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Interaction of Phospholipase A_2 and Its Zymogen with Divalent Metal Ions†

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ABSTRACT: Porcine pancreatic phospholipase A_2 as well as its zymogen bind calcium ions in a 1:1 molar ratio at calcium concentrations ranging from 5×10^{-3} to 5×10^{-5} M. Binding of calcium to both the zymogen and the active enzyme changes the protein absorption spectrum. The ultraviolet difference spectrum is characterized by a strong band at 242 nm and two smaller bands at 282 and 288 nm. The origin of the difference spectrum can be understood as a partial shielding of a tyrosine residue from the aqueous solvent and in addition, most likely, a charge effect on a histidine residue. Calcium does not influence the tryptophan fluorescence of either protein. However, addition of calcium enhances the 8-anilino-1-naphthalenesulfonate fluorescence induced by phospholipase A and its precursor. Besides the spectral changes binding of calcium protects the enzyme against inactivation by *p*-bromophenacyl bromide and diminishes its susceptibility to trypsin attack. These criteria suggest that the enzyme and the zymogen undergo a conformational change upon calcium binding. Substitution of calcium by barium or strontium results in a similar but somewhat smaller conformational change, in agreement with the behavior of these ions as competitive inhibitors. Magnesium, on the contrary, does not bind to the enzyme according to both kinetic and direct binding experiments. The dissociation constants of the various enzyme and zymogen metal complexes have been determined. Values were found ranging from 10^{-1} M at pH 4.0 to 2×10^{-4} M at pH 10.0. The data show a good agreement on using the various techniques and suggest that the metal ion binding site contains one or more carboxylates with an additional contribution of a residue with a pK of 6–7, presumably histidine.

Phospholipase A_2 (EC 3.1.1.4) catalyzes the hydrolysis of fatty acid ester bonds at the 2 position of 1,2-diacyl *sn*-phosphoglycerides (van Deenen and de Haas, 1964). The stimulating effect of calcium ions on the hydrolysis of phospholipids by phospholipase A_2 has long been recognized (Hayashi and Kornberg, 1954). The requirement of Ca^{2+} seems to be a general property of many phospholipases A isolated from such diverse sources as snake (Habermann, 1957) and bee venom (Shipolini *et al.*, 1971) and mammalian pancreatic tissue or juice (Figarella *et al.*, 1971). In those cases where calcium has been found to be an obligatory requirement for proper enzymatic action, the possibility of substitution by other divalent ions has been investigated (Long and Penny, 1957; Roholt and Schlamowitz, 1961; de Haas *et al.*, 1968; Wu and Tinker, 1969). From these studies it became clear that several phospholipases from snake and bee venom could be activated by a number of other divalent cations.

The high specificity of the porcine pancreatic enzyme for calcium suggests a specific function of the metal ion in catalysis. Investigations toward elucidation of the function of the

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Ca^{2+} ion are often impeded by the complex nature of the assay system. Many assay systems contain bile salts like deoxycholate or acidic phospholipids which give insoluble complexes with Ca^{2+}. In an attempt to generate phospholipid micelles in solution susceptible to phospholipase A hydrolysis without addition of bile salts, we shortened the fatty acid chains of the neutral phosphatidylcholine. Dihexanoyl- and diheptanoylphosphatidylcholine form micelles in solution which are readily attacked by phospholipase A. Using various other short-chain lecithins as substrate, de Haas *et al.* (1971) and Wells (1972) studied by kinetic experiments the metal ion binding of phospholipase A_2 from porcine pancreas and *Crotalus adamanteus*, respectively, and determined the affinity constant of the enzyme–Ca^{2+} complex. Possibly related to the different physicochemical state of the substrates used in these kinetic experiments (micellar state *vs.* monomeric state), no agreement exists as regards the kinetic mechanism of the enzyme. Whereas the Dutch group obtained evidence for a random mechanism in which Ca^{2+} and substrate bind to separate sites on the enzyme, Wells showed that the snake venom enzyme follows an ordered mechanism, in which Ca^{2+} has to be bound to the protein before the substrate can be attached. Therefore additional proof of the existence of separate binding sites for metal and substrate on the pancreatic enzyme is needed.

It is the aim of this paper to describe the interaction of Ca^{2+} with porcine pancreatic phospholipase A and its zymogen in the absence of substrate and to elucidate some of the structural changes in the protein as a consequence of this binding.

Experimental Section

Materials and Methods. Porcine pancreatic phospholipase A_2 was prepared by activation of the pure zymogen with trypsin as described previously by de Haas *et al.* (1968). The zymogen conversion was performed in the presence of 10^{-2} M $CaCl_2$ and 10^{-3} M Hepes[1] (pH 7.0). The enzyme had a specific activity of at least 1000 units per mg dry weight under standard assay conditions and was shown by atomic absorption spectrometry to contain less than 0.03 mol of Ca^{2+}/mol of enzyme.

Radioactive $^{45}CaCl_2$, specific activity 710 μCi/mmol, was obtained from the Radiochemical Centre, Amersham, England, and contained 28 μg of Ca/ml. 1,2-Dioctanoyl-*sn*-glycero-3-phosphorylcholine was prepared according to the method of Cubero Robles *et al.* (1967, 1969). The sodium salt of 8-anilino-1-naphthalenesulfonic acid was obtained from K and K laboratories and purified according to Thompson and Yielding (1968). *p*-Bromophenacyl bromide was obtained from Fluka A. G. All other reagents were of analytical grade.

Assay. Enzyme activities were determined using the titrimetric assay procedure with egg-yolk lipoproteins as substrate (de Haas *et al.*, 1968, 1971). Initial velocities were measured during the first 5% of the total reaction.

Equilibrium Gel Filtration. Measurements of metal binding to phospholipase A or its zymogen were performed according to a modification of the gel filtration technique described by Hummel and Dreyer (1962). Columns (46 × 1.1 or 30 × 0.6 cm) of Sephadex G-25 fine grade were equilibrated with 0.05 M buffer in 0.1 M NaCl (Hepes (pH 7–8); Tris (pH 8–9)) containing a known concentration of ^{45}Ca. In a typical experiment a 0.5-ml aliquot of metal-free protein in the metal ion buffer solution was applied to the column and eluted with the same elution buffer. Fractions of 300 μl were collected directly in weighed scintillation vials and the exact volume of the individual fractions was determined by weighing using the predetermined density of the elution medium. Subsequently 15 ml of scintillation liquid consisting of 7.0 g of 2,5-diphenyloxazole, 0.3 g of 2,2-*p*-phenylenebis(4-methyl-5-phenyloxazole), and 100 g of naphthalene in 1 l. of dioxane was added to each vial. Radioactivity was determined in a Packard Tri-Carb liquid scintillation counter. As a check on the radioactive determination of Ca^{2+}, the metal ion concentration was determined also by spectrophotometric titration with EDTA and murexide in alkaline solution according to Scarpa (1972). Protein concentrations were calculated from the absorbance at 280 nm with an $E_{1\,cm}^{1\%}$ of 13.0 and 12.5 for phospholipase A and its zymogen, respectively. The molecular weights of both proteins were derived from the amino acid sequence (de Haas *et al.*, 1971): 13.870 for phospholipase A and 14.630 for the zymogen.

Ultraviolet Difference Spectroscopy. Difference spectra were recorded on a Shimadzu MPS-50 double-beam spectrophotometer using 1-cm path-length cells. Protein concentrations never exceeded 70 μM, which gives an absorbance of about 1.8 at 240 nm. Metal ion stock solutions with a concentration of about 20 times the estimated dissociation constant at the appropriate pH were added to one cell and an equal volume of buffer to the reference cell. Buffer solutions used in the titrations were 50 mM in acetate, Hepes, Tris, and glycine for the pH regions 4–6, 6–7.5, and 8.5–10.0, respectively. All solutions were 0.1 M in NaCl. The nature of the buffer solutions used did not influence the spectroscopic data.

Fluorescence Measurements. Fluorescence spectra were obtained with a Perkin-Elmer MPF-3 spectrofluorimeter. Normal settings of excitation and emission slit widths were 10 nm. The increase in fluorescence intensity caused by the addition of metal ions to the protein solutions in the presence of the fluorescent probe ANS (60 μM) is expressed in arbitrary units. As a reference the same metal ion concentration was added to solutions containing only 60 μM ANS. Buffer solutions and NaCl concentration were the same as described under "ultraviolet difference spectroscopy."

Analysis of Data. Direct binding data on the interaction between (pro-) phospholipase A and divalent metal ions (Me) obtained by the three different techniques described above were treated according to the method of Fletcher *et al.* (1970) for the Scatchard (1949) model of ligand–protein interaction. For a single class of binding sites the following equation holds, where ν_{Me} is the saturation degree, n the number of

$$\nu_{Me}/[Me] = (n/K_{Me}) - (\nu_{Me}/K_{Me})$$

binding sites, K_{Me} the average dissociation constant of the enzyme–metal ion complexes, and [Me] the free metal ion concentration. Thus a plot of $\nu_{Me}/[Me]$ *vs.* ν_{Me} yields a straight line from which the value of n can be derived by extrapolation and the slope corresponds to $1/K_{Me}$. For a single gel filtration experiment as shown in Figure 1 the saturation degree ν_{Me} can be derived from the individual fractions or from the total peak and trough area according to $\nu_{Me} = [protein]_{bound}/[protein]_{total}$. Data obtained from ultraviolet difference spectroscopy and fluorescence measurements were treated in a similar manner, using the following equations: $\nu_{Me} = \Delta A/\Delta A_{max}$ and $\nu_{Me} = \Delta F/\Delta F_{max}$, where ΔA and ΔF are the absorbance and fluorescence intensity at a particular metal ion con-

[1] Abbreviations used are: dioctanoyllecithin, 1,2-dioctanoyl-*sn*-glycero-3-phosphorylcholine; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; ANS, 8-anilino-1-naphthalenesulfonic acid.

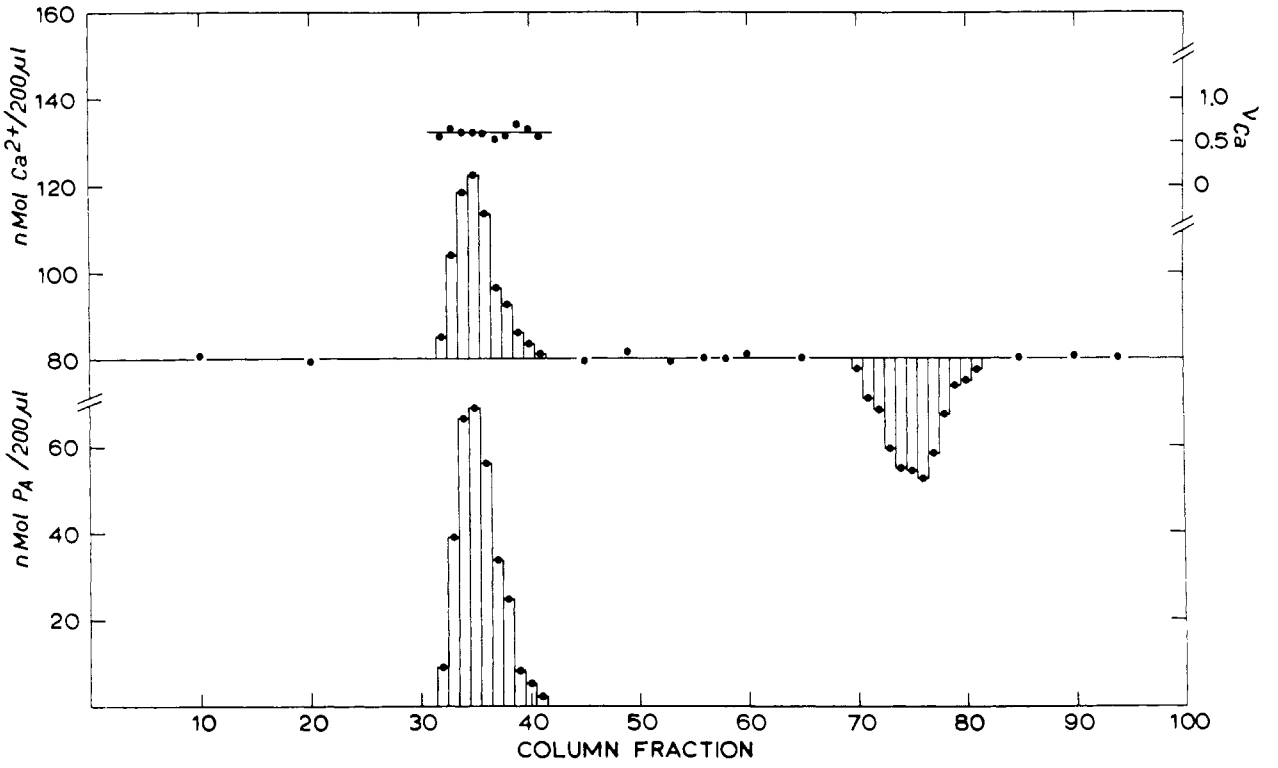

FIGURE 1: Typical elution profile for measurement of the binding of Ca^{2+} by phospholipase A on a Sephadex G-25 fine column (46 × 1.1 cm). The column was equilibrated with 4.0×10^{-4} M $CaCl_2$ in 0.05 M Tris and 0.1 M NaCl (pH 8.0); 0.48 µmol of phospholipase A was dissolved in 500 µl of elution buffer and developed along the column. Protein and calcium determinations were performed as described in the methods.

centration and ΔA_{max} and ΔF_{max} the maximal values for the absorbance and fluorescence intensity, respectively.

Metal Ion–Protein Interaction as Measured by Protection Against Irreversible Inactivation. As shown in the subsequent paper, *p*-bromophenacyl bromide inhibits phospholipase A and its zymogen through a 1:1 reaction with a single active site histidine residue. Calcium ions effectively protect both proteins against this modification, thereby allowing the determination of the dissociation constant of the protein–metal complex according to the method described by Scrutton and Utter (1965). Inactivation experiments in the absence or presence of metal ions were carried out in 0.1 M sodium cacodylate buffer–0.1 M NaCl, between pH 5.0 and 7.5 and at 30°. At zero time 20 µl of a 0.18 M solution of *p*-bromophenacyl bromide in acetone was added to 1 ml of buffer containing 1 mg of protein, and the loss of enzymatic activity was followed using the egg-yolk lipoprotein assay procedure.

As reported previously (de Haas *et al.*, 1970) a rapid loss of enzymatic activity is observed when phospholipase A is incubated with a small amount of trypsin. The protecting effect of calcium ions was studied by incubation of phospholipase A (0.25 mg/ml) with trypsin (10 µg/ml) in a 0.01 M Tris-HCl buffer (pH 9.0) at room temperature, both in the presence and absence of 9.0×10^{-4} M $CaCl_2$.

Results

Equilibrium Gel Filtration. Kinetic data reported previously (de Haas *et al.*, 1971) showed that the dissociation constant of the phospholipase A-Ca^{2+} complex possesses a minimal value at alkaline pH. Therefore an alkaline medium was chosen to study the interaction between (pro-)phospholipase and Ca^{2+} by means of Sephadex equilibrium filtration.

Figure 1 shows the typical elution pattern of 0.48 µmol of phospholipase A at pH 8.0 and an equilibrating Ca^{2+} concentration of 4.0×10^{-4} M. Similar results were obtained with prophospholipase instead of phospholipase A. From peak and trough areas the saturation degrees ν_{Ca} were calculated. The essential equivalence of peak and trough areas confirmed that equilibrium between protein and Ca^{2+} had been reached. For the experiment of Figure 1 the peak and trough areas yielded a saturation degree of 0.60 and 0.61, respectively. Figure 2 summarizes the data for phospholipase A obtained with various equilibrating Ca^{2+} concentrations at pH 8.8. A straight line is obtained for ν_{Ca} values between 0.2 and 0.8 indicating that in the corresponding Ca concentration range there exists only a single class of binding sites. Extrapolation to the abscissa gives a value $n = 1.03$; thus it can be concluded that this class consists of only one site. Similar results at pH 8.0 also showed the presence of one binding site for Ca^{2+}.

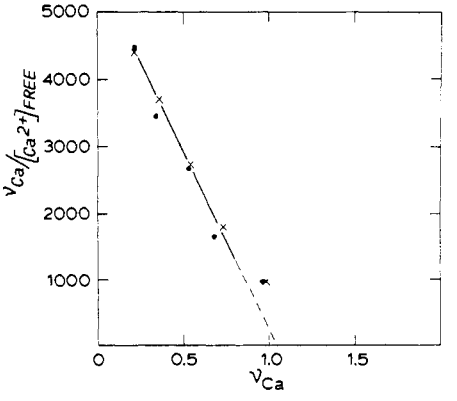

FIGURE 2: Scatchard plot for binding data at pH 8.8. ν_{Ca} values were obtained from peak and trough areas of the elution profiles as in Figure 1: (×) points derived from peak areas; (●) points derived from trough areas.

TABLE I: Dissociation Constants for the Interaction of Phospholipase A and Its Zymogen with Ca^{2+} from Equilibrium Gel Filtration.

	K_{Ca} (M)	
pH	Phospholipase	Prophospholipase
8.0	$2.5 (\pm 0.2) \times 10^{-4}$	$2.4 (\pm 0.2) \times 10^{-4}$
8.8	$1.9 (\pm 0.2) \times 10^{-4}$	$1.8 (\pm 0.2) \times 10^{-4}$

Essentially identical results were obtained with prophospholipase A. Table I summarizes the K_{Ca} values for both proteins derived from Sephadex equilibration experiments.

Ultraviolet Difference Spectroscopy. Figure 3 shows the Ca^{2+}-induced difference spectrum of phospholipase A. A distinctive but very small change appears in the 280–290-nm region whereas a much larger peak appears at 242 nm. Control experiments indicated that in the presence of a fixed high Ca^{2+} concentration (0.1 M) the spectral amplitudes were proportional to the enzyme concentration in the concentration range 10–60 μM. A qualitatively identical difference spectrum was obtained with prophospholipase and/or Ba^{2+} and Sr^{2+} instead of Ca^{2+}. On the contrary Mg^{2+} did not produce a difference spectrum nor interfered with the appearance of the normal Ca^{2+} difference spectrum.

Titration of a protein solution with increasing amounts of Ca^{2+} indicated a hyperbolic dependence of the absorbance at 242 nm (ΔA^{242}) on the amount of Ca^{2+} added. A double reciprocal plot of ΔA^{242} *vs.* $[Ca^{2+}]$ gave a straight line from which the maximal absorbance (ΔA^{242}_{max}) and the molar difference extinction coefficient were calculated. Table II gives the values for the molar difference extinction coefficients at saturating Ca^{2+} concentration and the corresponding dissociation constants for different metal ions at pH 6.0. The dissociation constants were calculated from Scatchard plots as described under Methods. Also these spectroscopic data indicate that there exists only a single site binding site for divalent metal ions in phospholipase A and prophospholipase A.

The spectroscopically determined K_{Ca} values at pH 8.0 were essentially identical with those obtained by Sephadex

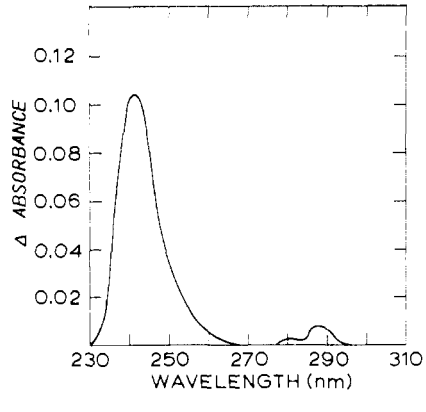

FIGURE 3: Ultraviolet difference spectrum of phospholipase A in the presence of 0.01 M calcium chloride at pH 8.0, 0.05 M Tris and 0.1 M NaCl. Enzyme concentration 54 μM. Cells of 10 mm path length containing the protein solutions were placed in the sample and reference compartments and spectra recorded with the 0–0.2 OD scale. A similar pattern as shown here is obtained with prophospholipase A instead of phospholipase A and/or barium instead of calcium.

TABLE II: Spectroscopic Parameters of the Interaction of Phospholipase A and Its Zymogen with the Alkaline Earth Metal Ions, Ca^{2+}, Sr^{2+}, and Ba^{2+}, at pH 6.0.

	Phospholipase A		Prophospholipase A	
Metal Ion	$K_{Me^{2+}}$ (mM)	E_{242} (cm^{-1} M^{-1})	$K_{Me^{2+}}$ (mM)	E_{242} (cm^{-1} M^{-1})
Ca^{2+}	2.5	2400	2.9	1920
Sr^{2+}	3.3	1930	3.2	1430
Ba^{2+}	2.2	1020	2.1	560

equilibrium filtration. Therefore the spectroscopic technique was adopted as the main tool for investigating the Ca^{2+}–phospholipase A association over a wide pH range. The results are included in Figure 8.

Fluorescence Spectroscopy. The fluorescence emission spectra of phospholipase A and its zymogen have been published recently (Abita *et al.*, 1972). The zymogen conversion is characterized by a blue shift in the tryptophan emission band from 348 to 342 nm and an increase in the quantum yield with a factor 2.3. However, the presence of Ca^{2+} does not influence the fluorescence spectra of both proteins. In contrast, the noncovalently bound fluorescent probe ANS is able to monitor the phospholipase A–Ca^{2+} interaction.

Figure 4 shows the native and phospholipase A induced ANS fluorescence spectra in the absence and presence of Ca^{2+}. Apparently the influence of Ca^{2+} on the ANS fluorescence is dependent on the presence of protein. The enhancement of the protein induced ANS fluorescence by Ca^{2+} was found to be specific for phospholipase A. Several other proteins including bovine serum albumin and lysozyme induced ANS fluorescence but no effect of Ca^{2+} was observed.

Similar results were obtained with Sr^{2+} and Ba^{2+} instead of Ca^{2+}. Mg^{2+} had no effect in agreement with results mentioned earlier.

A saturation curve was obtained when a phospholipase A solution containing ANS was titrated with Ca^{2+}. Therefore

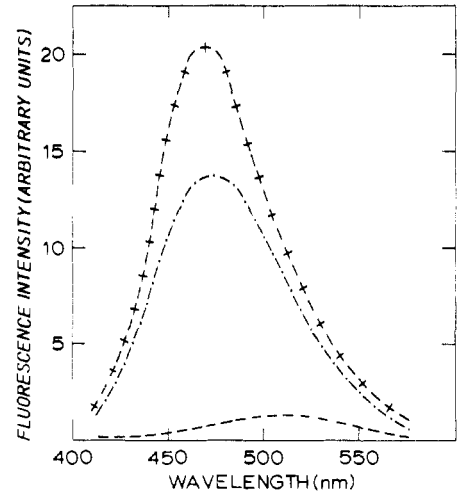

FIGURE 4: Influence of calcium on the native and protein-induced fluorescence of ANS. Conditions: to 3 ml of 0.05 M Tris–0.1 M NaCl (pH 8.0), enzyme was added to a final concentration of 20 μM and/or calcium to 1 mM: (- - - -) ANS with or without calcium, (·—·—·) ANS with phospholipase A, and (-+-+-) ANS with phospholipase A and calcium. A similar behavior was found for the zymogen.

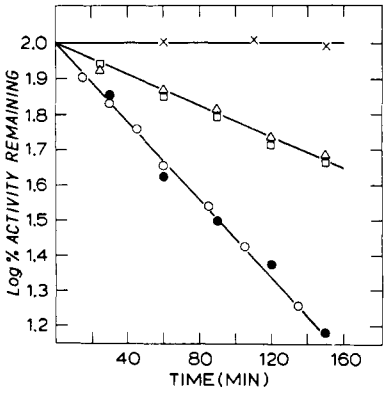

FIGURE 5: Effect of metal ions on the inactivation of phospholipase A by *p*-bromophenacyl bromide. The reaction was initiated by addition of 1 mg of *p*-bromophenacyl bromide dissolved in 20 μl of acetone to 1.0 ml of 0.1 M cacodylate–HCl buffer (pH 6.0) containing 0.1 M NaCl and 1 mg of protein: (×) no *p*-bromophenacyl bromide added; (○) no metal ion present; (●) in the presence of 50 mM $MgCl_2$; (□) in the presence of 5 mM $CaCl_2$; (△) in the presence of 5 mM $BaCl_2$.

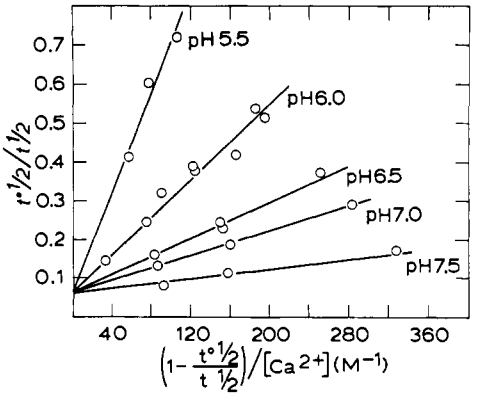

FIGURE 6: Determination of the dissociation constant for the enzyme–Ca^{2+} complex. $t_{1/2}$ values were obtained from plots similar to Figure 5. $t_{1/2}^0$ is the value obtained in the absence of $CaCl_2$. A 0.1 M cacodylate–HCl buffer containing 0.1 M NaCl was used for each pH.

values for K_{Ca} at different pH values could be determined in a similar manner as described in the previous section. The results are included in Figure 8.

Protection of Metal Ions Against Inactivation by p-Bromophenacyl Bromide. Phospholipase A as well as its precursor are completely inactivated through reaction of a single histidine residue with *p*-bromophenacyl bromide (Volwerk *et al.*, 1974). The modification reaction follows for both proteins first-order kinetics. Under these conditions rates of inactivation can be measured conveniently and expressed as half-time values ($t_{1/2}$) using a semilogarithmic plot or residual activity *vs.* time.

As is shown in Figure 5 a considerable increase in $t_{1/2}$ is observed when Ca^{2+} ions are present in the reaction mixture whereas Mg^{2+} ions have no effect. Ba^{2+} protects the enzyme also against inactivation and a similar effect of metal ions is found for the zymogen of phospholipase A.

In the presence of protecting metal ions the inactivation still follows essentially first-order kinetics. Therefore the method of Scrutton and Utter (1965) could be adopted to determine the dissociation constant of the enzyme–Ca^{2+} complex as a function of pH. At each pH and in the absence and presence of $CaCl_2$, $t_{1/2}$ values were measured and plotted as indicated in Figure 6. In this way a series of straight lines was obtained intersecting in a common point on the ordinate. The slope of the lines gives a direct estimate of the dissociation constant K_{Ca} for the enzyme–Ca^{2+} complex at each pH value. The results are included in Figure 8.

Protection of Calcium Against Tryptic Inactivation of Phospholipase A. Under the conditions described in the methods the inactivation of phospholipase A by trypsin follows essentially first-order kinetics. In the absence of Ca^{2+} ions the half-time for the inactivation is 65 min. In the presence of 10^{-3} M $CaCl_2$ the half-time for the inactivation increases to 215 min.

Kinetic Investigation of Metal Ion Binding. Mg^{2+}, Sr^{2+}, Ba^{2+}, Co^{2+}, and Mn^{2+} were investigated for their ability to act as metal ion activators for phospholipase A. Assay conditions were described previously (de Haas *et al.*, 1971) using L-dioctanoyllecithin as substrate at pH 7.0. No activity was found with Mg^{2+}, Mn^{2+}, and Co^{2+}, Ba^{2+} and Sr^{2+} gave activities ranging from 0.1 to 0.4% of the activity found with Ca^{2+} under identical conditions. Mg^{2+}, Mn^{2+}, and Co^{2+} had no influence on the enzymatic activity in the presence of Ca^{2+}, whereas Ba^{2+} and Sr^{2+} behaved as competitive inhibitors. This is illustrated in Figure 7 where Lineweaver–Burk (1934) plots of activity *vs.* Ca^{2+} concentration are represented. For different Ba^{2+} or Sr^{2+} concentration a common intercept on the ordinate is observed. Values for K_{Ba} and K_{Sr} were derived from the plot: K_{Ba} = 2.0 (± 0.4) × 10^{-4} M and K_{Sr} = 2.5 (± 0.4) × 10^{-4} M.

Discussion

Several pancreatic proteins like lipase (Benzonana, 1968), trypsinogen, trypsin, chymotrypsinogen (Abita *et al.*, 1969), and deoxyribonuclease (Price, 1972) share the ability of binding one or more calcium ions. As a consequence of this binding a conformational change takes place leading to a stabilization of the protein structure. The latter appears as a diminished susceptibility to denaturation, peptide bond cleavage by proteolytic enzymes, or reduction of disulfide bridges.

The experimental results described above indicate that similar phenomena occur in the case of porcine pancreatic phospholipase A_2 and its zymogen. In the pH range 4–10 the enzyme contains a single binding site for Ca^+ and the related ions Sr^{2+} and Ba^{2+}. Saturation of this site apparently produces a conformational change in the protein. This follows from (1) the appearance of a difference spectrum in the ultraviolet region and (2) the observation that the rate of tryptic

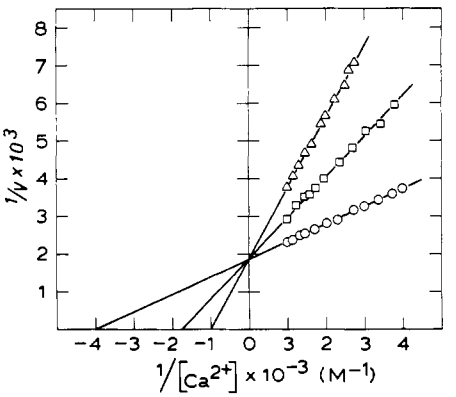

FIGURE 7: Lineweaver–Burk plots indicating the dependence of the enzymatic activity on Ca^{2+} and Ca^{2+}–Ba^{2+} mixtures. Substrate: 0.009 M dioctanoyllecithin. 0.1 M NaCl and 0.01 M Tris at pH 7.0 were held constant throughout the experiment: (○) no $BaCl_2$ present; (□) 2.0 × 10^{-4} M $BaCl_2$; (△) 7.0 × 10^{-4} M $BaCl_2$.

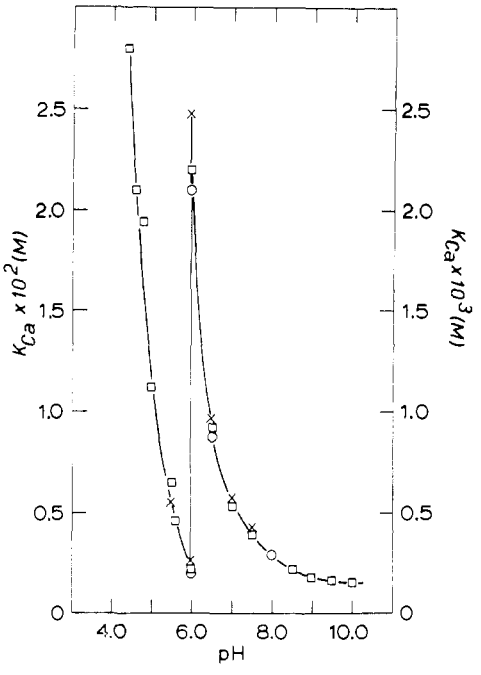

FIGURE 8: The calcium–phospholipase A dissociation constant, K_{Ca}, as a function of pH. The figure is divided in two parts; the values in the left part, below pH 6.0, are a factor of 10 larger in magnitude than those at the right part, above pH 6.0. The K_{Ca} *vs.* pH profile for the zymogen is virtually identical with the one presented here for the active enzyme: (□) points obtained from uv difference spectroscopy; (○) points obtained from fluorescence measurements; (×) points obtained from protection against the inactivation by *p*-bromophenacyl bromide.

inactivation of phospholipase A is substantially retarded in the presence of Ca^{2+}. Because Ca^{2+} ions have no influence on the rate of the tryptic zymogen–enzyme conversion it will be convenient to perform zymogen conversions in the presence of Ca^{2+}. This should minimize the occurrence of secondary splittings of peptide bonds leading to inactive enzyme species.

It is well known that only the aromatic side chains of Tyr, Phe, and Trp significantly contribute to the protein ultraviolet spectrum between 270 and 300 nm. Moreover these residues possess absorption bands in the 230-nm region where also the amide bonds absorb. For the Me^{2+} induced difference spectrum of phospholipase A the positions of the peaks in the 280–290-nm region indicate that most likely one or more tyrosine residues are perturbed. Donovan (1969) has deduced a correlation between the single tyrosine band at about 230 nm and the smaller bands in 280–290-nm region. Accepting this fixed relationship it appears that the band at 242 nm in the phospholipase–Me^{2+} difference spectrum cannot be ascribed exclusively to one or more perturbed tyrosine chromophores. The amplitude of the 242-nm band has about twice the value expected based on the conversion factors of Donovan. Therefore approximately 50% of the 242-nm band must originate from a chromophore not possessing concomitant bands in the 280–290-nm region. For the histidyl imidazole chromophore it is known that the charged form has a slightly higher molar extinction coefficient at 240 nm than the neutral form (Donovan, 1965). A similar phenomenon can be expected when positive charge accumulates on the histidyl imidazole ring, for example, due to the presence of a divalent metal ion. Therefore our tentative conclusion for the origin of the difference spectrum is that besides a shift of a tyrosine to a more polar environment a charge effect on a histidyl residue takes part.

The conclusion from the ultraviolet spectroscopy that in phospholipase A_2 and in the zymogen tyrosine is the only aromatic chromophore perturbed by Ca^{2+} is in agreement with fluorescence data. Apparently the conformational change, which follows Ca^{2+} binding, does not influence the single tryptophan located in the N-terminal region of the peptide chain. The interaction between phospholipase A and Ca^{2+} ions could be studied using the fluorescent probe ANS, which binds to hydrophobic regions in proteins (Aoe *et al.*, 1970). However, the apparently weak binding constant of phospholipase A with ANS did not permit an evaluation of the number of ANS molecules bound to phospholipase A either in the presence or absence of Ca^{2+}. The mechanism by which the ANS quantum yield is increased in the presence of Ca^{2+} ion therefore remains obscure. It seems likely, however, that Ca^{2+} either increases the number of bound ANS molecules or increases the quantum yield of already bound ANS.

As is shown in Figure 8 a very good agreement exists between the various direct binding techniques employed in this study to determine the phospholipase A–Ca^{2+} interaction. From the rapid increase of the dissociation constant in the acidic pH region we can conclude that one or more carboxylates participate in the metal ion binding site of the enzyme. However, still a tenfold decrease in K_{Ca} is observed from pH 6.0 to 9.0. This suggests that also a residue with a pK between 6 and 7, in particular a histidine, may participate in the metal ion binding. This is in agreement with the conclusion derived from the spectroscopic data that a histidine is partly responsible for the peak at 242 nm in the difference spectrum. Further evidence is obtained from the protection experiments described in this paper. As is shown in the subsequent paper phospholipase A and its zymogen contain an histidine (His-53) uniquely reactive toward the apolar halo ketone *p*-bromophenacyl bromide. The very efficient protection by Ca^{2+} and Ba^{2+} suggests that histidine-53 is located very close to the metal ion binding site.

The kinetic experiments described in this paper indicate that the metal ion requirement of phospholipase A is very specific for Ca^{2+}. None of the other divalent metal ions tested were capable of inducing substantial enzymatic activity. This suggests a specific role of Ca^{2+} in the catalytic mechanism of the enzyme, further supported by the close relation between Ca^{2+} and the active site residue histidine-53 (compare Volwerk *et al.*, 1974).

Of the divalent metal ions tested so far only Sr^{2+} and Ba^{2+} were found to be fully competitive with Ca^{2+}. The values for K_{Ba} and K_{Sr} derived from kinetics and from direct binding studies are in excellent agreement and equal in both cases K_{Ca}.

A comparison of the K_{Ca} values obtained from kinetics (de Haas *et al.*, 1971) and from the direct binding studies presented in this paper reveals that enzyme activity depends completely on saturation of the single metal ion binding site with Ca^{2+}. However, as shown in Table III, increasing discrepancy between the direct and kinetically determined K_{Ca} values exists at lower pH values. This might indicate that the presence of substrate increases the enzyme affinity for Ca^{2+} and thus that metal ion binding and substrate binding are not completely independent. This influence of substrate on the calcium binding was not detected in previous studies (de Haas *et al.*, 1971) and therefore further work is needed to elucidate the interdependence of substrate and metal ion binding.

However, it is to be concluded that phospholipase A can bind Ca^{2+} in the absence of substrate. In one of the accom-

TABLE III: Comparison of Direct Measured Phospholipase A Calcium Dissociation Constants with Kinetically Determined Values.

	K_{Ca} (mM)	
pH	Kinetics[a]	Direct
5.0	2.50	12.0
5.5	1.50	6.0
6.0	0.55	2.3
6.5	0.35	1.0
7.0	0.28	0.6
7.5	0.29	0.4
8.0	0.28	0.25

[a] de Haas *et al.* (1971).

panying papers (Pieterson *et al.*, 1974) it will be shown that the enzyme is also able to bind monomeric as well as micellar substrates in the absence of metal ions.

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