375-400.
Fox, G. E., Magrum, L. J., Balch, W. E., Wolfe, R. S., and Woese, C. R. (1977), *Proc. Natl. Acad. Sci. U.S.A. 74,* 4532-4541.
Ghisla, S., and Mayhew, S. G. (1976), *Eur. J. Biochem. 63,* 373-390.
Ghisla, S., Hartman, U., Hemmerich, P., and Muller, F. (1973), *Justus Liebigs Ann. Chem.,* 1388-1415.
Gibson, Q. H., Massey, V., and Atherton, N. M. (1962), *Biochem. J. 85,* 369-383.
Gunsalus, R. P., and Wolfe, R. S. (1977), *Biochem. Biophys. Res. Commun. 76,* 790-795.
Gurd, F. R. N., Lawson, P. S., Cochran, D. W., and Wenkert, E. (1971), *J. Biol. Chem. 246,* 3725-3730.
Krebs, K. G., Heusser, D., and Wimmer, H. (1969), in Thin Layer Chromatography, Stahl, E., Ed., New York, N.Y., Springer-Verlag, pp 854-909.
Johnson, L. R., and Jankowski, W. C. (1972), Carbon-13 NMR Spectra; A Collection of Assigned, Coded, and Indexed Spectra, New York, N.Y., Wiley-Interscience.
Marbach, E. P., and Weil, M. H. (1967), *Clin. Chem. 13,* 314-325.
Massey, V., and Hemmerich, P. (1978), *Biochemistry 17,* 9-17.
McBride, B. C., and Wolfe, R. S. (1971), *Biochemistry 10,* 2317-2324.
Müller, F., and Massey, V. (1969), *J. Biol. Chem. 244,* 4007-4016.
Rydon, H. N., and Smith, P. W. G. (1952), *Nature (London) 169,* 922-923.
Spencer, R., Fischer, J., and Walsh, C. (1976), *Biochemistry 15,* 1043-1053.
Spencer, R., Fischer, J., and Walsh, C. (1977), *Biochemistry 16,* 3586-3594.
Stankovich, M. T., and Massey, V. (1976), *Biochim. Biophys. Acta 452,* 335-344.
Taylor, C. D., and Wolfe, R. S. (1974), *J. Biol. Chem. 249,* 4879-4885.
Tzeng, S. F., Wolfe, R. S., and Bryant, M. P. (1975a), *J. Bacteriol. 121,* 184-191.
Tzeng, S. F., Bryant, M. P., and Wolfe, R. S. (1975b), *J. Bacteriol. 121,* 192-196.

Ca^{2+} Binding to Porcine Pancreatic Phospholipase A_2 and Its Function in Enzyme–Lipid Interaction†

A. J. Slotboom,* E. H. J. M. Jansen, H. Vlijm, F. Pattus, P. Soares de Araujo,‡ and G. H. de Haas*

ABSTRACT: In addition to the Ca^{2+} ion bound in the active site, porcine pancreatic phospholipase A_2 has been assumed to possess a second metal-ion binding locus, also specific for Ca^{2+}, which enables the enzyme to interact with organized lipid-water interfaces at alkaline pH [van Dam-Mieras, M. C. E., Slotboom, A. J., Pieterson, W. A., and de Haas, G. H. (1975), *Biochemistry 14,* 5387-5394]. Because this interaction in the absence of Ca^{2+} is governed by the pK of the α-NH_3^+ group of the N-terminal L-Ala[1] residue, the binding of this latter Ca^{2+} ion and its effect on the pK of the α-NH_3^+ group were studied. Titration studies of protons released during tryptic activation of the zymogen and of the ^{13}C chemical shift of a ^{13}C-enriched N-terminal L-Ala[1] residue in phospholipase A_2 showed that, specifically, Ca^{2+} ions increase the pK of the α-NH_3^+ group from 8.4 to 9.3. The pK values of the α-NH_3^+ groups of [D-Ala[1]]phospholipase A_2 and DL-Ala^{-1}-phospholipase A_2, however, decreased considerably upon the addition of Ca^{2+}. Ultraviolet difference spectroscopy suggested that the second Ca^{2+} ion binds close to the single Trp[3] residue and a $K_D \simeq 20$ mM was estimated at pH 7.5. In the presence of micelles of the substrate analogue *n*-hexadecylphosphorylcholine, it was found by equilibrium dialysis that at pH 8 phospholipase A_2 binds two Ca^{2+} ions/mol of enzyme, whereas 1-bromo-2-octanone-inhibited phospholipase A_2 binds only one Ca^{2+} ion/mol of protein. Similar experiments at pH 6 revealed binding of only one Ca^{2+} ion/mol of native phospholipase A_2, whereas no Ca^{2+} binding could be measured for 1-bromo-2-octanone-inhibited phospholipase A_2. Moreover, a strong synergistic effect of the micellar lipid on the binding of both Ca^{2+} ions was observed. Finally, the observations from microcalorimetry that at pH 10 1-bromo-2-octanone-inhibited phospholipase A_2, lacking the first Ca^{2+}-binding site, only binds to micelles in the presence of Ca^{2+} are in good agreement with the existence of a second Ca^{2+} binding site on phospholipase A_2.

Phospholipases A_2 (EC 3.1.1.4) from different origins catalyze the specific hydrolysis of fatty acid ester bonds at the 2 position of 3-*sn*-phosphoglycerides. The pancreatic enzyme shows an absolute requirement for Ca^{2+} ions which bind in a 1:1 molar ratio to the protein. Although Ba^{2+} and Sr^{2+} bind with the same affinity, these metal ions are pure competitive inhibitors and no hydrolytic activity is found. Mg^{2+} ions do not bind at all. The unusually high specificity of phospholipase A_2 for Ca^{2+} points to a specific function of this metal ion in the catalytic event. Spectroscopic evidence has been reported for the perturbation of histidine and tyrosine residues upon Ca^{2+} binding (Pieterson et al., 1974a). Active-site-directed irreversible inhibitors such as the halo ketones *p*-bromophenacyl

† From the Laboratory of Biochemistry, State University of Utrecht, Transitorium 3, "De Uithof", Padualaan 8, Utrecht, The Netherlands. *Received April 25, 1978.* These investigations were carried out under the auspices of the Netherlands Foundation for Chemical Research (SON), with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO), and from the Fundacão de Amparo a Pesquisa do Estado de São Paulo (Brazil) (P.S.d.A.).

‡ Present address: Department of Biochemistry, Institute of Chemistry, University of São Paulo, São Paulo, Brazil.

0006-2960/78/0417-4593$01.00/0

bromide and 1-bromo-2-octanone specifically react with His[48] [1] and this inactivation reaction is strongly inhibited by Ca^{2+} (Volwerk et al., 1974). Moreover, the inactivated enzymes, though conserving their binding capacity for organized lipid-water interfaces, are no longer able to bind Ca^{2+}. Therefore, it has been concluded that Ca^{2+} binds close to the active-site residue His[48].

The enzyme is secreted by the pancreas in a zymogen form which is activated by trypsin. It has been shown that the active center, where Ca^{2+} and monomeric substrate are bound and hydrolysis occurs, preexists in the zymogen (Pieterson et al., 1974b). The main difference between phospholipase A_2 and its zymogen is the presence of a so-called "interface recognition site" (IRS)[2] in the active enzyme (Verger et al., 1973; Pieterson et al., 1974b). From spectroscopic studies and protection experiments against specific tryptic hydrolysis, it has been demonstrated that at least the rather hydrophobic N-terminal sequence of the enzyme Ala[1]-Leu-Trp-Gln-Phe-Arg[6] is part of the IRS (van Dam-Mieras et al., 1975). This surface region, which is topographically distinct from the active center, allows the enzyme to penetrate into organized lipid-water interfaces. A consequence of this interaction is a reorganization of the active-center structure and an increase in the catalytic power of three to four orders of magnitude. It has been proposed that a functionally active IRS is stabilized by a specific ion pair between the α-NH_3^+ group of the N-terminal amino acid Ala[1] and a buried carboxylate function (van Dam-Mieras et al., 1975). Direct binding studies have led to the hypothesis that the enzyme can exist in at least two conformations E and E′. At neutral or slightly acidic pH, form E is present. This conformation, possessing the above-mentioned salt bridge and therefore an intact IRS, is able to interact with organized lipid-water interfaces. Form E′, which predominates at alkaline pH, where the α-NH_3^+ group is deprotonated, lacks the ion pair and no IRS is present. This form is unable to penetrate into lipid-water interfaces.

In certain kinetic studies (Verger et al., 1973; Pieterson, 1973), however, a high interface activity of the enzyme has been reported at pH values well above the pK of the α-NH_3^+ group where the salt bridge would be expected to be disrupted. These studies were performed at rather high Ca^{2+} concentrations, indicating the possibility of an additional metal-ion binding site on the enzyme at alkaline pH. Two hypotheses have been discussed to explain the stabilizing effect on the IRS by binding of this second Ca^{2+} ion (van Dam-Mieras et al., 1975): (1) The binding of Ca^{2+} causes an effective shielding of the salt bridge in a hydrophobic region. Such an apolar environment would shift the deprotonation of the α-NH_3^+ group to higher pH values, and the salt bridge would remain intact at alkaline pH. (2) The binding of Ca^{2+} stabilizes the conformation of the IRS even at pH values where the α-NH_3^+ group is deprotonated. In this hypothesis, the second Ca^{2+} ion is supposed to take over the stabilizing effect of the ion pair on the IRS.

It is the purpose of this study to demonstrate that at alkaline pH porcine pancreatic phospholipase A_2 possesses, indeed, an additional low-affinity Ca^{2+}-binding site, distinct from the active-center locus. Binding of this second Ca^{2+} ion shifts the pK of the N-terminal α-NH_3^+ group to higher values and stabilizes the enzyme form E at alkaline pH.

Experimental Section

Materials and Methods. Porcine pancreatic prophospholipase A_2 was purified from porcine pancreas and converted into phospholipase A_2 by limited proteolysis as described by Nieuwenhuizen et al. (1974). ϵ-Amidinated prophospholipase A_2 (AMPREC) and phospholipase A_2 (AMPA) were prepared as described previously (Slotboom and de Haas, 1975). [D]- and [L-[3-^{13}C]Ala[1]]AMPA and DL-[3-^{13}C]Ala^{-1}-AMPA containing 90% enriched ^{13}C were prepared as described previously (Slotboom et al., 1977). *n*-Hexadecylphosphorylcholine was prepared as described (van Dam-Mieras et al., 1975). 1-Bromo-2-octanone was synthesized essentially according to the procedure as described by Visser et al. (1971) and Mangold (1973). By using 1-bromo-2-[2-^{14}C]octanone, it was shown that upon complete inactivation of phospholipase A_2 1 mol of the inhibitor/mol of enzyme was incorporated with the concomitant loss of 1 His residue. Just as for *p*-bromophenacyl bromide (Volwerk et al., 1974) also 1-bromo-2-octanone specifically reacts with His[48]. Radioactive $^{45}CaCl_2$ and [1-^{14}C]heptanoic acid were obtained from the Radiochemical Centre, Amersham, England. Bovine trypsin was purchased from E. Merck AG, Darmstadt, Germany. All the other chemicals used were of the highest purity available.

Protein concentrations for phospholipase A_2 (and AMPA) and prophospholipase A_2 (and AMPREC) were calculated from the absorbances with an $E_{280nm}^{1\%,1cm}$ of 13.0 and 12.3, respectively. For the N-terminally modified as well as for the 1-bromo-2-octanone blocked phospholipase A_2 analogues a value of 13.0 was used. Enzyme activities were determined using the titrimetric assay procedure with egg-yolk lipoproteins as substrate (Nieuwenhuizen et al., 1974) or with short-chain lecithins (de Haas et al., 1971). Phospholipid concentrations were determined by phosphorus analysis according to the Fiske and Subbarow (1925) method, as modified by Bartlett (1959).

Fluorescence Measurements. Fluorescence spectra were recorded at 25 °C with a Perkin-Elmer MPF 3 spectrofluorimeter using 1-cm cells as described previously (van Dam-Mieras et al., 1975).

Ultraviolet Difference Spectroscopy. Ultraviolet difference spectra were obtained at 25 °C by means of a Shimadzu UV 210 double-beam spectrophotometer or an Aminco DW-2a spectrophotometer as described previously (Pieterson et al., 1974a; van Dam-Mieras et al., 1975). In order to avoid possible absorption due to the high metal ion concentrations, tandem cells (2 × 1 cm light path) were used. By using AGLA micrometer syringes a metal ion solution was titrated in the protein solution of the sample compartment and in the buffer solution of the reference compartment. The same volume of buffer was titrated in the protein solution of the reference compartment (to correct for dilution of the chromophore) and in the buffer solution of the sample compartment.

^{13}C NMR Spectroscopy. All ^{13}C spectra were obtained with a Bruker HX-360 spectrometer operating at 90.5 MHz equipped with a variable-temperature unit (accuracy ±1 °C) (SON Facility, University of Groningen, Groningen, The Netherlands). By using quadrature detection, 1000–2000 transients were accumulated with a repetition time of 0.82 s and a spectral width of 10 000 Hz. An exponential multiplication of the free induction decay corresponding to a line broadening of 6 Hz was applied to improve the signal to noise ratio. Chemical shifts were measured at 25 °C from benzene

[1] Numbering starts from the N-terminal L-Ala residue of phospholipase A_2.

[2] Abbreviations used are: IRS, interface recognition site; AMPREC, fully ϵ-amidinated prophospholipase A_2; AMPA, fully ϵ-amidinated phospholipase A_2; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; CMC, critical micellar concentration; NMR, nuclear magnetic resonance; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

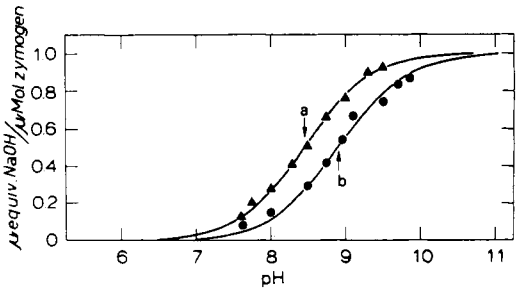

FIGURE 1: Titration curves of protons released during tryptic activation of prophospholipase A_2:

$$\underset{<\text{Glu}\ldots.\ \text{ArgCO-NHAla}\ldots.\ \text{Cys}}{\overset{-7\qquad -1\quad 1\qquad 124}{}} \xrightarrow[H_2O]{\text{trypsin}} \underset{<\text{Glu}\ldots.\ \text{ArgCOO}^- + H_3\text{NAla}\ldots.\ \text{Cys}}{\overset{-7\qquad\quad -1\qquad\quad +1\qquad 124}{}}$$

$$\underset{H_3\text{NAla}\ldots.\ \text{Cys} \rightleftharpoons H^+ + H_2\text{NAla}\ldots.\ \text{Cys}}{\overset{+1\quad 124\qquad\qquad 1\quad 124}{}}$$

Data obtained at 25 °C from the amount of standardized NaOH solution required to neutralize the protons released after the addition of trypsin to porcine pancreatic prophospholipase A_2: curve a, no Ca^{2+}; curve b, 50 mM Ca^{2+}. The drawn lines are calculated curves for pK values of 8.45 and 8.9, respectively.

(capillary). Routinely, 1.5 mL of 1.0–1.5 mM protein solutions in 50 mM Hepes or Tris buffer containing 10% of D_2O was measured using 10-mm diameter tubes equipped with a plug to prevent vortexing. The pH was measured with a Radiometer pH meter (PHM 62) equipped with a Radiometer electrode (GK 2321C) before and after each measurement. Adjustment of the pH was performed with 2 M NaOH or HCl in 10% of D_2O.

Equilibrium Dialysis. Equilibrium dialysis experiments were performed at 25 °C with a Dianorm dialysis apparatus (Diachema, Zürich, Switzerland) equipped with 10 two-compartment twin cells. Both compartments (0.2 mL) were separated through a dialysis membrane (molecular weight cutoff 5000). Stock solutions of ^{45}Ca were prepared by adding 10 μL of the $^{45}CaCl_2$ solution (4–30 mCi/mg of Ca) to 10 mL of $CaCl_2$ solutions ranging from 0.05 to 40 mM Ca^{2+} in 0.05 M buffer in 0.1 M NaCl [Tris (pH 8.0) and acetate (pH 6.0)]. Each of the different $^{45}Ca^{2+}$ stock solutions (0.2 mL) of known concentrations (containing 2×10^6 cpm) is added to one compartment and 0.2 mL of the protein solution in the same buffer to the other compartment. In the experiments with lipid micelles, *n*-hexadecylphosphorylcholine was present in both compartments at the same concentration. After 3 h of dialysis when equilibrium has been reached, the content of each compartment is recovered and 50-μL aliquots were added to 10 mL of a liquid scintillation cocktail (Packard Instagel). Radioactivity was determined in a Searle Isocap 300 liquid scintillation system (Nuclear Chicago Division). The experimental data were treated in the Scatchard representation according to the method of Klotz and Hunston (1971). The lower affinity constants were then calculated by successive readjustment to have the best fit between theoretical and experimental curves.

Microcalorimetry. Microcalorimetric measurements were performed as described by Rosseneu et al. (1974) and Donner et al. (1976) using a LKB batch microcalorimeter Type 2107, equipped with gold-plated cells and thermostated at 25 °C. Calorimetric cells were charged using Gilson P 5000 pipets which were gravimetrically calibrated to deliver 2.0 and 4.0 mL of the solutions used. In all the experiments, 2.0 mL of a 1-bromo-2-octanone-inhibited phospholipase A_2 solution (1.5 mg/mL) in 0.05 M buffer in 0.1 M NaCl [acetate (pH 6.0) and glycine (pH 10.0)] was placed in the compartment of the reaction vessel. The glycine buffer contained either 1 mM EGTA or different concentrations of Ca^{2+}. In the other compartment of the reaction vessel, 4.0 mL of an *n*-hexadecylphosphorylcholine solution at various concentrations in the same buffer was delivered. The reference vessels were charged in the same way, except that no protein was added. Separate dilution experiments were made with the reactants.

Proton Titrations. Ten microliters of freshly prepared trypsin solution (16.8 mg/mL) was added to a magnetically stirred solution of 2.7–3.4 μmol of prophospholipase A_2 in 4.0 mL of boiled distilled H_2O in an N_2 atmosphere. The protons released were titrated with standardized 0.0152 N NaOH solution at 25 °C using a Radiometer pH stat (Titrator TTT2, Titrigraph REA 300, and Autoburette ABU 12) equipped with a 0.25-mL burette and a combined electrode GK 2321 C. At the end of the reaction, the pH was lowered to 4.0 by the addition of acetic acid and the various batches were pooled. Upon chromatography on CM-cellulose (Nieuwenhuizen et al., 1974), only one symmetrical peak with a constant specific activity over the peak is obtained, indicating a complete activation without cleavage of other peptide bonds.

Results

Proton Titration. Figure 1 represents the proton release as a function of pH during the tryptic conversion of the porcine zymogen into active phospholipase A_2. Because trypsin specifically cleaves the peptide bond between Arg^{-1} and Ala^1, protons which are released upon the increase of pH originate exclusively from the α-NH_3^+ function of the N-terminus Ala^1. Without Ca^{2+} or in the presence of 2 mM Ca^{2+}, which is sufficient to saturate the high-affinity site in the zymogen and in the active enzyme (Pieterson et al., 1974a), one proton per mol of zymogen is released upon activation (Figure 1, curve a). The midpoint of the titration curve, representing the pK of the α-NH_3^+ group, is at pH 8.4, which is in good agreement with previously determined values (Janssen et al., 1972; Pieterson et al., 1974b). A similar zymogen activation but now in the presence of 50 mM Ca^{2+} still shows the release of one proton per mol of precursor (Figure 1, curve b). The midpoint of this titration curve, however, shifts to pH 8.9. Apparently, the pK of the proton-releasing group increases upon additional Ca^{2+} binding to phospholipase A_2 at alkaline pH.

^{13}C Nuclear Magnetic Resonance Spectroscopy. A more direct determination of the pK of the α-NH_3^+ group can be obtained by ^{13}C NMR spectroscopy. The chemical shift of ^{13}C probes is known to be sensitive to changes in charge around the ^{13}C nucleus (Gurd and Keim, 1973). Figure 2A shows the ^{13}C NMR titration of AMPA[3] in which the N-terminal amino acid L-Ala^1 has been replaced by a 90% enriched L-[3-^{13}C]Ala^1 residue (Slotboom et al., 1977). In the absence of Ca^{2+} or with Ca^{2+} concentrations just sufficient to saturate only the catalytic site (2–3 mM), the pK of the α-NH_3^+ group is 8.4 in agreement with the results of the proton titration. Increasing the Ca^{2+} concentration up to 200 mM gradually shifts the pK of the α-NH_3^+ group to 9.4.[4] This increase in pK is very spe-

[3] Chemical-modification studies of phospholipase A_2 involving the N terminus require protection of the ϵ-NH_2 groups of all lysine residues (Slotboom and de Haas, 1975). It has been shown that phospholipase A_2 in which the ϵ-NH_2 groups are transformed by acetamidination (AMPA) behaves in all respects very similar as compared to the native enzyme (Slotboom and de Haas, 1975).

[4] It has to be remarked that, in contrast to the curves in Figure 2B, the continuous lines drawn in Figure 2A are too steep for theoretical titration curves. Most probably, the enzyme forms E and E′ are in slow exchange on the NMR time scale (Bendall and Lowe, 1976).

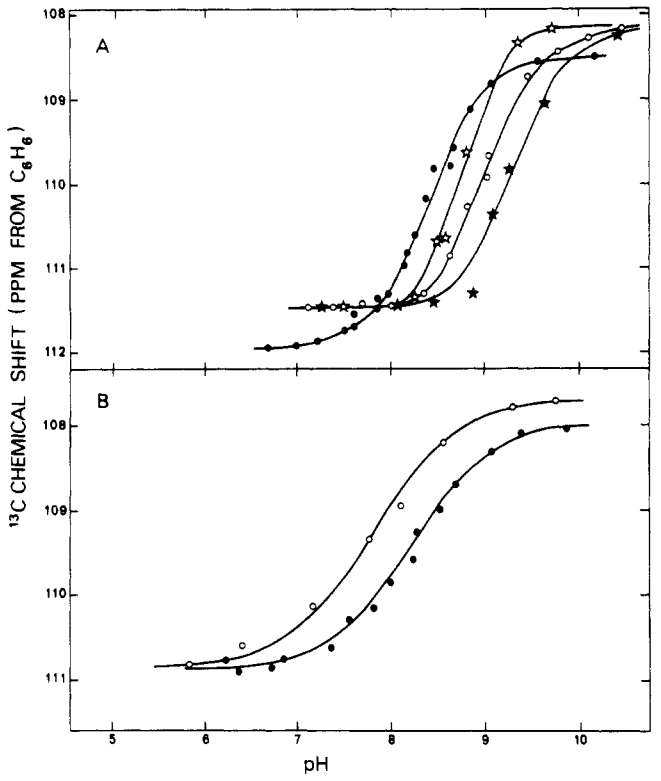

FIGURE 2: Plot of the chemical shift (ppm from C_6H_6) of the 90%-enriched ^{13}C nucleus in [L-[3-^{13}C]Ala^1]AMPA (A) and [D-[3-^{13}C]Ala^1]-AMPA (B) as a function of pH: (●) no Ca^{2+}; (☆) 25 mM Ca^{2+}; (○) 50 mM Ca^{2+}; (★) 200 mM Ca^{2+}. Data were obtained from spectra recorded at 90.5 MHz (1000–2000 transients) at 25 °C using 1–2 mM protein solutions.

cific for Ca^{2+}. Similar salt concentrations obtained by the addition of $BaCl_2$, $MgCl_2$, or NaCl do not change the pK value of the α-NH_3^+ group of L-Ala^1. Figure 2B emphasizes that the increase of the pK value of the α-NH_3^+ group of the N-terminal amino acid L-Ala^1 upon binding of a second Ca^{2+} ion is a highly specific phenomenon for the native enzyme. AMPA, in which the N-terminal L-Ala^1 residue is replaced by 90% enriched D-[3-^{13}C]Ala^1, is a protein which lacks the IRS (Slotboom and de Haas, 1975; Slotboom et al., 1977) and most probably cannot form the salt bridge essential for interface activity. In the presence of high salt concentrations of $CaCl_2$ as well as of NaCl, $BaCl_2$, or $MgCl_2$, the pK of the α-NH_3^+ group of D-Ala^1 in this protein decreases from 8.2 to 7.8. The same phenomenon is observed with phospholipase A_2 in which the polypeptide chain has been elongated with an additional amino acid, viz., DL-[3-^{13}C]Ala^{-1}-AMPA. Again the pK of the N-terminal amino acid, DL-Ala^{-1}, drops from 8.0 to 7.5 upon an increase of the ionic strength (cf. Table I). Such a behavior would be expected for a charged, exposed α-NH_3^+ group in an aqueous surrounding and, therefore, indicates that in the native enzyme the α-NH_3^+ group of L-Ala^1 must be located in a very specific microenvironment. Most probably, only this very particular conformation characterized by an ion pair between the α-NH_3^+ group and a buried carboxylate function allows the enzyme to bind a second Ca^{2+} ion. In this respect, the behavior of the enzyme in which the active site is switched off by blocking of His^{48} with 1-bromo-2-octanone is of considerable interest (Table I). In the "native" [L-[3-^{13}C]Ala^1] AMPA specific modification of His^{48} results in a protein which has lost its binding properties for Ca^{2+} in the active site and is devoid of catalytic power (Pieterson et al., 1974b). This modification, however, does not destroy the

TABLE I: pK Values[a] at 25 °C of the N-Terminal α-Amino Group of ϵ-Amidinated Phospholipase A_2 (AMPA) and Some Analogues from ^{13}C NMR.

	[Ca^{2+}] (mM)	
	0	50
[L-[3-^{13}C]Ala^1]AMPA[b]	8.4	9.0
[D-[3-^{13}C]Ala^1]AMPA[c]	8.0	7.8
DL-[3-^{13}C]Ala^{-1}-AMPA[c]	8.0	7.5
1-bromo-2-octanone inhibited:		
[L-[3-^{13}C]Ala^1]AMPA	9.0	9.4
[D-[3-^{13}C]Ala^1]AMPA	8.0	7.8

[a] The pK values reported were measured in 0.1 M NaCl in 10% of D_2O and have an accuracy of ±0.05 pH unit. [b] In the presence of 200 mM Ca^{2+}, a pK value of 9.4 was observed. 50 mM Ba^{2+} or Mg^{2+} had no measurable effect on the pK value. [c] In the presence of Ba^{2+} or Mg^{2+} (50 mM), the pK values drop to 7.8 and 7.6, respectively.

lipid-binding properties of the protein (Pieterson et al., 1974b) and the IRS and salt bridge remain intact. Therefore, at alkaline pH the inhibited protein is still able to bind the second Ca^{2+} ion, and again an increase in the pK of the N terminus is observed. The diastereoisomeric [D-[3-^{13}C]Ala^1]AMPA whose catalytic site is very similar to that of AMPA (Slotboom and de Haas, 1975) and which is inhibited in a comparable way by 1-bromo-2-octanone is known to have no lipid-binding properties because of the absence of the IRS. In this protein lacking both Ca^{2+}-binding sites, the pK of the α-NH_3^+ group of the N terminus is lowered by any increase in ionic strength.

Fluorescence Spectroscopy. The different conformation of the N-terminal part of the polypeptide chain, at least the different microenvironment of Trp^3 in the diastereoisomeric proteins [L-Ala^1]- and [D-Ala^1]AMPA, becomes evident also from their behavior in fluorescence spectroscopy as a function of pH. Figure 3 (curve A) demonstrates that Trp^3 in [D-Ala^1]AMPA must be located in a surrounding very similar to that of Trp in the zymogen of amidinated phospholipase (AMPREC) (Figure 3, curve B), a protein which lacks a free α-NH_3^+ group (de Haas et al., 1970; Puyk et al., 1977). This environment is different from that of Trp^3 in the active AMPA (curve C).

Ultraviolet Difference Spectroscopy. Figure 4 shows in a qualitative way how binding of Ca^{2+} to the first and second binding site influences the ultraviolet difference spectrum of the enzyme. Addition of small amounts of Ca^{2+} sufficient to saturate only the catalytic site results in a pure tyrosine perturbation spectrum[5] (curve 2). Upon further increasing the Ca^{2+} concentration, the difference spectrum gradually changes and shows additional Trp perturbation (curve 3) (Donovan, 1969). A similar titration using 1-bromo-2-octanone-inhibited phospholipase A_2 is shown in Figure 4, curve 5. The His^{48}-blocked phospholipase A_2 has lost its high-affinity Ca^{2+}-binding site, and in the presence of 2 mM Ca^{2+} no difference spectrum is observed (curve 4). However, 80.6 mM Ca^{2+} produces a clear Trp perturbation spectrum (curve 5), which confirms the results obtained with native phospholipase A_2 and indicates that binding of the second Ca^{2+} ion perturbs the microenvironment of Trp^3. Double-reciprocal plots of the ul-

[5] The height of the 242-nm peak relative to that of the peaks in the 280–290-nm region indicates a contribution at 242 nm of other residues than tyrosine (Donovan, 1969). It has been proposed by Pieterson et al. (1974a) that upon Ca^{2+} binding perturbation of His^{48} contributes also to the amplitude of the 242-nm band (cf. Donovan, 1965).

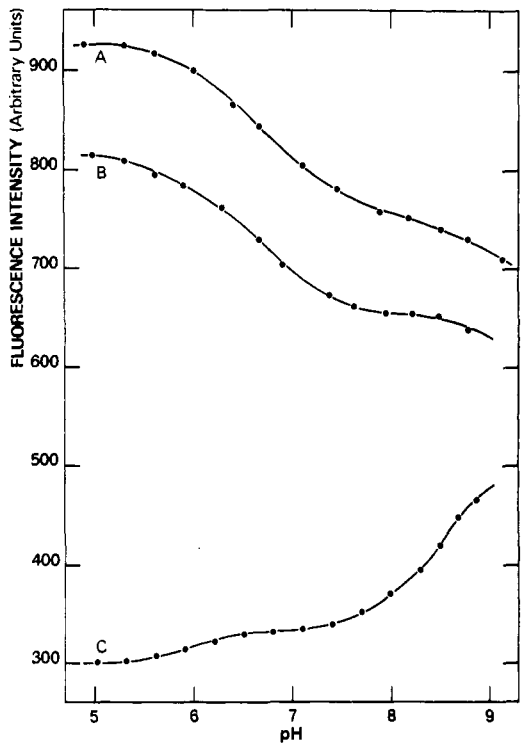

FIGURE 3: pH dependence of fluorescence intensities of [D-Ala^1]AMPA (A), AMPREC (B), and AMPA (C). Experimental conditions: 0.01 M sodium acetate, 0.01 M Tris, 0.1 M NaCl, 13.1 μM protein; excitation at 295 nm; intensities were measured at the maximal emission wavelengths (347, 347 and 342 nm, respectively) at 25 °C.

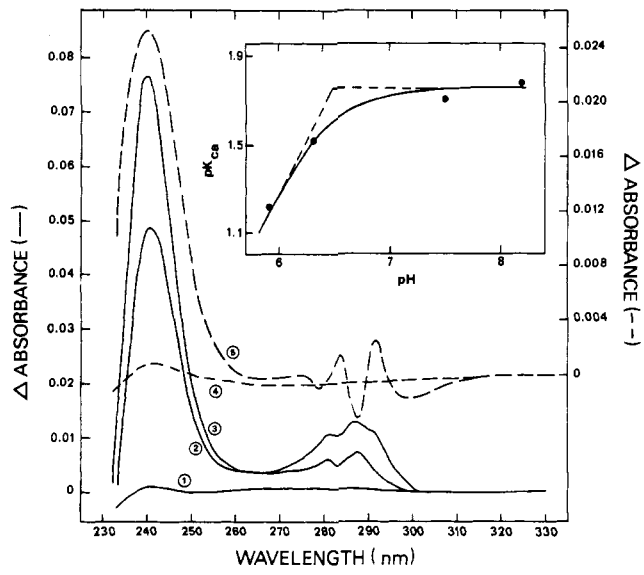

FIGURE 4: Ultraviolet difference spectra produced by the interaction of phospholipase A_2 (—) and 1-bromo-2-octanone-inhibited phospholipase A_2 (- - -) with Ca^{2+}. Experimental conditions: 0.05 M Tris, 0.1 M NaCl (pH 7.5) at 25 °C. 33.7 μM phospholipase A_2 (—): curve 1, no Ca^{2+}; curve 2, 2.9 mM Ca^{2+}; curve 3, 73.6 mM Ca^{2+}. 40.2 μM 1-bromo-2-octanone-inhibited phospholipase A_2 (- - -): curve 4: no Ca^{2+} and 3.0 mM Ca^{2+}; curve 5, 80.6 mM Ca^{2+}. Inset: Dixon plot of the effect of pH on the interaction of 1-bromo-2-octanone-inhibited phospholipase A_2 with Ca^{2+} obtained from ultraviolet difference spectroscopy.

traviolet difference absorptions at 242 nm[6] against Ca^{2+} concentrations show two straight lines with different slopes from which the dissociation constants for the first and second Ca^{2+} binding sites can be calculated. The results are summarized in Table II. With the exception of the His^{48}-blocked enzyme, which has lost its first Ca^{2+} binding site, the other proteins are characterized by a dissociation constant of 0.3–0.5 mM for the first Ca^{2+} binding site at pH 7.5. This value agrees favorably with values reported earlier (Pieterson et al., 1974a). As regards the Ca^{2+} binding constant for the second site, weak affinity was observed only for native phospholipase A_2, AMPA, and the His^{48}-blocked enzyme. The zymogen as well as [D-Ala^1]AMPA do not possess a second Ca^{2+} binding site. It is of interest to remark that the Ca^{2+} binding ligand of the second site, which most probably is a carboxylate group, ionizes at a rather high pH. Already at pH 6.0 it was difficult to detect the second site in phospholipase A_2 and in its His^{48}-blocked derivative. A rough estimate of the pK of the Ca^{2+} binding ligand can be obtained from the Dixon plot given in the inset of Figure 4.

Equilibrium Dialysis. From the above it is evident that the affinity for Ca^{2+} of the second binding site is very weak and difficult to measure accurately in the lipid-free enzyme. In the presence of organized lipid–water interfaces, however, such as the micellar substrate analogue *n*-hexadecylphosphorylcholine, not only does the Ca^{2+} affinity of the first site improve (Pieterson et al., 1974a) but there is an even stronger synergistic effect on the second site (van Dam-Mieras et al., 1975). This allows us to determine an apparent Ca^{2+} binding constant of the second site in the presence of saturating lipid with better

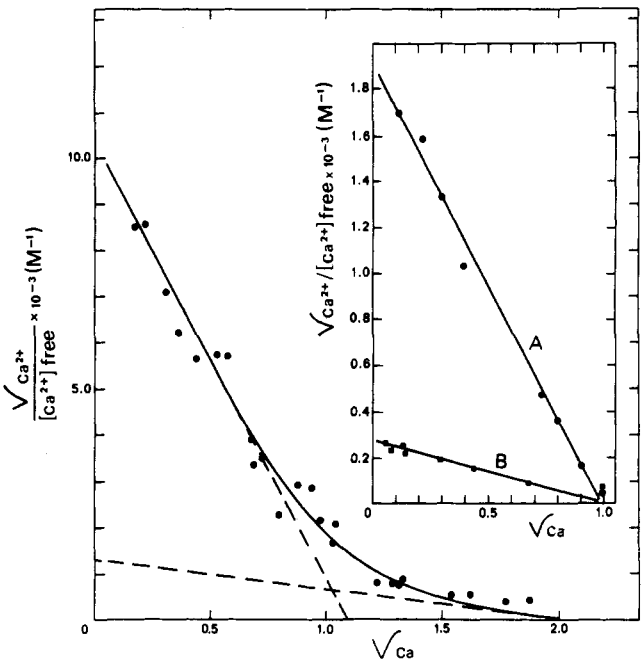

FIGURE 5: Scatchard plots for binding data of Ca^{2+} to phospholipase A_2 at pH 8.0 (●–●) and 6.0 (inset, curve A, ●–●) and to 1-bromo-2-octanone-inhibited phospholipase A_2 (inset, curve B, ■—■) from equilibrium dialysis at 25 °C in the presence of micellar *n*-hexadecylphosphorylcholine. Experimental conditions: 0.05 M buffer in 0.1 M NaCl [Tris (pH 8.0) and acetate (pH 6.0)]. Initial Ca^{2+} concentrations varied from 0.05 to 40 mM (one compartment). Protein concentrations (other compartment): 164 μM, 1400 μM (inset, curve A), 921 μM (inset, curve B). *n*-Hexadecylphosphorylcholine concentration (both compartments): 37.6 mM, 37.6 mM (inset, curve A), 94 mM (inset, curve B).

accuracy. Figure 5 shows a Scatchard plot of Ca^{2+} binding to native phospholipase A_2, obtained by equilibrium dialysis in the presence of micelles of *n*-hexadecylphosphorylcholine. At pH 8.0, the Ca^{2+} binding curve to native phospholipase A_2 and

[6] This wavelength was chosen because of the larger difference signal. It has been checked that the variations at 242 nm correspond to the changes in absorption in the more specific aromatic region between 270 and 295 nm.

TABLE II: Spectroscopic Parameters of the Interaction of Phospholipase A_2, Its Zymogen, and Some Modified Phospholipases A_2 with Ca^{2+}.

	pH	$K_{Ca^{2+}}$ (mM)[a] 1st site	$K_{Ca^{2+}}$ (mM)[a] 2nd site	ΔA_{max}^{242} (cm^{-1} M^{-1}) 1st site	ΔA_{max}^{242} (cm^{-1} M^{-1}) 2nd site
phospholipase A_2	7.5	0.4	20	1730	890
phospholipase A_2	6.0	3.6	≥50	2150	
prophospholipase A_2	7.5	0.5		1770	
ε-amidinated phospholipase A_2	7.5	0.3	20	1760	740
ε-amidinated [D-Ala^1]phospholipase A_2	7.5	0.3		1100	
1-bromo-2-octanone-inhibited phospholipase A_2	7.5		25		870
1-bromo-2-octanone-inhibited phospholipase A_2	6.0		≥50		

[a] The reported $K_{Ca^{2+}}$ values have an accuracy of ±8% and ±25% for the first and second site, respectively. In those cases where no $K_{Ca^{2+}}$ values are reported, no measurable interaction could be detected.

TABLE III: Dissociation Constants for the Interaction of Phospholipase A_2, Fully ε-Amidinated Phospholipase A_2, and 1-Bromo-2-octanone-Inhibited Phospholipase A_2 with Ca^{2+} from Equilibrium Dialysis in the Presence[a] and Absence of Micellar *n*-hexadecylphosphorylcholine.

	pH	[protein] (μM)	[*n*-hexadecylphosphorylcholine] (mM)	$K_{Ca^{2+}}$ (mM)[b] 1st site	$K_{Ca^{2+}}$ (mM)[b] 2nd site
phospholipase A_2	8.0	1478	0	0.3	+[c]
phospholipase A_2	8.0	164	37.6	0.1	3
phospholipase A_2	8.0	278	18.8	0.1	3
phospholipase A_2	6.0	1400	37.6	0.5	+[c]
ε-amidinated phospholipase A_2	8.0	286	18.8	0.1	4
1-bromo-2-octanone-inhibited phospholipase A_2	8.0	1400	0		+[c]
1-bromo-2-octanone-inhibited phospholipase A_2	8.0	921	94		3
1-bromo-2-octanone-inhibited phospholipase A_2	6.0	286	18.8		

[a] $K_{Ca^{2+}}$ values reported in the presence of micellar *n*-hexadecylphosphorylcholine have to be considered as apparent values. [b] In those cases where no $K_{Ca^{2+}}$ values are reported, no measurable interaction could be detected. The reported $K_{Ca^{2+}}$ values have an accuracy of ±8% and ±25% for the first and second site, respectively. [c] From the Scatchard plots it could be concluded that at high Ca^{2+} concentrations a second metal-ion binding site becomes evident. However, the experimental points do not allow an estimation of the exact number of binding sites nor the calculation of the average dissociation constant of these sites.

AMPA is biphasic, and apparent dissociation constants for the first and second site are 0.1 and 3 mM, respectively. With this technique, no quantitative value of the second Ca^{2+} binding constant could be determined at pH 6.0 notwithstanding high protein concentrations (inset Figure 5, curve A), which is in agreement with the extremely weak Ca^{2+} binding as found by the ultraviolet difference technique. The 1-bromo-2-octanone-inhibited phospholipase A_2 has lost its first Ca^{2+} binding site, but its lipid-binding properties are very similar to those of the native enzyme. At alkaline pH the second Ca^{2+} binding site in this His^{48}-blocked protein is intact and in the presence of micellar lipids again an apparent dissociation constant for the binding of Ca^{2+} to the second site is found to be 3 mM (inset Figure 5, curve B). The results of these measurements are summarized in Table III.

Microcalorimetry. The role of Ca^{2+} ions in the binding process between phospholipase A_2 and micellar lipid-water interfaces at alkaline pH can also be shown by microcalorimetry. In order to obtain specifically information on the second Ca^{2+} binding site of the enzyme, 1-bromo-2-octanone-inhibited phospholipase A_2, which has lost its high-affinity Ca^{2+}-binding site, was studied. Just like native phospholipase A_2, also the His^{48}-blocked inactive protein interacts with micelles of *n*-hexadecylphosphorylcholine at pH 6.0 in the absence of Ca^{2+}, and the binding process is exothermic (Figure 6, ● - ●). At pH 10 and in the absence of Ca^{2+}, addition of lipid micelles to the protein does not produce a measurable heat effect (Figure 6, ▲ - -▲). However, upon the addition of increasing amounts of Ca^{2+} to a mixture of 1-bromo-2-octanone-inhibited phospholipase A_2 and lipid micelles at pH 10, a growing heat release is observed and at 20 mM Ca^{2+} the heat effect is very similar to that produced at pH 6.0 in the absence of Ca^{2+} (Figure 6, ⚠ → ⚠). A double-reciprocal plot of heat release vs. Ca^{2+} concentration is shown in the inset of Figure 6 from which an apparent Ca^{2+} dissociation constant of 4 mM could be calculated. This value competes favorably with the dissociation constant found for the second Ca^{2+} binding site by equilibrium dialysis (3 mM).

Discussion

At neutral or slightly acidic pH, porcine pancreatic phospholipase A_2 strongly interacts with micellar lipid-water interfaces even in the absence of Ca^{2+}. This binding is caused by a hydrophobic interaction between the IRS, involving the apolar N-terminal sequence of the enzyme, and the fatty acyl chains of the lipid substrate. A high ionic strength of the medium improves the lipid-protein binding, and a considerable exothermic heat effect is observed. Under these conditions, a high catalytic activity is found when a single Ca^{2+} ion is bound to the active site of the enzyme close to His^{48}. This metal-ion binding locus is called the high-affinity site. Irreversible blocking of His^{48} by active-site-directed inhibitors such as 1-bromo-2-octanone prevents this Ca^{2+} binding and the hydrolytic activity is lost, but the lipid-binding properties of the protein (IRS) remain fully intact. A functionally active IRS owes its stability most probably to an internal salt bridge between the α-NH_3^+ group and a buried carboxylate function (Fersht, 1972; Mavridis et al., 1974; Kossiakoff et al., 1977). The p*K* of the α-NH_3^+ group of Ala^1 is 8.4. Therefore, at alkaline pH values, deprotonation of the α-NH_3^+ occurs, the salt bridge is disrupted, and the IRS is destroyed. Under these conditions, the enzyme is no longer able to penetrate into or-

ganized lipid–water interfaces.

Previously it has been reported that high Ca^{2+} concentrations enable the enzyme to interact with micellar lipids also at alkaline pH, and it was suggested that binding of a second Ca^{2+} ion to the enzyme stabilizes the IRS at higher pH values (van Dam-Mieras et al., 1975). The present results of the proton titration (Figure 1) and in particular those of the ^{13}C NMR (Figure 2) clearly show that a low Ca^{2+} concentration, just sufficient to saturate the catalytic, high-affinity site (2–3 mM), has hardly any influence on the pK of the α-NH_3^+ group. However, in the presence of higher Ca^{2+} concentrations (up to 200 mM), the pK of the N terminus gradually shifts from 8.4 to 9.3. Comparable concentrations of Ba^{2+}, Mg^{2+}, or Na^+ have no effect, whereas the slightly modified proteins [D-Ala^1]AMPA and DL-Ala^{-1}-AMPA do not show this shift upon the addition of Ca^{2+}. The fact that this shift is very specific for Ca^{2+} and is observed only with the native enzyme strongly suggests that pancreatic phospholipase A_2 possesses a second low-affinity Ca^{2+} binding site located close to the N terminus of the chain. Most probably the Ca^{2+} liganding groups are carboxylates, and from the Dixon plot given in the inset of Figure 4 one would expect that they are located in a rather hydrophobic microenvironment close to the salt bridge. Ionization of these carboxylates makes binding of a second Ca^{2+} possible and drives the salt bridge into a more apolar surrounding. In this respect, it is of interest to note that recent studies on specific carboxylate modification of phospholipase A_2 showed that 2 out of the 16 carboxylates present in the enzyme are accessible only after complete oxidation of all disulfide bridges (E. A. M. Fleer, personal communication). Minor changes in the N-terminal sequence of the enzyme, such as D-Ala^1 replacing L-Ala^1 or chain elongation with an additional Ala, perturb the three-dimensional conformation of the N terminus (cf. Figures 2 and 3), the salt bridge is no longer possible, and no second Ca^{2+} binding site is present. Any increase of ionic strength with these latter proteins results in a lowering of the pK of the N terminus, and this phenomenon is not specific: Ca^{2+} as well as other bi- or monovalent cations have the same effect.

From Tables II and III it is clear that irreversible blocking of the active-site residue His^{48} prevents binding of Ca^{2+} to the catalytic site. However, the His^{48}-modified protein in which the IRS is intact (Pieterson et al., 1974b) still possesses the second Ca^{2+} binding site: addition of Ca^{2+} increases the pK of the α-NH_3^+ in the L-Ala^1 protein, whereas in the D-Ala^1 protein the pK drops (see Table I). It is of interest to compare the pK values of the α-NH_3^+ group in the native enzyme and the His^{48}-blocked protein: 8.4 and 9.0, respectively. Although the active-site area around His^{48} and the IRS (N-terminal region) are known to be topographically distinct (Pieterson et al., 1974b; van Dam-Mieras et al., 1975) the presence of an apolar C-8 chain on His^{48} apparently induces a more hydrophobic environment around the α-NH_3^+ group. This means that, in the active enzyme, His^{48} and the N terminus most probably are not far apart. This tentative conclusion is sustained also by the fluorescence increase around pH 6.0 (Figure 3, curve C). The increase in quantum yield disappears upon acylation of His^{48} with 1-bromo-2-octanone. The results obtained by ultraviolet difference spectroscopy in Figure 4 indicate that binding of a second Ca^{2+} ion in phospholipase A_2 perturbs the microenvironment of Trp^3 and confirms the tentative conclusion from the ^{13}C NMR that the second Ca^{2+}-binding site must be located close to the N-terminus of the polypeptide chain. Notwithstanding the fact that weak dissociation constants are experimentally difficult to quantitate, the NMR results of Figure 2 and the ultraviolet results of Figure 4 are in reasonable agreement and point to a dissociation constant for the second Ca^{2+} binding site in lipid-free phospholipase A_2 of about 20 mM at pH 8.0. In the presence of organized lipid–water interfaces, however, the affinity of the enzyme for binding a second Ca^{2+} ion considerably increases (Table III and Figure 6) and this allows the enzyme to bind its substrate and perform effective catalysis at alkaline pH.

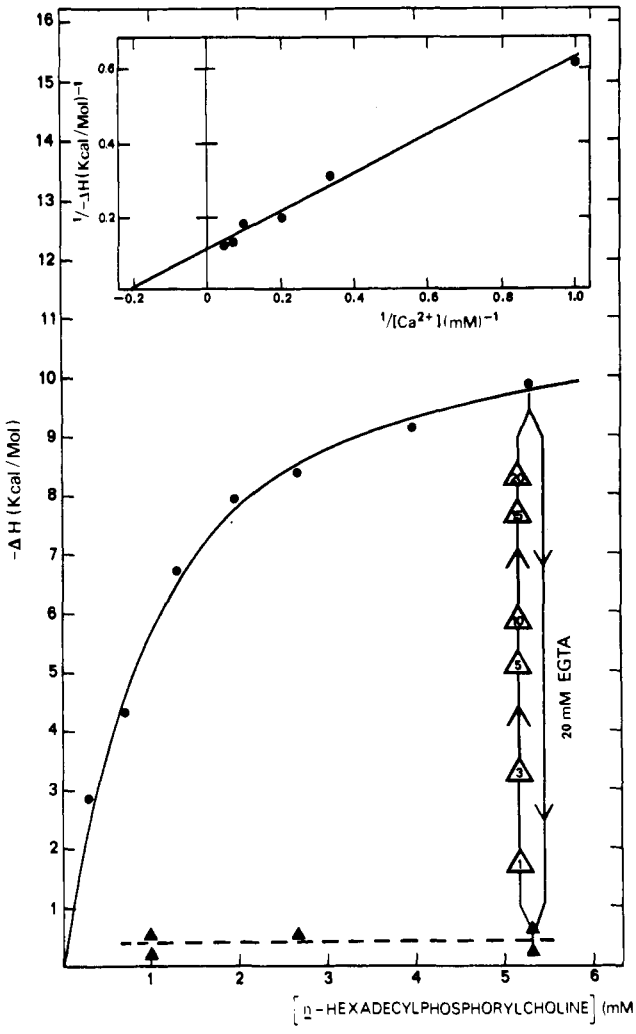

FIGURE 6: Enthalpy change of binding of micellar *n*-hexadecylphosphorylcholine to 1-bromo-2-octanone-inhibited phospholipase A_2 at pH 6.0 (●–●) and 10.0 (▲ - - ▲). Experimental conditions: 35.7 μM protein, 0.05 M buffer in 0.1 M NaCl [acetate (pH 6.0) and glycine (pH 10.0)] at 25 °C. At pH 10.0, EGTA (1 mM) was present (▲) or increasing concentrations of Ca^{2+} [(△) 1 up to (△) 20 mM). Inset: Double-reciprocal plot of the change in enthalpy vs. Ca^{2+} concentration in the presence of 5.33 mM *n*-hexadecylphosphorylcholine at pH 10.0.

Acknowledgments

The authors thank Drs. H. M. Verheij and J. J. Volwerk for preparing 1-bromo-2-octanone and Mr. R. Dijkman for the preparation of the various phospholipids.

References

Bartlett, G. R. (1959), *J. Biol. Chem. 234*, 466–468.

Bendall, M. R., and Lowe, G. (1976), *Eur. J. Biochem. 65*, 493–502.

de Haas, G. H., Slotboom, A. J., Bonsen, P. P. M., van Deenen, L. L. M., Maroux, S., Puigserver, A., and Desnuelle, P. (1970), *Biochim. Biophys. Acta 221*, 31–53.

de Haas, G. H., Bonsen, P. P. M., Pieterson, W. A., and van Deenen, L. L. M. (1971), *Biochim. Biophys. Acta 239*,

252-266.
Donnér, J., Spink, C. H., Borgström, B., and Sjöholm, I. (1976), *Biochemistry 15,* 5413-5417.
Donovan, J. W. (1965), *Biochemistry 4,* 823-829.
Donovan, J. W. (1969), *J. Biol. Chem. 244,* 1961-1967.
Fersht, A. R. (1972), *J. Mol. Biol. 64,* 497-509.
Fiske, G. H., and Subbarow, Y. (1925), *J. Biol. Chem. 66,* 375-379.
Gurd, F. R. N., and Keim, P. (1973), *Methods Enzymol. 27D,* 836-911.
Janssen, L. H. M., de Bruin, S. H., and de Haas, G. H. (1972) *Eur. J. Biochem. 28,* 156-160.
Klotz, I. M., and Hunston, D. L. (1971), *Biochemistry 10,* 3065-3069.
Kossiakoff, A. A., Chambers, J. L., Kay, L. M., and Stroud, R. M. (1977), *Biochemistry 16,* 654-664.
Mangold, H. K. (1973), *Chem. Phys. Lipids 11,* 244-253.
Mavridis, A., Tulinsky, A., and Liebman, M. N. (1974), *Biochemistry 13,* 3661-3666.
Nieuwenhuizen, W., Kunze, H., and de Haas, G. H. (1974), *Methods Enzymol. 32B,* 147-154.
Pieterson, W. A. (1973), Ph.D. Thesis, State University, Utrecht, The Netherlands.
Pieterson, W. A., Volwerk, J. J., and de Haas, G. H. (1974a), *Biochemistry 13,* 1439-1445.
Pieterson, W. A., Vidal, J. C., Volwerk, J. J., and de Haas, G. H. (1974b), *Biochemistry 13,* 1455-1460.
Puyk, W. C., Verheij, H. M., and de Haas, G. H. (1977), *Biochim. Biophys. Acta 492,* 254-259.
Rosseneu, M. Y., Soetewey, F., Blaton, V., Lievens, J., and Peeters, H. (1974), *Chem. Phys. Lipids 13,* 203-214.
Slotboom, A. J., and de Haas, G. H. (1975), *Biochemistry 14,* 5394-5399.
Slotboom, A. J., van Dam-Mieras, M. C. E., and de Haas, G. H. (1977), *J. Biol. Chem. 252,* 2948-2951.
van Dam-Mieras, M. C. E., Slotboom, A. J., Pieterson, W. A., and de Haas, G. H. (1975), *Biochemistry 14,* 5387-5394.
Verger, R., Mieras, M. C. E., and de Haas, G. H. (1973), *J. Biol. Chem. 248,* 4023-4034.
Visser, L., Sigman, D. S., and Blout, E. R. (1971), *Biochemistry 10,* 735-742.
Volwerk, J. J., Pieterson, W. A., and de Haas, G. H. (1974), *Biochemistry 13,* 1446-1454.

UDP-Glucuronosyltransferase: Phospholipid Dependence and Properties of the Reconstituted Apoenzyme†

Jeffrey P. Gorski‡ and Charles B. Kasper*

ABSTRACT: The effect of membrane lipid on the stability and catalytic activity of microsomal UDP-glucuronosyltransferase (EC 2.4.1.17) from rat liver was examined. Ninety-eight percent of membrane phospholipid was separated from total microsomal protein by gel filtration on a deoxycholate-equilibrated Sephadex G-50 column. Lipid removal reduced transferase activity, using *p*-nitrophenol as acceptor, to 0-6% that of a deoxycholate-treated control preparation. Incubation of the apoenzyme with liposomes, prepared from microsomal lipid, restored 30-44% of the original activity. Synthetic and naturally occurring lecithins as well as lysolecithin also reconstituted transferase function; however, dioleoyl- and bovine lecithins were more active than either dipalmitoyl- or plant lecithins. Bovine phosphatidylserine was without effect. *p*-Nitrophenol conjugation catalyzed by the reconstituted transferase exhibited similar responses to ionic strength, temperature, and pH as did the nondelipidated deoxycholate solubilized enzyme, although the reconstituted enzyme displayed a more restricted pH range (7.3-7.6 vs. 7.3-8.2) over which maximum activity was expressed. Ability to reactivate the apoenzyme decreased rapidly upon storage with a half-life of approximately 1 day. Storage in the presence of ethylene glycol markedly stabilized the apoenzyme; however, even in the presence of glycol, capacity of the apoenzyme to be reactivated decayed at a faster rate than did the activity of the fully reconstituted enzyme. Collectively, our results establish the phospholipid requirement of microsomal UDP-glucuronosyltransferase for maximal catalytic activity and implicate the involvement of lipid in the stabilization of the active enzyme.

Since the demonstration by Fleischer and Klouwen (1961) of the lipid dependence of mitochondrial electron transport, many other membrane-bound enzymes, such as glucose-6-phosphatase (Garland & Cori, 1972), cytochrome oxidase (Hinkle et al., 1972), β-hydroxybutyrate dehydrogenase (Nielsen & Fleischer, 1973), cytochrome b_5 reductase (Rogers & Strittmatter, 1973), lipopolysaccharide glycosyltransferases (Rothfield & Romeo, 1971), adenosine triphosphatase (Kimelberg & Papahadjopoulos, 1972; Hilden et al., 1974), and cytochrome P-450 (Lu et al., 1972), have been shown to require association with phospholipid for enzymatic function. A similar characterization of rat liver microsomal UDP-glucuronosyltransferase, *p*-nitrophenol as acceptor, has been hampered due to its inactivation upon membrane disruption. As a result, the bulk of current knowledge is based upon work utilizing particulate, microsomal membrane as a source of transferase activity. A number of investigations have shown that digestion of intact microsomes with phospholipase A or

† From the McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin 53706. *Received May 26, 1978.* Supported by Grants CA-07175 and CA-17300 from the National Cancer Institute. This work was submitted by J.P.G. in partial fulfillment of the Ph.D. degree requirements of the University of Wisconsin, Madison, on August, 1975.

‡ Current address: Department of Molecular Immunology, Scripps Clinic and Research Foundation, La Jolla, California 92037.

0006-2960/78/0417-4600$01.00/0