cannot be obtained. In case of Ca^{2+} binding, a theoretical Z_{II} value

was deduced from eq 6 and is used as a reference point.

It has to be noted that especially the $-dpH/d\bar{Z}_H$ values of Max 2 can be subject to error in case the step size of addition is too large. Although usually very small amounts are added (1 μL), we will restrict ourselves to merely a comparison of the heights of Max 2 of the several studied proteins and not occupy ourselves with the absolute values. This comparison is justified because all experiments were carried out under almost identical conditions (enzyme concentration, step size of additions, etc.).

Titration Curves of the Acidic Residues and the Histidines in PLA. The titration curve of the glutamic and aspartic acids can be derived from eq 2:

$$n_{COOH}\alpha_{COOH} = Z_{max} - \bar{Z}_H - \sum n_i\alpha_i \qquad (7)$$

where Z_{max} and the number of titratable groups can be derived from Table I. It is assumed that the n_i groups in class i are intrinsically identical. Usually the pK_{int} of the C-terminal COOH group is set to 3.6, the pK_{int} of the lysines to 10.3, and that of the tyrosines to 9.6 (Tanford, 1962). The pK values for the α-NH_2 are taken from Jansen et al. (1979), Jansen (1979), and Slotboom et al. (1978). The values for the histidines were taken from Aguiar et al. (1979) and Janssen et al. (1972). A pK of 8.9 is assumed for the deprotonation of the His^{48} side chain in porcine [Oct-His^{48}]PLA (Verheij et al., 1980).

The titration curves of the histidines can be derived from eq 7 only by replacing the $\alpha_i(pK_{int})$ and the number of histidines by the corresponding values for the acidic groups.

The titration curve of the carboxylates and histidines is characterized by an average apparent pK value, $pK_{1/2}$, which is defined as $pK_{1/2}$ = pH at which $\alpha_i = 0.5$. The corresponding $pK_{int}^{(i)}$ value can then be determined from

$$pK_{int}^{(i)} = pK_{1/2}^{(i)} + 0.868w\bar{Z}_H \qquad (8)$$

where $\bar{Z}_H$ is the proton charge at the pH with $\alpha_i = 0.5$.

A separate pK_{int} value for either the Glu's or Asp's can be derived from linearization of the titration curve. This is achieved by plotting pH − log $[\alpha/(1-\alpha)]$ vs. $\bar{Z}_H$, where α is the degree of dissociation of either the Glu's or Asp's. α can be derived from eq 7. The pK_{int} can be derived from the intercept at the point $\bar{Z}_H = 0.0$ which is a representative value for the acidic residues when the plot is truly linear, i.e., when the residues are intrinsically identical.

Calculation of Individual pK_{int} Values. For the determination of individual pK_{int} values a program was written in Basic for an iterative nonlinear regression analysis based on the least-squares principle. Data input consists of the values for the proton charges of the protein molecule, the corresponding pH values, data necessary for the calculation of w (ionic strength, partial specific volume, and molecular weight of the protein) and the Z_{max} value. The minimization procedure can be done in any desired pH region. The pK_{int}'s and the number of groups titrating in that pH region have to be given as input including the initial guessed values of the pK_{int}'s to be determined. The unweighted data are essentially fitted to eq 2. The experimental and calculated function values are

$$Q_{calcd} = \sum n_j\alpha_j \qquad (9)$$

with $\alpha_j = 10^B/(1 + 10^B)$ and $B = pH - pK_j + 0.868w\bar{Z}_H$ and

$$Q_{measd} = Z_{max} - \bar{Z}_H - \sum_{i \neq j} n_i\alpha_i \qquad (10)$$

where the pK_j's are the desired parameters. The program can be used for the determination of as many pK_{int}'s as desired. However, a one- or two-parameter variation appeared to give the most reliable values.

Kinetic Studies. Enzymatic hydrolysis of 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine was followed by continuous titration of the liberated fatty acids at pH 6 and pH 8 at 25 °C with a Radiometer pH-stat equipment. The assay system containing Ca^{2+} in various amounts (0.1–26 mM) was standarized to 2 mL total volume with a buffered solution containing 10.0 mM lecithin, 100 mM NaCl, and 10 mM sodium acetate (pH 6) or 0.5 mM Tris (pH 8). Titrations with 10.5 mM NaOH were carried out under nitrogen. In each assay, less than 1 μg of the enzyme dissolved in a 1% albumin (Calbiochem, pro analysi) solution was used.

UV Absorption Difference Spectroscopy. UV absorption difference spectra were recorded on an Aminco Model DW-2-a spectrophotometer equipped with a Midan data analyzer coupled to an Apple II desk top computer. A 12-bit paralleled data transfer from the Midan to the computer was accomplished, by using either the "PLOT" mode (slow transfer 1 min) or "CRT" mode (fast transfer 30 ms) of the Midan. Transferred spectra were stored on floppy disk (1.5 kilobytes/spectrum). Measurements were done and binding parameters were obtained as previously described by de Araujo et al. (1979) and Hille et al. (1981). Experimental conditions are 50 mM sodium acetate, Hepes, Tris, or glycine–NaOH containing 100 mM sodium chloride and 1 mM EDTA at pH values above 6.

Results and Discussion

Proton Titrations of Porcine, Bovine, and Equine PLA. The proton titration curves of three pancreatic PLA's are shown in Figure 1A. The differential curves show a relative increase in the height of Max 2 at pH 8–9 ($\bar{Z}_H = -1$ to -3) in the series from porcine to bovine and equine PLA. This behavior can qualitatively be explained by eq 5, taking into account the decrease of the number of histidines in the series porcine (3) – bovine (2) – equine (1) PLA and the differences in the $pK_{1/2}$ values of the α-NH_2 being 8.4, 8.9, and 8.82, respectively (Janssen et al., 1972; Jansen et al., 1979; Jansen, 1979). The increase of the $-dpH/d\bar{Z}_H$ values at pH 5–6 ($\bar{Z}_H$ around 0–3) in the same series is mainly due to the difference in the number of histidines. A comparison of theoretically determined Z_{II} values (eq 6) and the experimental ones (see Table II) revealed no significant differences, the values deviating from integers mainly due to the α-NH_2 titrating in the pH region where Max 2 is located.

To investigate whether or not the above proteins contain abnormally high titrating carboxylates, the titration curves of their acidic residues were determined according to eq 7 (see Figure 1B). The titration curves are characterized by their average $pK_{1/2}$ values (see Materials and Methods), which are far below the values for aspartic (pK = 4.08) or glutamic groups (pK = 4.5) present in small model compounds, i.e., 3.53, 3.63, and 3.93 for porcine, bovine, and equine PLA, respectively. This demonstrates that the acidic groups are titrating in a strongly positive protein molecule as is clear from Figure 1A. For bovine PLA the end point of titration of the acidic groups is reached around pH 6, while for porcine and equine PLA a deprotonation end point is observed at a significantly higher pH value of about 7.5. This indicates that one or more abnormal carboxylates are present in the latter two enzymes. When the nonlinear regression analysis was used for both enzymes (two-parameter variation), at most one abnormal pK_{int} value could be determined for both PLA's, being 5.9 for porcine PLA and 6.0 for equine PLA (see Table III). Application of the same procedure for the bovine enzyme revealed

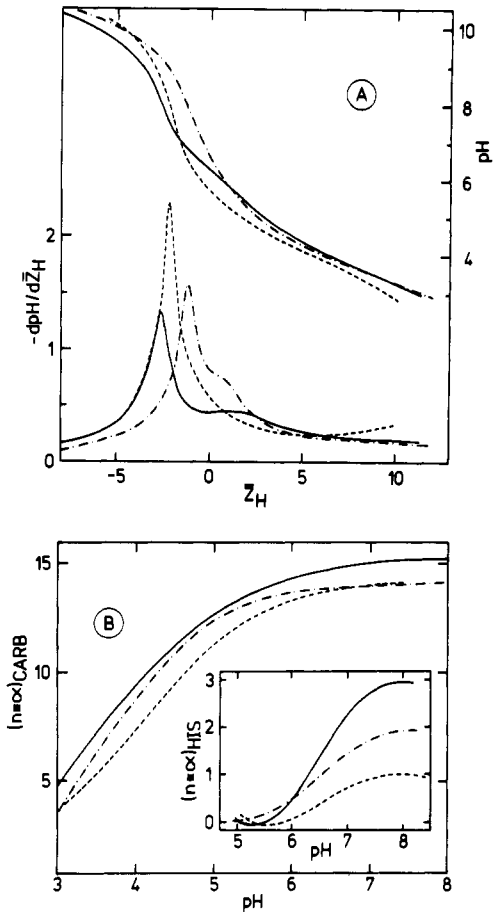


FIGURE 1: (A) Normal and differential titration curves for native PLA's at I = 0.1 M (100 mM KCl) and 25 °C. (—) Porcine PLA; (···) bovine PLA; (- - -) equine PLA. The reference point ($\bar{Z}_H$ = 0) is the pH of the isoionic solution (Table II). (B) Titration curves of carboxylates and histidines (inset) of native PLA. (—) Porcine PLA; (···) bovine PLA; (- - -) equine PLA.

Table II: Titration Data for Porcine, Bovine, and Equine Phospholipases A_2[a]

enzyme	IIP	parameters of Max 2[b] pH	Z_{II}	$-dpH/d\bar{Z}_H$
pig				
PLA	6.35	8.05	−2.2	1.4
PLA (50 mM Ca^{2+})	6.00	8.00	−2.1	1.8
PLA (250 mM Ca^{2+})	6.00	7.80	−2.0	1.5
PLA (750 mM KCl)	6.35	8.05	−2.2	1.4
PREC	5.93	8.25	−3.0	2.6
PREC (50 mM Ca^{2+})	5.60	7.91	−3.0	2.9
iso-PLA	5.95	7.88	−2.0	2.1
[Oct-His^{48}]PLA	6.75	8.04	−1.5	1.4
ox				
PLA	6.59	7.85	−1.1	1.7
PLA (50 mM Ca^{2+})	6.10	7.40	−1.0	2.4
PREC	6.40	8.10	−1.0	3.0
horse				
PLA	5.70	7.82	−2.1	2.3
PLA (50 mM Ca^{2+})	5.40	7.49	−2.0	3.3

[a] At 25 °C and 100 mM KCl unless otherwise indicated. [b] The standard deviations determined from duplicate and triplicate experiments were approximately 1%, 5%, and 5% for pH, Z_{II}, and $-dpH/d\bar{Z}_H$, respectively.

no acidic residues having a pK_{int} above 4.5. Figure 2 shows theoretical and experimental titration curves for native porcine PLA.

For the determination of the histidine titration curves of bovine PLA (Figure 1B, inset), two separate pK_{int} values for

Table III: pK_{int} Values of Carboxylic Residues of Phospholipases A_2 Titrating at Abnormally High pH Values[a]

enzyme	pK_{int}[b,c]
pig	
PLA	5.9
PLA (50 mM $CaCl_2$)	5.5
PLA (750 mM KCl)	5.5
PREC	6.2
PREC (50 mM $CaCl_2$)	5.3
AMPA	5.8
[Oct-His^{48}]PLA	6.6
horse	
PLA	6.0
PLA (50 mM $CaCl_2$)	5.5

[a] Experimental conditions: 100 mM KCl (or indicated otherwise) at 25 °C. [b] The pK_{int} values were obtained from nonlinear regression analysis. For either one- or two-parameter variation only one group was found having the abnormally high pK_{int} value. For the other carboxylic groups the pK_{int} values were assumed to be 4.08 (Asp) and 4.5 (Glu). pK_{int} values for the other titrating groups were taken from literature as mentioned under Materials and Methods. [c] Standard deviations are approximately 5%.

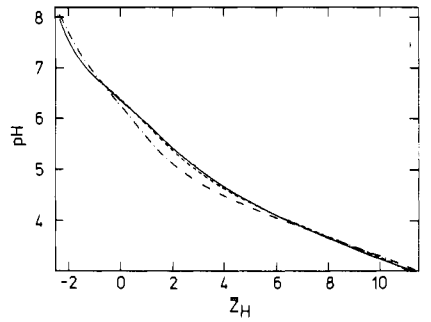


FIGURE 2: Representation of the results of the nonlinear regression analysis for native porcine PLA. (—) Experimental titration curve, for the pH region between pH 3 and pH 8.2; (···) theoretical titration curve obtained from eq 2. The carboxylate groups were assumed to have a pK_{int} of 4.08 (Asp's) and 4.5 (Glu's) according to Tanford (1962). The pK_{int} values for the other titrating groups were taken from literature as mentioned under Materials and Methods. (- - -) Theoretical curve obtained from eq 2 by using the same pK_{int} values as for the previous curve, only replacing the pK_{int} of one glutamic acid by the value determined in the regression analysis for the abnormally high titrating carboxylate (pK_{int} = 6).

the classes of Glu's and Asp's were used. These values were derived from linearization of the titration curve of the enzyme (see Materials and Methods) and were found to be 4.5 for the class of Glu's and 4.1 for the class of Asp's. For the acidic residues in porcine and equine PLA, the values from Table III were used together with the values of 4.1 and 4.5 for the residual aspartic and glutamic acids, respectively. The average $pK_{1/2}$ of the histidines appeared to be 6.6 for all three enzymes, which is in excellent agreement with the ^1H NMR results of Aguiar et al. (1979) and the proton titration results of porcine PLA of Janssen et al. (1972).

Influence of Ca^{2+} on Porcine, Bovine, and Equine PLA. The effect of Ca^{2+} on the titration behavior of the various PLA's studied is shown in Figure 3A. The titration curves for all enzymes show an increase of the height in Max 2 of 40–50% relative to Max 2 in the absence of Ca^{2+}. Furthermore, a shift of Max 2 to lower pH values is observed (see Table II). The $-dpH/d\bar{Z}_H$ values around pH 5 decrease by about 20% upon addition of Ca^{2+}. These results indicate a pK shift for several residues in the pH region around pH 5–7. This is also demonstrated by the differences in IIP's of the proteins titrated either in the absence or in the presence of Ca^{2+} (see Table II). The IIP's of the Ca^{2+}-bound proteins were deduced from their

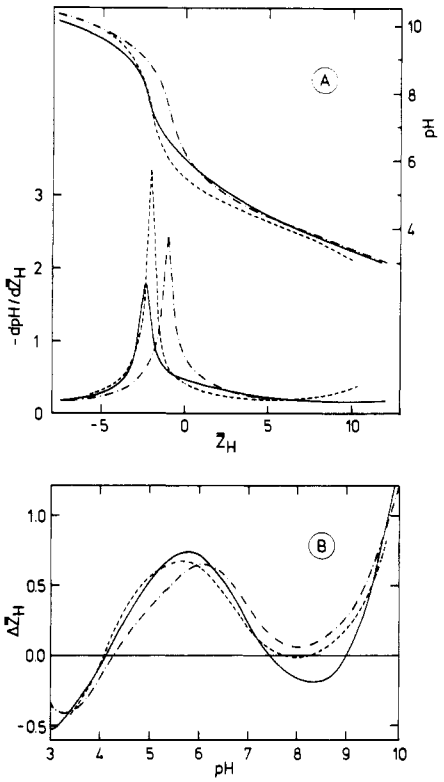


FIGURE 3: (A) Normal and differential titration curves for native PLA's at 50 mM $CaCl_2$ and at 25 °C. (–) Porcine PLA; (·-·) bovine PLA; (---) equine PLA. The reference point ($\bar{Z}_H = 0$) is the IIP derived from the theoretical Z_{II} value (Table II). (B) Difference titration curves obtained by subtracting the titration curve of the enzyme in the presence of Ca^{2+} from the one in the absence of Ca^{2+}. (—) Porcine PLA; (·-·) bovine PLA; (---) equine PLA. The calculation of the difference curves was based on a linear interpolation method.

theoretical Z_{II} values (Table II) assuming that at the pH of Max 2 the Z_{II} value of the protein at 50 mM $CaCl_2$ is only deviating from the value at 100 mM KCl due to an effect of Ca^{2+} on the $pK_{1/2}$ of the α-NH_2 (Slotboom et al., 1978; Jansen, 1979).

The effect of Ca^{2+} on the phospholipases A_2 can be seen most clearly in the difference curves as presented in Figure 3B. These curves stress the almost identical effect of 50 mM Ca^{2+} on the titration behavior of the three enzymes. It is known (Aguiar et al., 1979) that binding of Ca^{2+} to the catalytic site of PLA induces a pK shift of the active site residue His^{48} from 6.5 to 5.7. This pK shift mainly accounts for the behavior of the difference curves between pH 4.5 and pH 7.5. The small dip in the curve of the porcine enzyme between pH 7.5 and pH 9 is explained by the pK shift of the α-NH_2 going from 8.4 to 9.03 upon addition of 50 mM Ca^{2+} (Slotboom et al., 1978). For both other enzymes this effect is almost negligible (Jansen, 1979). Besides the known effects of Ca^{2+} on His^{48} and the amino terminal, Figure 3B reveals a third effect. Between pH 4 and 6 the ΔZ values for porcine and equine PLA are significantly higher than for the bovine enzyme. This points to an additional pK shift of most probably an acidic residue in the former two enzymes. Close examination of the titration curves of the acidic residues of porcine and equine PLA (see Materials and Methods) reveals that addition of Ca^{2+} shifts the deprotonation end point from around pH 7.5 to pH 7. The titration end point of the acidic residues in bovine PLA is not affected by Ca^{2+}. Application of the iterative nonlinear regression analysis shows that the shifts of the titration end points can be explained by a pK shift of the abnormally high titrating carboxylate from a value of 6.0 to 5.5 (Table III).

Ca^{2+} Binding Studies on Native Porcine PLA. The above experiments at 50 mM Ca^{2+} showed a specific but small effect on the pK_{int}'s of the abnormally high titrating carboxylates in porcine and equine PLA's. Apart from Ca^{2+} binding to the active site residue Asp^{49} (Fleer et al., 1981; Dijkstra et al., 1981), another, low-affinity, Ca^{2+} binding site has been proposed for porcine PLA (Pieterson, 1973; van Dam-Mieras et al., 1975; Slotboom et al., 1978; Andersson, 1981). At 50 mM Ca^{2+} the active site is saturated by Ca^{2+} at pH values above 6 (K_{Ca} = 2.3 mM at pH 6; Pieterson et al., 1974a), while the low-affinity site is not ($K_{Ca} \simeq$ 50 mM at pH 6; Slotboom et al., 1978). Because the pK_{int} shift at 50 mM Ca^{2+} is rather small, it seems unlikely that the high titrating carboxylate should be assigned to the carboxylate group of Asp^{49} (Tanford, 1962). A further identification of the unknown carboxylate was obtained by adding higher Ca^{2+} concentrations. At 250 mM Ca^{2+} small pK_{int} shifts of all carboxylates were found ($\Delta pK_{int} \simeq -0.1$). However, the strongest pK_{int} shift was observed for the abnormally high titrating carboxylate ($\Delta pK_{int} \simeq 1.5$). This indicates that the carboxylate is one of the Ca^{2+} ligands in the second, low-affinity, Ca^{2+} binding site. A control experiment was carried out at 750 mM KCl (ionic strength identical with that of 250 mM $CaCl_2$). Again small pK_{int} shifts were found for the normal acidic residues ($\Delta pK_{int} \simeq -0.1$). The pK_{int} shift found for the carboxylate assigned to the low-affinity Ca^{2+} binding site was –0.5. This effect is larger than experienced by normally titrating carboxylates, due to the unique environment of this specific carboxylate. This environment must be either strongly hydrophobic in nature or strongly negatively charged. The control experiment further demonstrates that the pK_{int} shift of the unique carboxylate induced by addition of Ca^{2+} is not due to a salt effect alone.

The results of the above experiments are summarized in Table III.

Further Identification of the Second Ca^{2+} Binding Site in Porcine PLA. A further discrimination between Ca^{2+} binding to the active site residue Asp^{49} and to another carboxylate in porcine PLA was obtained by using modified enzyme. Porcine [Oct-His^{48}]PLA does not bind Ca^{2+} to the active site and is catalytically inactive (Pieterson et al., 1974b). However, lipid binding properties of this protein are not affected by the modification or its ability to bind Ca^{2+} to a low-affinity site (Slotboom et al., 1978). Proton titrations of this protein in the absence of Ca^{2+} showed that it still contains a carboxylate group having an abnormally high pK_{int} (Table III). The pK_{int} of the unusual carboxylate is even higher in the modified enzyme than in the native porcine PLA (pK_{int} = 6.6). It was previously found that modification of His^{48} also shifted the pK of the N-terminal Ala^1 to higher pH values ($pK_{1/2}$ = 9.0; Jansen, 1979). Jansen (1979) obtained evidence from ^{13}C NMR titrations that addition of 50 mM Ca^{2+} to the modified enzyme not only shifts the $pK_{1/2}$ of the N-terminal amino group of Ala^1 ($pK_{1/2}$ = 9.4; Slotboom et al., 1978) but also affects a group close to the N-terminal Ala^1 having a $pK_{1/2}$ around 6.5. All these data justify the conclusion of Slotboom et al. (1978) that the low-affinity Ca^{2+} binding site is located near the N-terminal region of porcine PLA. As high-resolution X-ray diffraction data are still lacking for porcine pancreatic PLA, the molecular model of the bovine enzyme was used to search for carboxylate groups near the N-terminal region of PLA. Residue 71, being Asn in bovine PLA and Glu in both porcine and equine PLA's, might meet the requirements mentioned above. Although it is tempting to conclude at this

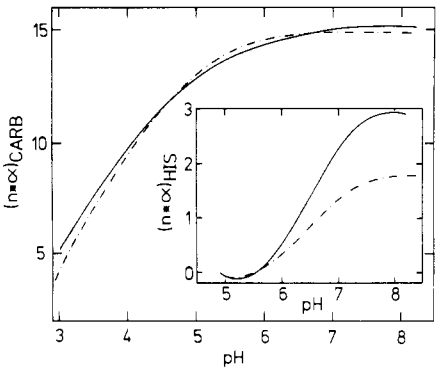


FIGURE 4: Titration curves of carboxylates (Glu's and Asp's) and histidines (inset) for porcine PLA (—) and porcine iso-PLA (···).

stage that the abnormal carboxylate found in proton titrations of porcine and equine PLA's, being absent in bovine PLA, should be assigned to Glu^{71}, the evidence is still too weak. The several pancreatic PLA's studied exhibit high sequence homology (around 80%), but there are too many possible substitutions involving carboxylate groups apart from the one at location 71. Advantage could be made of the presence of an iso-PLA present in porcine pancreas, which has been sequenced (Puijk et al., 1979) and found to differ from PLA in four residues, only two of which involve a carboxylate: His^{17} (PLA) → Asp^{17} (iso-PLA) and Glu^{71} (PLA) → Asn^{71} (iso-PLA). Thus, the isoenzyme lacks the residue of interest, being Glu^{71}, and contains Asn^{71} instead, just as the bovine enzyme does. The results of proton titration experiments are shown in Figure 4. As compared to porcine PLA, the isoenzyme indeed lacks a carboxylate having a pK_{int} of around 6. This was confirmed by the regression analysis. Therefore it can be concluded that this carboxylate is Glu^{71} as suggested above. The additional carboxylate (Asp^{17}) in porcine iso-PLA has a normal pK_{int} value.

Second Ca^{2+} Binding Site in Porcine PREC. Slotboom et al. (1978) showed that binding of Ca^{2+} to the low-affinity site of porcine PLA perturbs the microenvironment of Trp^3 and shifts the $pK_{1/2}$ of the α-NH_3^+ group of Ala^1 from 8.4 (100 mM KCl) to 9.3 (200 mM Ca^{2+}). They concluded that the second Ca^{2+} ion binds close to the N-terminal part of the protein. The same studies showed that addition of high concentrations of Ca^{2+} to the porcine zymogen did not affect Trp^3. By specific modification of L-Ala^1 of ϵ-amidinated phospholipase A_2 (AMPA), Slotboom et al. (1978) demonstrated that chain shortening or elongation by one residue or merely the replacement of L-Ala^1 by D-Ala^1 yielded proteins in which even very high concentrations of Ca^{2+} could not shift the $pK_{1/2}$ of the N-terminal α-NH_3^+ group or affect Trp^3. From these results it was concluded that both the precursor and the N-terminally modified enzymes are devoid of a low-affinity Ca^{2+} binding site. However, by fluorescence spectroscopy measurements, the same authors demonstrated the different microenvironment of Trp^3 in the diastereoisomeric proteins [D-Ala^1]- and [L-Ala^1]AMPA. Furthermore, it was shown that Trp^3 in [D-Ala^1]AMPA must be located in a surrounding very similar to that of Trp in the zymogen (AMPREC), while this environment is different from that of Trp^3 in the active AMPA. Therefore, another plausible explanation of the above experiments is that the second Ca^{2+} binding site is still present in the zymogen and the modified enzymes but the effect of Ca^{2+} on Trp^3 and the amino terminal is absent due to a different conformation of the N-terminal part in these proteins compared to native porcine PLA. This assumption was verified by proton titration studies on porcine PREC in the absence

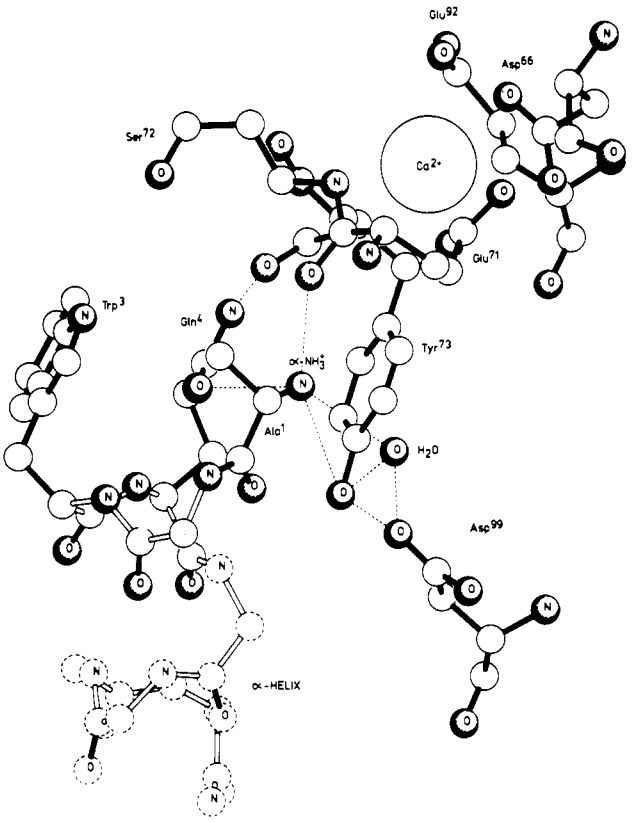


FIGURE 5: Three-dimensional representation of the N-terminal part and proposed second Ca^{2+} binding site of porcine and equine phospholipase A_2. The coordinates are derived from the X-ray diffraction data of bovine PLA (Dijkstra et al., 1981). The side chain N and O atoms and also the α-NH_3^+ group of Ala^1 are indicated by darkly shaded atoms. The N and O atoms of the backbone are indicated by the lightly shaded atoms. The dotted lines connect atoms which lie within a distance of 2.5–4.5 Å of each other and which are most probably important for the functioning of the enzyme.

and presence of 50 mM Ca^{2+}. The results presented in Table III show that porcine PREC indeed contains a second low-affinity site for Ca^{2+}.

Proton titration experiments of porcine AMPA and AM-PREC revealed no significant differences between the proton titration behavior of these enzymes and that of the corresponding unmodified enzymes (PLA and PREC). Porcine AMPA also contains an abnormal carboxylate (Table III).

Bovine PREC (Table II) appeared to have no abnormal carboxylates in the high pH range, their titration curve overlapping completely with the one for the acidic residues in bovine PLA.

UV Difference Absorption Spectroscopy. The proton titration experiments point to Glu^{71} as the Ca^{2+} ligand in the low-affinity site of equine PLA and porcine PLA, PREC. This residue is assumed to have a pK_{int} of around 6, being abnormally high most probably due to the presence of several acidic residues such as Asp^{66} and Glu^{92} which are present in both porcine and equine phospholipases A_2 (see also Figure 5).

Previous micelle binding studies (Donné-Op den Kelder et al., 1981) showed that binding of native porcine PLA to micelles of the substrate analogue $C_{18}PN$ in the absence of Ca^{2+} is controlled by a group having a $pK_{1/2}$ of 6.25 [Figure 6 (●)]. As at that time nothing was known about a possible presence of abnormally high titrating carboxylates in porcine PLA, the above group was assigned to His^{48} (with a rather normal $pK_{1/2}$ of 6.3), a residue which is hydrogen bonded via a H_2O molecule to the carboxylate group of Asp^{49}. As addition of 100 mM Ca^{2+} appeared to restore the micelle binding

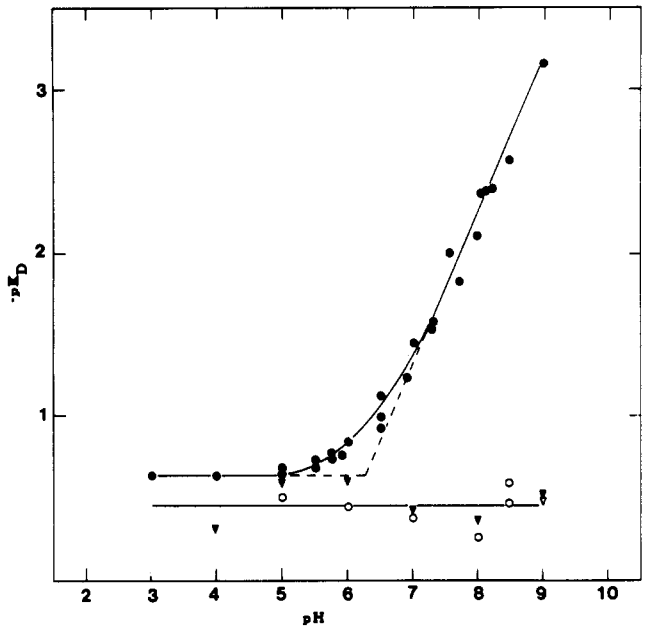


FIGURE 6: Dixon's plots of the effect of pH on the interaction of porcine phospholipase A_2 with micelles of *n*-octadecylphosphocholine. (●) Porcine PLA; (○) porcine PLA at 100 mM Ca^{2+}; (▼) iso-PLA; (▽) iso-PLA for all Ca^{2+} concentrations from 5 to 50 mM. K_D (μM) is defined as the dissociation constant of the complex. The standard deviation in K_D was about 15%. For porcine PLA, the lipid/enzyme ratio of the complex (N) was determined to be around 50.

capacity of the enzyme at neutral and alkaline pH [Figure 6 (○)], it was concluded that a positive charge in the microenvironment of His^{48} and Asp^{49} is extremely important for optimal micelle binding. However, one argument could be raised against His^{48} controlling micelle binding in native porcine PLA. This argument is that only very high concentrations of Ca^{2+} were able to restore optimal micelle binding. Ca^{2+} concentrations just enough to saturate the high-affinity site in native PLA (5 mM Ca^{2+}) were insufficient to restore the maximum binding capacity of the enzyme. It now seems very likely that it is Glu^{71} instead of His^{48} which controls binding of the porcine enzyme to organized lipid–water interfaces. Neutralization of the negative charge of the carboxylate group of Glu^{71} either by a proton or by binding of a Ca^{2+} ion should then be essential for optimal binding. It is to be expected that enzymes such as bovine PLA and porcine iso-PLA will exhibit micelle binding independent of pH. As micelle binding of bovine PLA cannot be measured very accurately ($K_S \sim 50$ mM;[2] Fleer, 1980), the pH-titration profile of porcine iso-PLA was studied. Indeed, micelle binding appeared to be independent of pH up to pH values around 9 [Figure 6 (▼)]. The curve coincides with the one for native porcine PLA in the presence of 100 mM Ca^{2+} [Figure 6 (○)]. The K_S value has an averaged value of 0.23 mM. Saturation of the high-affinity Ca^{2+} binding site in porcine iso-PLA by addition of 5 mM Ca^{2+} at pH 9 improved micelle binding by about 8.5%, while higher concentrations of Ca^{2+} appeared to have no further effect. These results confirm the above assumption that it is indeed Glu^{71} which is controlling micelle binding in the absence of Ca^{2+}. A negative charge on location 71 perturbs the enzyme–micelle interaction forces.

Kinetic Experiments. The effect of the presence of Glu^{71} as a possible Ca^{2+} ligand on the kinetics of the conversion of micellar substrates is shown below. Five phospholipases A_2 were tested: porcine and equine PLA both containing Glu^{71}, porcine iso-AMPA and bovine PLA containing Asn on this location, and human PLA having a histidine at position 71. As presented in Figure 7, at pH 6 the dependence of the velocity on the Ca^{2+} concentration appears to be a rectangular hyperbola for all Ca^{2+} concentrations and for all studied proteins. At higher pH values and Ca^{2+} concentrations of about 1 mM, deviations from the initially straight Hanes' plots are observed for porcine and equine PLA. From this it can be concluded that these enzymes contain a second Ca^{2+} binding site which becomes kinetically important at alkaline pH values, i.e., when Glu^{71} is deprotonated. In addition, the proteins lacking a carboxylate at position 71 do not contain a kinetically important second Ca^{2+} binding site. Table IV summarizes the results of the kinetic experiments derived from unweighted fits to Hanes' plots of the data. No data are given for those enzymes that bind a second Ca^{2+} ion giving rise to nonlinear Hanes' plots at alkaline pH values (Figure 7A). A semiquantitative description of the events taking place under these conditions is that the first Ca^{2+} ion is strongly bound, K_{Ca} being 0.25 mM and 0.43 mM at pH 8 for porcine and equine PLA, respectively (Pieterson et al., 1974a; H. M. Meijer, personal communications). Low apparent V_{max} values can be estimated from the initially straight portions of the Hanes' plots, being 425 and 1038 units/mg for porcine and equine PLA, respectively. These values are low because the enzyme is not satu-

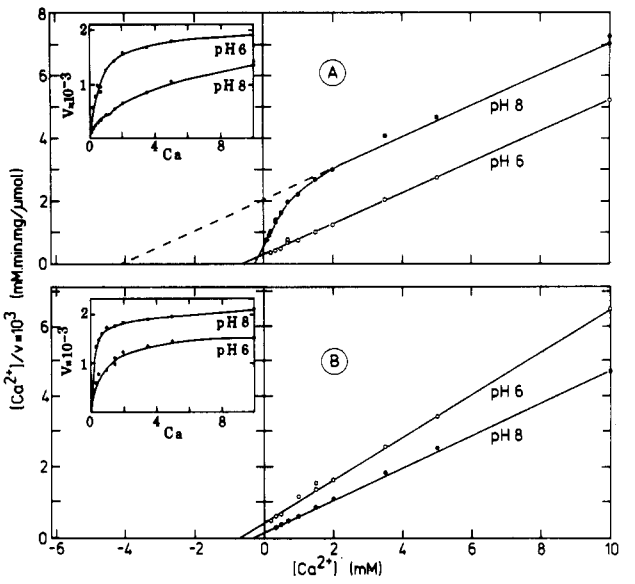


FIGURE 7: Hanes' plots of the phospholipase A_2 hydrolysis of micellar di-C_8-PC as a function of Ca^{2+} concentration at pH 6 and pH 8 at 25 °C. (A) Porcine PLA; (B) porcine iso-AMPA; (inset) corresponding saturation curves.

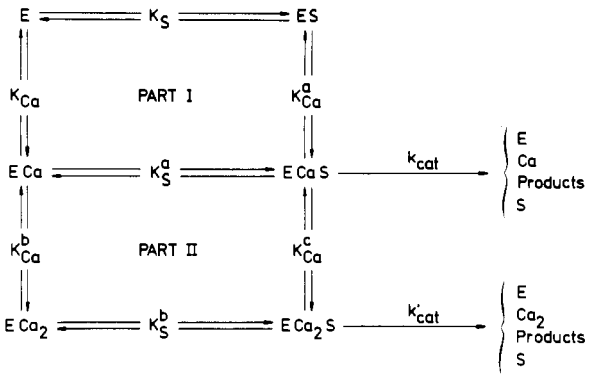
Table IV: Kinetic Parameters from Hanes' Plots of Ca^{2+} Binding to Asp^{49} to Several Phospholipases A_2 at 25 °C[a]

source	pH 6 K_{Ca}^{app} (mM)	pH 6 V_{max}^{app} (units/mg)	pH 8 K_{Ca}^{app} (mM)	pH 8 V_{max}^{app} (units/mg)
porcine PLA	0.64	2038		
equine PLA	0.79	6671		
porcine iso-AMPA	0.71	1648	0.30	2170
bovine PLA	8.1	2910	2.0	2725
human PLA	0.42	1693	0.013	573

[a] Enzyme activities are expressed in units/mg (μmol min^{-1} mg^{-1}) of product formation from micellar di-C_8-PC (at 10 mM). Standard deviations are ±10% for K_{Ca}^{app} and ±2% for V_{max}^{app}.

[2] K_S is defined here as $K_D N$, where K_D is the dissociation constant of the lipid–protein complex and N the lipid/protein molar ratio of the complex.

Scheme I



rated with micelles of the substrate under these conditions. A decrease of the slope of the Hanes' plots at high Ca^{2+} concentrations indicates that binding of the second Ca^{2+} ion leads to higher V_{max} values. When straight lines are fitted to these portions of the Hanes' plots, apparent V_{max} values of 1958 and 11 200 units/mg are obtained for porcine and equine PLA, respectively. The apparent K_{Ca} values one finds under these conditions are 4.1 mM and 15 mM, respectively. The value found for porcine PLA is in excellent agreement with the microcalorimetry and equilibrium dialysis values of Slotboom et al. (1978) and as determined by monolayer kinetics (Pattus et al., 1979b).

Generally, at pH 8 lower K_{Ca} values are found than at pH 6 for Ca^{2+} binding to the first site. It should be noted that the concentration of substrate micelles present is saturating for all PLA's, except for those enzymes that need a second Ca^{2+} for optimal binding of the substrate. A more quantitative description of the events taking place when PLA's bind two Ca^{2+} ions at high pH is given in the Appendix.

Most probably, no physiological significance can be ascribed to the second Ca^{2+} binding site in porcine and equine pancreatic phospholipase A_2. One reason is that not all phospholipases A_2 contain this second site (Glu^{71}). Moreover, Ca^{2+} binding to this site only becomes kinetically important at pH values above 7.5, and high Ca^{2+} concentrations are needed to restore full micelle binding. On the other hand, the physiological conditions of Ca^{2+} concentrations and pH along the intestine are not known in detail, and the naturally occurring phospholipid dispersions during digestion are difficult to mimic in vitro.

Appendix

A reaction mechanism that may describe the observed kinetics for PLA's binding to Ca^{2+} ions at high pH is shown in Scheme I. If all equilibria are supposed to be fast relative to the catalytic steps k_{cat} and k_{cat}', it can be derived that

$$\frac{V_{max}}{\nu} = \frac{1 + \frac{K_S^b}{[S]} + \frac{K_{Ca}^c}{[Ca]}\left[1 + \frac{K_S^a}{[S]} + \frac{K_{Ca}^a}{[Ca]}\left(1 + \frac{K_S}{[S]}\right)\right]}{1 + (k_{cat}/k_{cat}')(K_{Ca}^c/[Ca])} \quad (11)$$

where [S] is the micellar lipid concentration and $V_{max} = k_{cat}'E_0$, E_0 being the total enzyme concentration. At low Ca^{2+} concentrations, such that Ca^{2+} binding to the second site is negligible, eq 11 can be approximated by

$$\frac{V_{max}}{\nu} \simeq 1 + K_S^a/[S] + (K_{Ca}^a/[Ca])(1 + K_S/[S]) \quad (12)$$

The apparent K_{Ca} for the first site was found to be 0.25 mM for porcine PLA at pH 8. This apparent value should be equal to

$$K_{Ca}^{app} = K_{Ca}^a(1 + K_S/[S])/(1 + K_S^a/[S])$$

As the apparent value is approximately identical with that of K_{Ca} found previously (Pieterson et al., 1974a), it can be concluded that $K_S \simeq K_S^a$ (based on the relation $K_S K_{Ca}^a = K_S^a K_{Ca}$) and that $K_{Ca}^a \simeq 0.25$ mM.

According to the proposed mechanism and the assumptions made, the apparent V_{max} is equal to $V_{max}/(1 + K_S^a/[S])$. If one assumes that $k_{cat} \simeq k_{cat}'$, it follows that $K_S^a/[S] \simeq 4$, by comparing the apparent V_{max} and the maximum rate obtained when two Ca^{2+} ions are bound and the porcine enzyme is consequently saturated with substrate micelles. Thus, for 10 mM substrate $K_S^a \simeq K_S \simeq 40$ mM (expressed as lipid monomer concentration) for the porcine enzyme.

At high Ca^{2+} concentrations, such that Ca^{2+} only binds to the second site, the first site being saturated, eq 11 can be approximated by

$$\frac{V_{max}}{\nu} \simeq \frac{1 + (K_{Ca}^c/[Ca])(1 + K_S^a/[S])}{1 + (K_{Ca}^c/[Ca])(k_{cat}/k_{cat}')} \quad (13)$$

In this equation it is assumed that the enzyme becomes gradually saturated by substrate micelles upon increasing Ca^{2+} concentrations; thus $[S] \gg K_S^b$. The above equation will not yield linear Hanes' plots or double-reciprocal plots. These plots will appear linear at high Ca^{2+} concentrations only when $K_S^a/[S] \gg k_{cat}'/k_{cat}$. Since $K_S^a/[S]$ was found to be approximately 4 (for porcine PLA) and about 9 (for equine PLA), the above assumption seems justified if one again assumes that $k_{cat} \simeq k_{cat}'$. Then the apparent Ca^{2+} binding constant for the second site at pH 8 will be approximately $K_{Ca}^c(K_S^a/[S])$. The values for K_{Ca}^c thus derived will then be about 1 mM and 1.6 mM for porcine and equine PLA, respectively.

Registry No. Phospholipase A_2, 9001-84-7; L-glutamic acid, 56-86-0; Ca, 7440-70-2.

References

Aguiar, A., de Haas, G. H., Jansen, E. H. J. M., Slotboom, A. J., & Williams, R. J. P. (1979) *Eur. J. Biochem. 100*, 511–519.

Andersson, T. (1981) Ph.D. Thesis, State University of Lund, Lund, Sweden.

de Araujo, P. S., Rosseneu, M. Y., Kremer, J. M. H., van Zoelen, E. J. J., & de Haas, G. H. (1979) *Biochemistry 18*, 580–586.

de Bruin, S. H., & van Os, G. A. J. (1968) *Recl. Trav. Chim. Pays-Bas 87*, 861–872.

Cubero Robles, E., & van den Berg, D. (1969) *Biochim. Biophys. Acta 187*, 520–526.

Dijkstra, B. W., Kalle, K. H., Hol, W. G. J., & Drenth, J. (1981) *J. Mol. Biol. 147*, 97–123.

Donné-Op den Kelder, G. M., Hille, J. D. R., Dijkman, R., de Haas, G. H., & Egmond, M. R. (1981) *Biochemistry 20*, 4074–4078.

Donné-Op den Kelder, G. M., de Haas, G. H., & Egmond, M. R. (1982) *Anal. Biochem. 125*, 149–155.

Evenberg, A., Meyer, H., Verheij, H. M., & de Haas, G. H. (1977) *Biochim. Biophys. Acta 491*, 265–274.

Fleer, E. A. M. (1980) Ph.D. Thesis, State University of Utrecht, Utrecht, The Netherlands.

Fleer, E. A. M., Verheij, H. M., & de Haas, G. H. (1978) *Eur. J. Biochem. 82*, 261–270.

Fleer, E. A. M., Verheij, H. M., & de Haas, G. H. (1981) *Eur. J. Biochem. 113*, 283–288.

Grataroli, R., de Caro, A., Guy, O., & Figarella, C. (1981) *Biochimie 63*, 677–684.
Grataroli, R., Dijkman, R., Dutilh, C. E., van der Ouderaa, F., de Haas, G. H., & Figarella, C. (1982) *Eur. J. Biochem. 122*, 111–117.
Hille, J. D. R., Donnê-Op den Kelder, G. M., Sauve, P., de Haas, G. H., & Egmond, M. R. (1981) *Biochemistry 20*, 4068–4073.
Jansen, E. H. J. M. (1979) Ph.D. Thesis, State University of Utrecht, Utrecht, The Netherlands.
Jansen, E. H. J. M., van Scharrenburg, G. J. M., Slotboom, A. J., de Haas, G. H., & Kaptein, R. (1979) *J. Am. Chem. Soc. 101*, 7397–7399.
Janssen, L. H. M., de Bruin, S. H., & de Haas, G. H. (1972) *Eur. J. Biochem. 28*, 156–160.
Linderstrøm-Lang, K. (1924) *C.R. Trav. Lab. Carlsberg 15*, 1–29.
Nieuwenhuizen, W., Kunze, H., & de Haas, G. H. (1974) *Methods Enzymol. 32B*, 147–154.
Pattus, F., Slotboom, A. J., & de Haas, G. H. (1979a) *Biochemistry 18*, 2691–2697.
Pattus, F., Slotboom, A. J., & de Haas, G. H. (1979b) *Biochemistry 18*, 2698–2702.
Pieterson, W. A. (1973) Ph.D. Thesis, State University of Utrecht, Utrecht, The Netherlands.
Pieterson, W. A., Volwerk, J. J., & de Haas, G. H. (1974a) *Biochemistry 13*, 1439–1445.
Pieterson, W. A., Vidal, J. C., Volwerk, J. J., & de Haas, G. H. (1974b) *Biochemistry 13*, 1455–1460.
Puijk, W. C., Verheij, H. M., & de Haas, G. H. (1977) *Biochim. Biophys. Acta 492*, 254–259.
Puijk, W. C., Verheij, H. M., Wietzes, P., & de Haas, G. H. (1979) *Biochim. Biophys. Acta 580*, 411–415.
Slotboom, A. J., & de Haas, G. H. (1975) *Biochemistry 14*, 5394–5399.
Slotboom, A. J., Jansen, E. H. J. M., Vlijm, H., Pattus, F., Soares de Araujo, P., & de Haas, G. H. (1978) *Biochemistry 17*, 4593–4600.
Tanford, C. (1962) *Adv. Protein Chem. 17*, 96–165.
van Dam-Mieras, M. C. E., Slotboom, A. J., Pieterson, W. A., & de Haas, G. H. (1975) *Biochemistry 14*, 5387–5394.
van Wezel, F. M., & de Haas, G. H. (1975) *Biochim. Biophys. Acta 410*, 299–309.
Verger, R., & de Haas, G. H. (1973) *Chem. Phys. Lipids 10*, 127–136.
Verheij, H. M., Volwerk, J. J., Jansen, E. H. J. M., Puijk, W. C., Dijkstra, B. W., Drenth, J., & de Haas, G. H. (1980) *Biochemistry 19*, 743–750.
Volwerk, J. J., Pieterson, W. A., & de Haas, G. H. (1974) *Biochemistry 13*, 1446–1454.

Phospholipids Chiral at Phosphorus. Preparation and Spectral Properties of Chiral Thiophospholipids†

Karol Bruzik, Ru-Tai Jiang, and Ming-Daw Tsai*

ABSTRACT: The thiophospholipid 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphocholine (DPPsC) was shown to be a mixture of two diastereomers by ^{31}P nuclear magnetic resonance. The isomer that resonates at the lower field in $CDCl_3$ (56.12 ppm) was designated as isomer A and the other (resonates at 56.07 ppm) as isomer B. Phospholipase A_2 from four different sources (bee venom, *Naja naja* venom, *Crotalus adamanteus* venom, and porcine pancreas) was shown to hydrolyze the isomer B of DPPsC specifically, whereas phospholipase C from two different sources (*Bacillus cereus* and *Clostridium perfringens*) hydrolyzes isomer A specifically. So that the two diastereomers could be separated, DPPsC(A+B) was first digested with phospholipase A_2 to give 1-palmitoyl-*sn*-glycero-3-thiophosphocholine (MPPsC) (which is designated as isomer B of MPPsC) and the unreacted DPPsC(A). Reacylation of MPPsC(B) gave pure DPPsC(B). The properties of DPPsC(A) and DPPsC(B) were investigated by ^{31}P, ^{13}C, ^{1}H, and ^{14}N nuclear magnetic resonance (NMR). ^{1}H and ^{13}C NMR showed that both isomers in methanol solution have conformational properties similar to those of the natural phospholipid, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine. On the other hand, the two isomers A and B showed small but significant differences in the chemical shifts of the carbon in the chiral carbon center and the phosphorus in the chiral phosphorus center.

During the past 5 years, various chirally labeled nucleotides (in which P–O bonds are substituted by P–^{17}O, P–^{18}O, or P–S) have been synthesized and widely used in mechanistic studies of enzyme-catalyzed reactions (Buchwald et al., 1982; Cleland, 1982; Cohn, 1982; Eckstein, 1979; Eckstein et al., 1982; Frey, 1982; Frey et al., 1982; Knowles, 1980; Tsai, 1982; Tsai & Bruzik, 1983; Villafranca & Raushel, 1980; Webb, 1982). However, an important class of biophosphates, the phospholipids, has been ignored. We have therefore initiated the stereochemical study of phospholipids, aiming at probing the mechanism of phospholipase-catalyzed reactions and the roles of the phosphate head group of phospholipids in protein–lipid interactions and in other membrane functions. In preliminary papers (Bruzik & Tsai, 1982; Bruzik et al., 1982; Tsai et al., 1982), we have reported synthesis of phospholipids chirally labeled with ^{18}O and chiral thiophospholipids and use of these compounds to elucidate the stereochemical course of transphosphatidylation catalyzed by phospholipase D and the stereospecificity of phospholipases A_2 and C toward the two diastereomers of DPPsC.[1] In this paper, we report detailed

† From the Department of Chemistry, The Ohio State University, Columbus, Ohio 43210. *Received December 17, 1982.* This work was supported by National Institutes of Health Research Grant GM 30327. The NMR facilities used were funded in part by the following grants: NIH GM 27431 and NSF CHE 7910019. This paper is part 3 in the series "Phospholipids Chiral at Phosphorus". The results have been presented (Tsai et al., 1982) at the 66th Annual Meeting of the American Society of Biological Chemists, New Orleans, LA, April 18–21, 1982. A preliminary account of this work has also been published (Bruzik et al., 1982; part 2 of this series).

0006-2960/83/0422-2478$01.50/0