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The Interaction of Phospholipase A_2 with Micellar Interfaces. The Role of the N-Terminal Region†

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ABSTRACT: The localization of the previously postulated interface recognition site (IRS) in porcine pancreatic phospholipase A_2, required for a specific interaction between the enzyme and organized lipid–water interfaces, was investigated by ultraviolet difference spectroscopy, by measurements of the intrinsic fluorescence of the unique Trp residue, and by protection experiments against specific tryptic hydrolysis. Using the enzymically nondegradable substrate analogues: $C_nH_{(2n+1)}$-O-P(O^-)$OCH_2CH_2N^+(CH_3)_3$-(H,OH), it is shown that the rather hydrophobic N-terminal sequence of the enzyme, viz., Ala-Leu-Trp-Gln-Phe-Arg, is directly involved in the interaction with the lipid–water interface. Besides hydrophobic probably also polar interactions contribute to the binding process. At neutral or acidic pH the presence of a salt bridge between the N-terminal α-NH_3^+ group and a negatively charged side chain stabilizes the interface recognition site and allows the enzyme to penetrate micellar surfaces, even in the absence of metal ion. At alkaline pH, interaction of the enzyme with micellar interfaces requires the presence of Ca^{2+} (or Ba^{2+}) ions.

Pancreatic phospholipase A_2 (EC 3.1.1.4) catalyzes the specific hydrolysis of fatty acid ester bonds at the 2 position of 3-*sn*-phosphoglycerides (de Haas et al., 1968a). The enzyme has an absolute requirement for Ca^{2+} ions which bind in a 1:1 molar ratio to the enzyme molecule. Ba^{2+} and Sr^{2+}, which behave as competitive inhibitors, bind with the same affinity to the enzyme as Ca^{2+} (Pieterson et al., 1974a). The high specificity of the pancreatic enzyme for Ca^{2+} suggests a specific function of the metal ion in catalysis, which is further supported by the close relation between this Ca^{2+} ion and the active site residue His_{53} (Volwerk et al., 1974). Phospholipase A_2 belongs to the class of lipolytic enzymes, which are esterases hydrolyzing in vivo water-insoluble substrate molecules. Although such enzymes are able to hydrolyze substrate molecules present in monomeric dispersion (Roholt and Schlamowitz, 1961; de Haas et al., 1971; Wells, 1972), the same substrate present as an organized lipid–water interface is degraded at a much higher rate.

Pancreatic phospholipase A_2 is known to be secreted by the porcine gland not directly in the active form but as a zymogen (de Haas et al., 1968b) which is not able to hydrolyze substrates organized in lipid–water interfaces. Calcium ions also bind in a 1:1 molar ratio to the zymogen and the affinity of the metal ion to both proteins appears to be the same (Pieterson et al., 1974a). In addition, both proteins bind monomeric substrates with a comparable affinity and they catalyze the hydrolysis of the two-ester function with a comparable efficiency (Pieterson et al., 1974b). These results have been interpreted as follows: both phospholipase A_2 and its zymogen contain a rather similar active site construction and their main difference consists of a specific site, the so-called interface recognition site (IRS),[1] which is present in the enzyme and not in the zymogen.

As was shown before (Abita et al., 1972), during the limited tryptic hydrolysis of the Arg_7–Ala_8 bond which transforms the zymogen into the active enzyme, a small conformational change takes place in which the newly formed protonated α-amino group of the N-terminal alanine forms a salt bridge with a negatively charged side chain, presumably a carboxylate function. The essential role of this salt bridge in stabilizing the structure of a functionally active IRS has been discussed (Pieterson et al., 1974b). This presumably hydrophobic region is thought to interact specifically with certain lipid–water interfaces with a concomitant *optimization* of the active site architecture.

Earlier studies of the enzymatic activity of phospholipase A_2 toward short-chain 1,2-diacyl-*sn*-glycero-3-phosphorylcholines spread as a monolayer led already to the assumption of the existence of a specific site on the enzyme molecule for the recognition of interfaces (Verger et al., 1973). From these experiments it became evident that Ca^{2+} ions

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[1] Abbreviations used are: IRS, interface recognition site; cmc, critical micellar concentration. AMPA, ϵ-amidinated phospholipase A_2; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone.

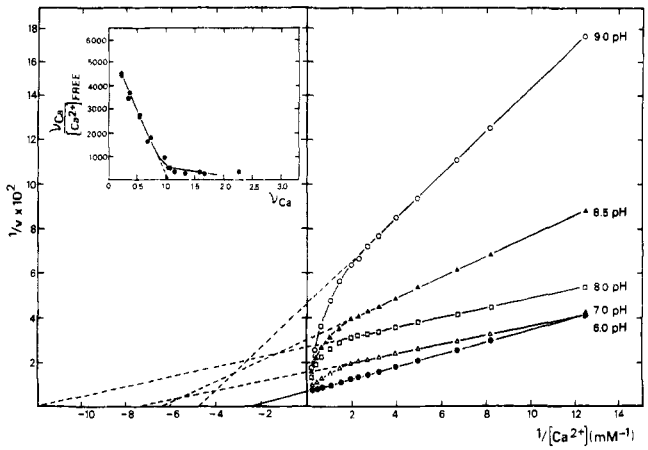


FIGURE 1: Lineweaver-Burk plots of the phospholipase A hydrolysis of 1,2-diheptanoyl-*sn*-glycero-3-phosphorylcholine as a function of Ca^{2+} concentration at different pH values. Assay conditions: 0.5 m*M* Tris or acetate buffer, 0.1 *M* NaCl, and saturating lecithin concentration (13.4 m*M*). The dotted lines represent the extrapolated linear parts of those reciprocals which show at higher Ca^{2+} concentrations a deviation from linearity. The abscissa intercepts of the extrapolated reciprocals give $1/K_{Ca}$ values (apparent) which represent the affinity of the catalytic metal ion site for Ca^{2+}. Inset: Scatchard plot for binding data of phospholipase A_2 and Ca^{2+} at pH 8.8 in the absence of lipid-water interfaces. Values were obtained by equilibrium gel filtration as described earlier (Pieterson et al., 1974a).

have a facilitating influence upon the interaction of the enzyme with lipid-water interfaces at alkaline pH values. A second indication for a functional role of Ca^{2+} ions in the recognition process at alkaline pH was obtained from kinetic experiments with micellar substrates by Pieterson (1973). As is shown in Figure 1, only at pH 6.0 the dependence of the velocity on the Ca^{2+} concentration appears to be a rectangular hyperbola for all Ca^{2+} concentrations. At higher pH values and Ca^{2+} concentrations of about 1 m*M*, deviations from the initially straight Lineweaver-Burk plots become increasingly important and the presence of a second Ca^{2+} binding site in the enzyme which becomes operative at alkaline pH has been suggested. Equilibrium gel filtration experiments of phospholipase A_2 on Sephadex G-25 in the presence of different equilibrating Ca^{2+} concentrations also indicated that at alkaline pH more than one Ca^{2+} ion is bound per enzyme molecule. Compare the Scatchard plot shown in the inset of Figure 1. This paper will provide evidence that the N-terminus region of the phospholipase A molecule is directly involved in the interaction process with micellar lipid-water interfaces. The role of metal ions in this process will be discussed.

Experimental Section

Materials and Methods. Porcine pancreatic phospholipase A_2 and its zymogen were prepared as described previously (Nieuwenhuizen et al., 1974). ε-Amidinated phospholipase A_2 (AMPA) and [Ala⁸,Leu⁹,Phe¹⁰]AMPA were prepared as described in the accompanying paper (Slotboom and de Haas, 1975). L-1-Tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin was obtained from Serva (Germany). The chemicals used were of p.a. quality. All solutions were made in distilled water.

1-Dodecanoyl-*sn*-glycero-3-phosphorylcholine (dodecanoyllysolecithin) was prepared from the corresponding diacyl compound by phospholipase A_2 breakdown. The diacyl compounds were prepared as described previously (Bonsen et al., 1972). *n*-Alkylphosphorylcholines were prepared by phosphorylation of the respective alcohols with 2-bromoethylphosphoric acid dichloride as described by Hirt and Berchtold (1958) and subsequent quaternization with trimethylamine according to the procedure as described by Eibl et al. (1967) and Weltzien and Westphal (1967). The end products were purified by chromatography on silicic acid and their purity was checked by thin-layer chromatography using various solvent systems.

Table I: Monomeric Dissociation Constant of Various Phospholipid–Phospholipase A_2 Complexes at pH 6.0.

Phospholipid	*K* (m*M*)	cmc (m*M*)
1-Decanoyl-*sn*-glycero-3-phosphorylcholine	1.2;[b] 1.4[a]	6
n-Decylphosphorylcholine	1.0[a]	10
1-Dodecanoyl-*sn*-glycero-3-phosphorylcholine	0.2;[a] 0.2[b]	0.4
n-Dodecylphosphorylcholine	0.2;[c] 0.4[b]	1.1

[a] Values obtained from protection against irreversible inhibition (Volwerk et al., 1974). [b] Values obtained from ultraviolet difference spectroscopy (Pieterson, 1973, and this paper). [c] Values obtained from equilibrium gel filtration (Pieterson et al., 1974b).

Protein concentrations were calculated from the absorbance at 280 nm with an $E_{1\,cm}$ (1%) of 13.0 and 12.3 for phospholipase A and its zymogen, respectively. Enzyme activities were determined using the titrimetric assay procedure with egg-yolk lipoproteins as substrate (*Nieuwenhuizen* et al., 1974) unless stated otherwise.

Critical micellar concentrations (cmc) of the phospholipids were determined with the Wilhelmy plate method (Davies and Rideal, 1961). Kinetic experiments were performed with micellar diheptanoyl-*sn*-glycero-3-phosphorylcholine as described previously (de Haas et al., 1971). Gel filtration experiments were carried out as described before (Pieterson et al., 1974a).

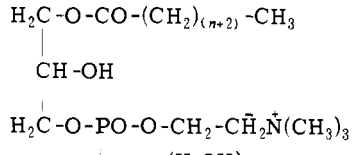
Ultraviolet Difference Spectroscopy. Ultraviolet difference spectra were obtained at 25° by means of a Shimadzu U.V. 200 or a Shimadzu M.P.S. 50L[2] double beam spectrophotometer. Titrations with phospholipids and metal ions were performed directly in the cells using Agla micrometer syringes. The pH was determined before and after each experiment. When the interaction enzyme-phospholipid was studied, tandem cells (2 × 1 cm light path) were used because of the absorption of the phospholipid. Lipid 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. When the enzyme-Ca^{2+} binding was measured, normal cells (1-cm path length) were used. A Ca^{2+} solution was titrated in the sample cell and an equal volume of buffer in the reference cell. Protein concentrations never exceeded 90 μ*M*, which gives an absorbance of about 2.3 at 240 nm. In this concentration range absorbance differences were found to obey Beer's law. Lipid and Ca^{2+} stock solutions had a concentration of about 20 times the estimated dissociation constant. The buffers used in direct binding measurements contained 0.05 *M* Tris and 0.1 *M* NaCl or 0.05 *M* sodium acetate and 0.1 *M* NaCl.

[2] This spectrophotometer equipped with two identical end-on photomultipliers is much less sensitive for light-scattering effects due to lipid dispersions.

Fluorescence Measurements. Fluorescence spectra were measured at 25° with a Perkin-Elmer MPF3 spectrofluorimeter using 1-cm cells and a thermostated cell holder. The setting of excitation and emission slit width was 5 or 7 nm and excitation was performed at 295 nm. Titrations with phospholipids and metal ions were performed directly into the cells. In the fluorimetric titration experiments a 10–15-ml protein solution in 0.001 *M* Tris-acetate buffer containing 0.1 *M* NaCl was titrated with 0.2 or 12 *N* NaOH, depending upon the pH region studied. The pH was continuously measured with a combined electrode. After adjustment of the pH, 3-ml samples were taken and the spectrum was recorded. The fluorescence maxima were accurate to within 0.5 nm of the quoted values.

Results

As has been described earlier (de Haas et al., 1971; Bonsen et al., 1972; Zografi et al., 1971; Verger et al., 1973; Pieterson et al., 1974b), the activity of pancreatic phospholipase A_2 toward substrates organized in a lipid–water interface is very much influenced by the physicochemical parameters of the interface. Therefore, these parameters should be kept constant when the interaction between the enzyme and the lipid–water interface is being measured. So this interaction and the influence of the activator Ca^{2+} hereupon cannot be studied with the natural substrates, the 1,2-diacyl-*sn*-glycero-3-phosphorylcholines (L-α-lecithins), because of the change in the physicochemical state of the interface upon release of products during enzymatic breakdown. At the high enzyme concentrations required in direct binding studies even short-chain 2,3-diacyl-*sn*-glycero-1-phosphorylcholines (D-α-lecithins), which are pure competitive inhibitors of the specific substrates (de Haas et al., 1971), or 1-acyl-*sn*-glycero-3-phosphorylcholines (L-α-lysolecithins, I), which are hydrolysis products of the enzymatic



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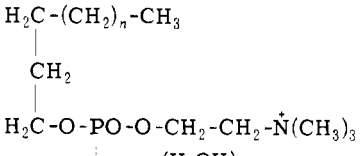

II

reaction, are difficult to handle, because, especially in the presence of Ca^{2+} ions, a slow hydrolysis is often observed. Therefore lysolecithin analogues have been synthesized which are completely resistant to phospholipase A hydrolysis (II, viz., decyl-, dodecyl-, and hexadecylphosphorylcholines, n = 6, 8, and 12, respectively). These lipids gave qualitatively the same ultraviolet difference spectra with the enzyme in the monomeric and micellar region of "substrate" concentrations as lysolecithins (Figure 2) and short-chain diacyllecithins (Pieterson, 1973). From Table I it is clear that also quantitatively the dissociation constants between enzyme and lysolecithin monomers or enzyme and alkylphosphorylcholine monomers are rather similar. Based on equilibrium gel filtration, ultraviolet difference spectroscopy, and protection against irreversible inhibition (Pieterson et al., 1974a; Volwerk et al., 1974), substrate analogues having comparable critical micellar concentration values also show similar affinities to the enzyme (L-decanoyllysolecithin vs. *n*-decylphosphorylcholine; L-dodecanoyllysolecithin vs. *n*-dodecylphosphorylcholine.[3] Moreover, both lysolecithins and *n*-alkylphosphorylcholines in the monomeric region were shown to protect effectively the active site residue His-53 against irreversible modification by active site directed inhibitors in the same way as the pure competitive D-lecithin (Volwerk et al., 1974). Therefore use of these alkylphosphorylcholines in interaction studies with phospholipase A as substitutes for lysolecithins or D-α-lecithins seems to be justified. The rather high cmc values of *n*-dodecylphosphorylcholine makes this lipid very convenient to study monomer interaction. On the other hand, the *n*-hexadecylphosphorylcholine, possessing the extremely low cmc value of about 0.01 m*M*, has been used to study micellar interaction.

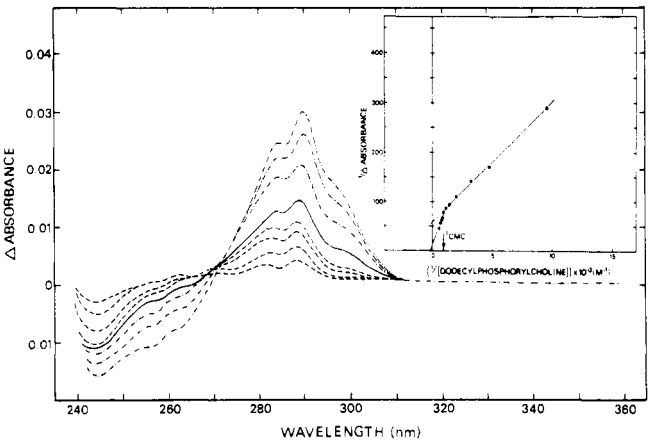

FIGURE 2: Ultraviolet difference spectra produced by the interaction of phospholipase A_2 with increasing amounts of *n*-dodecylphosphorylcholine. Experimental conditions: 0.05 *M* sodium acetate, 0.05 *M* $CaCl_2$, 0.1 *M* NaCl (pH 6.0), and 28 μ*M* phospholipase A. Phospholipid concentrations were: (- - -) 0.105, 0.209, 0.511, and 0.897 m*M*; (—) 1.174 m*M* (cmc = 1.1 m*M*); (----) 1.353, 1.528, and 1.699 m*M*. Inset: Lineweaver–Burk plot for binding date of phospholipase A (28 μ*M*) with *n*-dodecylphosphorylcholine obtained from ultraviolet difference spectroscopy. Δ*A*'s were measured at the absorption maxima.

Ultraviolet Spectroscopy. Figure 2 shows the ultraviolet difference spectra obtained when increasing amounts of *n*-dodecylphosphorylcholine are added to a solution of phospholipase A. Lipid concentrations up to the cmc produce difference spectra in which the absorption is dominated by tyrosine:[4] two positive absorption peaks appear at 282 and 288 nm and another positive absorption peak at about 230

[3] Although the total number of carbon atoms in the lysolecithin is three higher than in the corresponding *n*-alkylphosphorylcholine only a relatively small difference in cmc value is found. This may be explained by the fact that the ester and secondary alcohol function in the lysolecithin confer more polar properties to this lipid.

[4] This conclusion is based on comparative studies with the modified enzyme [Ala[8],Leu[9],Phe[10]]AMPA lacking the single Trp residue and possessing the same affinity constant for monomeric substrates as the native enzyme (Slotboom, and de Haas, 1975). In this case the pure tyrosine difference spectrum obtained on monomer binding displays rather similar absorption peaks and heights as found for the native phospholipase A_2 (Figure 2) and AMPA. Therefore monomer binding to the enzyme only slightly perturbs the Trp environment.

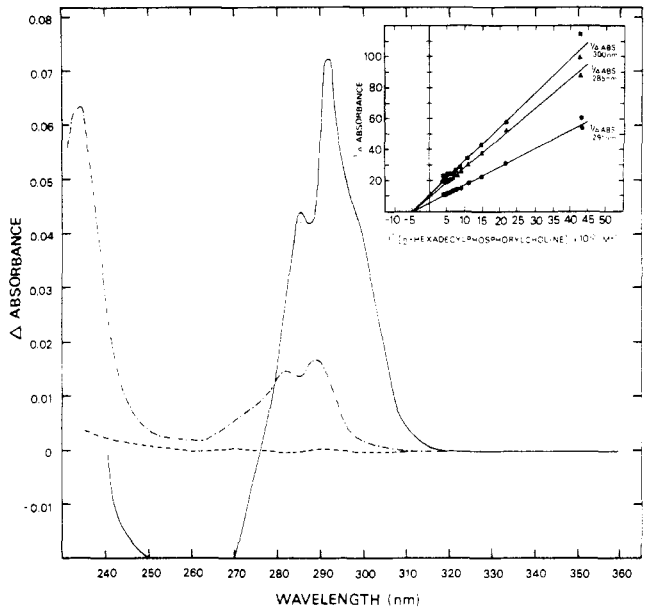

FIGURE 3: Ultraviolet difference spectra produced by the interaction of phospholipase A_2, its zymogen, and [Ala^8,Leu^9,Phe^{10}]AMPA ($E_{1\,cm}$(1%) at 280 nm = 10.1) with *n*-hexadecylphosphorylcholine. Experimental conditions: 0.05 *M* sodium acetate, 0.05 *M* $CaCl_2$, and 0.1 *M* NaCl (pH 6.0). (- - -) 45.0 μM zymogen + 2.0 m*M* phospholipid. (·····) 44.7 μM [Ala^8,Leu^9,Phe^{10}]AMPA + 2.0 m*M* phospholipid; (—) 44.7 μM phospholipase A_2 + 2.0 m*M* phospholipid. ΔA_{288} = 756 cm^{-1} M^{-1} at saturating lipid concentration for [Ala^8,Leu^9,Phe^{10}]AMPA. ΔA_{292} = 3138 cm^{-1} M^{-1} at saturating lipid concentration for phospholipase A and AMPA. Inset: Lineweaver-Burk plots for binding data of phospholipase A with *n*-hexadecylphosphorylcholine at three different wavelengths as obtained from ultraviolet difference spectroscopy.

nm. However, peaks in the 230-nm region cannot easily be ascribed to the perturbation of a particular amino acid residue because their shape is less characteristic than in the 280-nm region and several amino acid residues are known to absorb in the far-ultraviolet. The height of the absorption peaks increases with lipid concentration and the interaction shows a saturation character. As can be seen in Figure 2, when the cmc is passed the difference spectrum produced by the enzyme-lipid interaction suddenly changes. The shoulder around 300 nm on the absorption peak and the trough in the 260-nm region become more pronounced. In addition red shifts of the 282- and 288-nm peaks to 284 and 291 nm, respectively, occur and a disproportional increase of the absorption peaks is observed (cf. inset to Figure 2). These changes in the difference spectrum when the cmc of the phospholipid is passed and which are pH independent are indicative of a change in the microenvironment of the single tryptophan residue in the enzyme (Donovan, 1969; Andrews and Forster, 1972; Strickland et al., 1972). These changes are not seen in similar experiments using [Ala^8, Leu^9,Phe^{10}]AMPA which confirms that the interaction with micellar interfaces perturbs the Trp surrounding. Similar titrations of the zymogen with *n*-dodecylphosphorylcholine in the monomeric region give rise to difference spectra similar to those shown in Figure 2, although the maximal amplitudes of the zymogen peaks are smaller as compared to those of the active enzyme (ΔA_{288} values are 374 and 640, respectively, expressed in cm^{-1} M^{-1} at saturating lipid concentrations). As can be derived from the double reciprocal plot the dissociation constant of the zymogen is larger than that of the enzyme (2.3 and 0.6 m*M*, respectively). Upon passing the cmc, however, the absorption peaks at 282 and 288 nm do not shift to longer wavelengths and the disproportional increase in absorption does not occur. With the zymogen a straight line is observed indicating that the spectroscopic signal corresponds to a single process: the saturation of the monomer binding site. From these observations, it is obvious that with the enzyme a second type of interaction, in addition to monomer binding, starts when phospholipid-water interfaces are formed in the system. This difference between enzyme and zymogen against organized lipid-water interfaces was also found in kinetic and gel filtration experiments (Pieterson et al., 1974b) where it was shown that the enzyme interacts with both monomeric and micellar substrates while the precursor only interacts with monomeric substrates. The apparent dissociation constant for the interaction enzyme-micelles, calculated from the micellar part of the double reciprocal plot for *n*-dodecylphosphorylcholine, has no meaning because of the interference by monomer binding in this system where the monomer concentration is high. Furthermore it should be kept in mind that in calculating the affinity of phospholipase A for lipid-water interfaces, the lipid molecules are no longer monodisperse. So it should not be concluded from the double reciprocal plots that the enzyme shows a weaker affinity toward micelles than it does toward monomers.

In order to study the enzyme-*interface* interaction more carefully, use was made of a higher homologue, viz., *n*-hexadecylphosphorylcholine, which possesses such a low cmc value that the protein lipid monomer binding could be expected not to perturb the protein-interface interaction. As shown in Figure 3, addition of this substrate analogue to a solution of phospholipase A_2 gives an ultraviolet difference spectrum which is very similar to those found with the *n*-dodecylphosphorylcholine in the micellar region (Figure 2). The zymogen, however, notwithstanding its comparable affinity for monomers as the active enzyme, undergoes no perturbation by high concentrations of *n*-hexadecylphosphorylcholine. Therefore it can be concluded that the zymogen has no affinity for micellar lipid-water interfaces which confirms previous results obtained by kinetic studies and equilibrium gel filtration experiments (Pieterson et al., 1974b). Figure 3 also shows the pure tyrosine perturbation spectrum caused by addition of *n*-hexadecylphosphorylcholine to [Ala^8,Leu^9,Phe^{10}]AMPA, an enzyme which possesses a very similar affinity for lipid-water interfaces as native phospholipase A (see Slotboom and de Haas, 1975). From these spectra, obtained with the same molar concentrations of proteins and lipid, it can be concluded that upon micellar interaction with phospholipase A besides Trp perturbation also a change in Tyr environment occurs. Upon titration of phospholipase A_2 with increasing amounts of *n*-hexadecylphosphorylcholine and plotting of the ΔA's as a function of lipid concentration in a double reciprocal way (cf. inset Figure 3) the apparent dissociation constant[5] (K_D) of the enzyme for the micellar interface can be obtained.

In order to get more insight into the identity of the residues involved in the enzyme-interface interaction, the pH dependence of this interaction and the influence of the activator Ca^{2+} hereupon were studied by ultraviolet difference spectroscopy using micellar *n*-hexadecylphosphorylcholine

[5] Expressed in the usual way as millimoles/liter. Notwithstanding the fact that the enzyme binds in a stoichiometrically unknown ratio to micelles, the values obtained will probably reflect rather correctly the dissociation constants for enzyme-micelle binding, because of the proportionality between number of micelles and molar concentration.

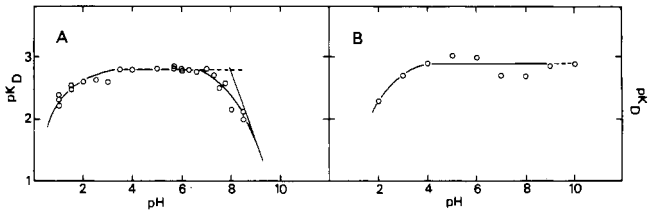

FIGURE 4: Dixon plots of the effect of pH on the interaction of phospholipase A_2 with *n*-hexadecylphosphorylcholine micelles in the absence (A) and presence of 0.05 *M* $CaCl_2$ (B). Experimental conditions: 0.05 *M* sodium acetate or 0.05 *M* Tris–0.1 *M* NaCl. K_D values were obtained from ultraviolet spectroscopy.

as lipid. The spectra show the same characteristics over the whole pH range studied: perturbation of Tyr and Trp absorption although the amplitude of the difference peaks changes somewhat with pH. The pH dependence of the dissociation constants is shown in the Dixon plots of Figure 4A and B in the absence and in the presence of 0.05 *M* $CaCl_2$, respectively. In the acidic range the interaction is weaker than at neutral pH. The p*K* of the group(s) that govern(s) the interaction is (are) around 2, but could not be determined exactly. This change in affinity might be related to the known acidic isomerization which occurs in the enzyme at pH 2.1 (Abita et al., 1972). As could be expected, Ca^{2+} has no influence on the affinity of the protein for lipid–water interfaces at acidic pH, because below pH 4 the affinity of the enzyme for metal ions becomes extremely weak (Pieterson et al., 1974a). At alkaline pH the interaction enzyme–phospholipid interface becomes weaker with increasing pH. In the absence of Ca^{2+} ions, the interaction in the alkaline pH region is governed by the protonation of a group with a p*K* of about 8.1, as is shown in Figure 4A. This value has been determined previously also by gel filtration experiments and was related to the p*K* of the N-terminal α-amino group of the enzyme (Pieterson et al., 1974b; Abita et al., 1972). The presence of Ca^{2+} ions, however, has a distinct influence on the enzyme–lipid interface interaction at alkaline pH values. The dissociation constant for the interaction enzyme–phospholipid interface is not only somewhat smaller, but seems to remain constant up to pH 10 in the presence of 0.05 *M* $CaCl_2$ (Figure 4B).[6]

Fluorescence Spectroscopy. As can be seen in Figure 5 both the enzyme and the zymogen give only a very low increase in quantum yield upon interaction with increasing concentrations of lipid monomers. Apparently, monomer binding to these proteins scarcely perturbs the environment of the Trp residue. This is confirmed by the constant maximum emission wavelength of the fluorescence which is identical with that of the lipid-free proteins (cf. Figure 5). Upon passing the cmc, however, the fluorescence quantum yield produced by the phospholipase A–micelle complex sharply increases while the emission maximum is shifted at the same time to shorter wavelengths. These drastic changes do not appear in the zymogen-lipid system. So from these fluorescence measurements it is clear that upon passing the cmc, the microenvironment of the single Trp residue in the enzyme becomes less polar. When the fluorescence quantum yield is taken as a measure for the enzyme–lipid interface interaction, the same problems are encountered as in

[6] Because ultraviolet spectroscopic studies above pH 9 are hampered by spectral changes due to tyrosine ionization, the values of the dissociation constants obtained between pH 9 and 10 are represented by a dotted line.

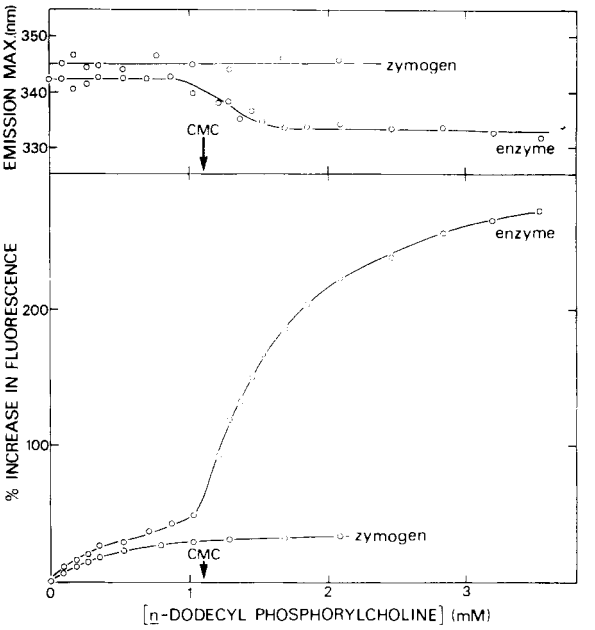

FIGURE 5: Percent increase in fluorescence intensity and shift in the maximal emission wavelength produced upon interaction of phospholipase A_2 (15.1 μM) and its zymogen (15.2 μM) with *n*-dodecylphosphorylcholine. Experimental conditions: 0.05 *M* sodium acetate, 0.05 *M* $CaCl_2$, and 0.1 *M* NaCl (pH 6.0).

the ultraviolet difference spectroscopy above pH 9 because of the strong quenching effect of ionized Tyr residues on the tryptophan fluorescence. The blue shift of the emission maximum, however, is less sensitive to these events and can be followed until higher pH values. Therefore measurement of the shift of the maximal emission makes it possible to study the interaction of phospholipase A with the lipid–water interface and the influence of Ca^{2+} hereupon at pH values above pH 9. When the shift was measured as a function of lipid concentration the dissociation constant could be determined in the same way as described above using ultraviolet spectroscopy. The dissociation constants obtained from such fluorescence experiments are consistent with the values obtained from ultraviolet difference spectroscopy. In Figure 6A the pH dependence of the interaction of phospholipase with the lipid–water interface is shown in the absence and presence of Ca^{2+}. In the absence of Ca^{2+} ions this interaction is governed by the protonation of a group of p*K* about 8.4, ascribed above to the α-NH_2 function. In the presence of increasing amounts of Ca^{2+}, however, the enzyme remains bound to the interface until much higher pH values. In the presence of 40 m*M* $CaCl_2$ the interaction seems to be governed by (a) function(s) having an apparent p*K* above 11.0. This effect appeared to be specific for Ca^{2+} ions. As is shown in Figure 6B, Ba^{2+} ions are also able to keep the enzyme bound to the phospholipid–water interface until pH values higher than 8, but the Ba^{2+} concentrations needed are much higher than the required Ca^{2+} concentration.

Protection by Lipid–Water Interfaces against Tryptic Inactivation of Phospholipase A. The important role of the N-terminal sequence of phospholipase A in the specific interaction of the enzyme with lipid–water interfaces could be confirmed by the following experiments. Under certain conditions it is possible to inactivate phospholipase A by a specific tryptic cleavage of one single peptide bond located in the N-terminal sequence of the enzyme. If the latter region is indeed of fundamental importance for interfacial activity,

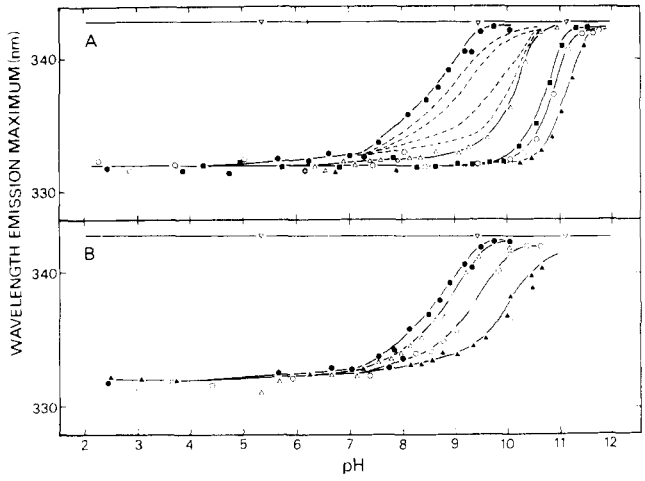

FIGURE 6: Effect of pH on the wavelength of maximal fluorescence emission in the system phospholipase A-*n*-hexadecylphosphorylcholine micelles. Experimental conditions: 10^{-3} *M* Tris, 0.1 *M* NaCl, 14.2 μM phospholipase A, and 9.4 m*M* *n*-hexadecylphosphorylcholine. (A) (▽—▽) Phospholipase A in the absence of lipids and metal ions; (●—●) 5×10^{-3} *M* EDTA; (△—△) 10^{-3} *M* $CaCl_2$; (■—■) 5×10^{-3} *M* $CaCl_2$; (○—○) 10^{-2} *M* $CaCl_2$; (▲—▲) 4×10^{-2} *M* $CaCl_2$. For the sake of clearness, the measurements at Ca^{2+} concentrations below 1 m*M* are given as dotted lines without symbols. From the left to the right these curves have been measured at Ca^{2+} concentrations of 5×10^{-5}, 10^{-4}, 3.5×10^{-4}, and 10^{-4} *M*, respectively. (B) (▽—▽) Phospholipase A in the absence of lipids and metal ions; (●—●) 5×10^{-3} *M* EDTA; (△—△) 10^{-3} *M* $BaCl_2$; (○—○) 10^{-2} *M* $BaCl_2$; (▲—▲) 5×10^{-2} *M* $BaCl_2$.

one might expect that the presence of organized lipid–water interfaces will exert a protecting effect against the tryptic inactivation of phospholipase A. In the accompanying paper (Slotboom and de Haas, 1975) it is shown that when phospholipase A_2, in which all ϵ-amino groups have been amidinated (AMPA) and which shows still 60–70% of the activity of the native enzyme, is submitted to tryptic hydrolysis, only the Arg_{13}–Ser_{14} bond of the polypeptide chain is cleaved. The remaining protein molecule is completely inactive. As can be seen in Table II, lipid monomers, Ca^{2+} ions, or lipid–water interfaces in the absence of metal ions scarcely protect against tryptic hydrolysis at pH 8.9. In the presence of 50 m*M* $CaCl_2$, however, the protection of the lipid–water interface against tryptic inactivation is almost complete. At the same $BaCl_2$ concentration only a very weak protection is observed. These results confirm the spectroscopic evidence discussed above that the enzyme requires Ca^{2+} in order to be able to interact with organized lipid–water interfaces at alkaline pH. In addition they strongly suggest that besides Trp_{10} also Arg_{13} belongs to the interface recognition site.

Discussion

In agreement with previously reported kinetic results (Pieterson et al., 1974b), ultraviolet difference spectroscopy also shows that both the zymogen and the active phospholipase A_2 interact in a similar way with monomeric substrate analogues. Moreover, the kinetically observed specific interaction of phospholipase A_2 with certain organized lipid–water interfaces which does not occur with the zymogen, could be confirmed by ultraviolet spectroscopy. These facts led to the proposal that lipolytic enzymes, in addition to a catalytically active center where monomers are bound and hydrolyzed, possess a topographically distinct region required to interact specifically with lipid–water interfaces.

Table II: Half-Time Values ($t_{1/2}$) for the Tryptic Inactivation of ϵ-Amidinated Phospholipase A_2 at pH 8.9 and the influence of substrate Analogues, Ca^{2+} and Ba^{2+}.[a]

Addition	$t_{1/2}$ (min)
None	13
$CaCl_2$ 5 m*M*	17
$CaCl_2$ 50 m*M*	22
n-Dodecylphosphorylcholine + $CaCl_2$ 50 m*M*	32
n-Hexadecylphosphorylcholine	21
n-Hexadecylphosphorylcholine + $BaCl_2$ 5 m*M*	28
n-Hexadecylphosphorylcholine + $CaCl_2$ 5 m*M*	140
n-Hexadecylphosphorylcholine + $BaCl_2$ 50 m*M*	70
n-Hexadecylphosphorylcholine + $CaCl_2$ 50 m*M*	1500

[a] Incubations were carried out at 25° in 1 ml of Tris buffer (10 m*M*) containing 100 m*M* NaCl, 1 mg of ϵ-amidinated phospholipase A_2, and 0.2 mg of TPCK-trypsin. [*n*-Hexadecylphosphorylcholine], 9.4 m*M*; [*n*-dodecylphosphorylcholine], 0.68 m*M*.

The additional information given by the present spectroscopic techniques deals with the nature of the amino acid residues involved in lipid binding. Monomer binding, known to occur in the vicinity of the active site residue His_{53} (Volwerk et al., 1974) involves one or more tyrosines in both the zymogen and active enzyme. However, the specific interaction with interfaces given only by the active enzyme involves perturbation of the single Trp residue of the polypeptide chain and of one or more tyrosine residues. The fluorescence measurements of the lipid–protein interaction show a large increase in fluorescence quantum yield upon mixing of phospholipase A and micellar lipids. In agreement herewith is the observed considerable blue shift of the maximum emission wavelength from 340 to about 330 nm upon interaction with micelles. Therefore, either the Trp in the phospholipase A–micelle complex is shielded from the aqueous environment because it is buried in a more hydrophobic environment of the enzyme itself, or, and this seems much more probable, the Trp residue forms part of the interface recognition site (IRS) and is buried in the lipid–water interface. Two extreme situations have to be considered. The fluorescence maximum around 332 nm is characteristic of a hydrophobic environment and it might be argued that the α-helical N-terminus region of the enzyme molecule interdigitates between the fatty acyl chains. The fact that very high NaCl concentrations improve the apparent affinity constant of the enzyme for micelles (de Haas et al., 1971) would be compatible with such a hydrophobic interaction. On the other hand, the shape of the ultraviolet difference spectra obtained upon interaction of the enzyme with micellar lipids indicates a polar perturbation of the Trp side chain: polar interactions involving Trp are reported to induce a red shift of the ^1L_a band producing a difference spectrum extending into the 300-nm region and a broad negative band around 260 nm appears (Andrews and Forster, 1972; Strickland et al., 1972). Therefore our results do not allow a definite conclusion about the exact location of the Trp side chain in the interface. Previously discussed direct binding data obtained by equilibrium gel filtration in the absence of Ca^{2+} (Pieterson et al., 1974b) showed that the specific interaction between phospholipase A and lipid–water interfaces is governed by the protonation of a residue having a pK around 8.1. It seems likely that this amino acid residue is identical with the N-terminal Ala_8 of the chain (Janssen et al., 1972) which forms a salt bridge with a buried carboxylate group (Abita et al., 1972). Above pH 8, the

N-terminal α-NH_3^+ group looses its proton, the salt bridge opens, and the interaction with lipid-water interfaces is lost. The results of ultraviolet difference spectroscopy, obtained in the absence of Ca^{2+} and shown in Figure 4A, are in good agreement with the gel filtration data. From Figure 4B, however, it is evident that in the presence of Ca^{2+} ions the enzyme remains able to interact with the micelles until higher pH values. Using the fluorescence data given in Figure 6, it might even be concluded that a functionally active IRS remains intact up until pH values above 10 in the presence of Ca^{2+}. This is in agreement with kinetic data showing a considerable enzyme activity toward micellar substrates at pH 10. One might argue that at these extreme pH values the lipid-water interface becomes somewhat negatively charged and that Ca^{2+} could function as a bridge between the protein and the phosphate groups of the lipid micelle. It should be realized, however, that the Ca^{2+} effect at high pH is rather specific. Although Ba^{2+} ions, which in the absence of lipids bind with the same affinity to the enzyme as Ca^{2+}, seem also able to stabilize the IRS in alkaline medium, the Ba^{2+} concentration required for keeping the enzyme in the interface is much higher. From Figures 6A and B it can be derived that at pH 9.4 the apparent dissociation constant for Ba^{2+} is 50 times higher than that for Ca^{2+} (app. $K_{Ca^{2+}}$ = 0.3 m*M*; app. $K_{Ba^{2+}}$ = 15 m*M*). These results clearly demonstrate that at alkaline pH the enzyme must first bind Ca^{2+} (or Ba^{2+}) before interaction with the lipid-water interface becomes possible and might be explained by assuming that a second metal ion binding site exists on the protein which has a much higher affinity for Ca^{2+} than for Ba^{2+} ion:

$$E + Ca^{2+} \rightleftharpoons E{\cdot}Ca^{2+} + \text{micelles} \rightleftharpoons E{\cdot}Ca^{2+}{\cdot}\text{micelles}$$

$$E + Ba^{2+} \rightleftharpoons E{\cdot}Ba^{2+} + \text{micelles} \rightleftharpoons E{\cdot}Ba^{2+}{\cdot}\text{micelles}$$

Although an additional metal ion binding site on the protein could explain the results obtained by the kinetic and gel filtration experiments (Figure 1), it has not been possible to give direct evidence for the existence of such a site.

Confirmation of the above results was obtained by studying the role of Ca^{2+} in reinforcing the protective effect of lipid-water interfaces against specific tryptic inactivation of phospholipase A_2 at alkaline pH (cf. Table II). The most obvious conclusion therefore is that phospholipase A specifically binds to lipid-water interfaces through its hydrophobic IRS. This site most probably embraces the full N-terminal sequence Ala-Leu-Trp-Gln-Phe-Arg and owes its stability to a salt bridge between the N-terminal α-ammonium group and an unknown acidic side chain.

There are two possible explanations for the stabilizing effect of Ca^{2+} ions. In the first hypothesis the binding of Ca^{2+} causes an effective shielding of the salt bridge in the hydrophobic region of the lipid-water interface. Such an apolar environment would be expected to shift the deprotonation of the α-NH_3^+ group to much higher pH values. Therefore the salt bridge and consequently the IRS would remain intact up until higher pH values. A similar interpretation has been proposed by Bender and Wedler (1967) to explain the activity of chymotrypsin at pH values where the salt bridge in the enzyme in the absence of substrates or inhibitors is deprotonated.

The second hypothesis assumes that, when Ca^{2+} is bound to the enzyme in the presence of lipid-water interfaces, the IRS can remain intact even at pH values when the salt bridge is disrupted. In this hypothesis the Ca^{2+} ion takes over the stabilization of the IRS at high pH values where the α-NH_3^+ group is deprotonated. An analogous explanation has been proposed by Garel and Labouesse (1970, 1973) to understand the activity of chymotrypsin at high pH. Further work is needed to demonstrate which hypothesis is the right one.

Acknowledgments

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Specific Transformations at the N-Terminal Region of Phospholipase A_2†

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ABSTRACT: Treatment of porcine pancreatic prophospholipase A_2 with methyl acetimidate converted all lysine residues into ϵ-acetimidolysine residues. Enzymatically active ϵ-amidinated phospholipase A_2 (AMPA) was obtained from the ϵ-amidinated zymogen by limited tryptic proteolysis cleaving the Arg_7–Ala_8 bond. AMPA was used to prepare des-Ala^8-, des-(Ala^8,Leu^9)- and des-(Ala^8,Leu^9,Trp^{10})-AMPA by successive Edman degradations, and des-(Ala^8–Arg^{13})-AMPA by selective splitting of the Arg_{13}–Ser_{14} bond by trypsin. Structural analogues of AMPA with different N-terminal amino acid residues, viz., D-Ala, β-Ala, and Gly, have been prepared by reacting des-Ala^8-AMPA with the corresponding *N*-*t*-Boc-*N*-hydroxysuccinimide esters of these amino acids. Similarly, the only Trp_{10} residue has been substituted for Phe by coupling of des-(Ala^8,-Leu^9,Trp^{10})-AMPA with *N*-*t*-Boc-L-Ala-L-Leu-L-Phe-*N*-hydroxysuccinimide ester. The feasibility of these substitutions has been proven unambiguously by the retroconversion of des-Ala^8-AMPA and of [Ala^7]AMPA into AMPA having identical enzymatic activity as the starting AMPA. The single Trp_{10} residue in native phospholipase A_2 and its zymogen was specifically sulfenylated using *o*-nitrophenylsulfenyl chloride. The homogeneous proteins were kinetically analyzed using short-chain lecithins in the monomeric and micellar region. All modified AMPA analogues, except those in which two or more of the N-terminal amino acid residues are removed, show enzymatic activities toward monomeric substrate comparable to that of AMPA, indicating that the active site region is still intact. Only [Gly^8]-, [β-Ala^8]-, and [Ala^8,Leu^9,Phe^{10}]AMPA exhibit a dramatic increase in enzymatic activity similar to that of AMPA upon passing the critical micellar concentration (cmc) of the substrate. From these results it can be concluded that the N-terminal region of the enzyme requires a very precise architecture in order to interact with lipid–water interfaces and consequently to display its full enzymatic activity.

Phospholipase A_2 (EC 3.1.1.4) specifically catalyzes the hydrolysis of fatty acid ester bonds at the 2 position of 3-*sn*-phosphoglycerides (van Deenen and de Haas, 1964). Although porcine pancreatic phospholipase A_2 has some activity toward substrates present in monomeric solutions there is a tremendous increase in enzymatic activity when substrate is present as an organized lipid–water interface (de Haas et al., 1971). In contrast, however, prophospholipase A_2, although possessing about 50% of the activity of the enzyme toward monomeric substrate, does not show the increase in enzymatic activity when the substrate concentration passes the critical micellar concentration. This fundamental difference between the zymogen and the active enzyme has been postulated to be due to the presence of an interface recognition site (IRS)[1] in the enzyme which is not present in the zymogen (Verger et al., 1973; Pieterson et al., 1974). This site is supposed to give a specific interaction with certain organized lipid–water interfaces, followed by a conformational change in the enzyme with concomitant optimization of the active site architecture. Furthermore it has been demonstrated that the formation of the IRS is controlled by the protonation of the α-amino group of the N-terminal Ala_8 (Figure 1) having a p*K* value close to 8.1 (Pieterson et al., 1974) and which presumably forms an ion pair with a buried carboxylate group (Abita et al., 1972). In agreement with earlier observations that the IRS is not only functionally but also topographically distinct from the active site (Pieterson et al., 1974), the results of spectroscopic studies and protection against tryptic attack provide strong evidence that the hydrophobic N-terminal part of the enzyme, Ala_8-Leu-Trp-Gln-Phe-Arg_{13} (Figure 1), is directly involved in the IRS (van Dam-Mieras et al., 1975).

In order to delineate further the role of the amino acids involved in the IRS, N-terminally modified and substituted enzyme analogues are required. The main purpose of the present paper is to describe convenient methods to prepare such analogues by various chemical and enzymatic procedures (Figure 2).

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[1] Abbreviations following the IUPAC–IUB Commission on Biochemical Nomenclature recommendations (*Biochemistry 6,* 362 (1967); *Biochemistry 6,* 3287 (1967); *Biochemistry 11,* 1726 (1972)) were used throughout. Other abbreviations used are: *t*-Boc, *tert*-butyloxycarbonyl; Ptc, phenylthiocarbamoyl; NPS, *o*-nitrophenylsulfenyl; Dip-F, diisopropyl phosphorofluoridate; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; IRS, interface recognition site; AMPA, ϵ-amidinated phospholipase A_2; cmc, critical micellar concentration.