Zymogen-Catalyzed Hydrolysis of Monomeric Substrates and the Presence of a Recognition Site for Lipid–Water Interfaces in Phospholipase A_2†

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ABSTRACT: Short-chain lecithins producing clear micellar solutions in water are very effectively degraded by porcine pancreatic phospholipase A (EC 3.1.1.4) whereas the zymogen of this enzyme is not able to attack such interfacial structures. In the present study it is shown that the enzyme also hydrolyzes these lecithins at substrate concentrations where only monomeric species exist though the maximal rate of breakdown amounts to only a few per cent of that observed for the same substrate in micellar form. Such molecularly dispersed lecithins are hydrolyzed by the zymogen as well at a rate of about 50% of that found with the enzyme itself. This indicates that the architecture of the active site in phospholipase A and the zymogen must be very similar. Direct binding experiments using equilibrium gel filtration and difference spectroscopy show that both proteins possess similar affinities for monomeric lecithin molecules. The fundamental difference between phospholipase A and the zymogen becomes evident if the substrate is present in an organized lipid–water interface. Evidence is presented that phospholipase A contains an additional site, a so-called "recognition site," which is not present in the zymogen. This site is essential for interaction with organized structures and its induction during zymogen activation seems to be related to the formation of an ion pair between the newly formed N-terminal amino group and a buried carboxylate. It is shown that the recognition site is functionally and topographically distinct from the monomer binding and catalytic site.

Phospholipase A_2 (EC 3.1.1.4) catalyzes the specific hydrolysis of fatty acid ester bonds at the 2 position of 1,2 diacyl *sn*-phosphoglycerides (de Haas *et al.*, 1968a,b). The protein belongs to the class of lipolytic enzymes, which are esterases hydrolyzing *in vivo* water-insoluble substrates. Although such enzymes are able to hydrolyze substrate molecules present in monomeric dispersion (de Haas *et al.*, 1971; Wells, 1972; Roholt and Schlamowitz, 1961), the same substrate present as an organized lipid–water interface is degraded at a much higher rate. In bulk studies, the tremendous influence on phospholipase activity of a changing lipid–water interface has been demonstrated recently by use of a homologous series of short-chain lecithins as substrate (de Haas *et al.*, 1971; Bonsen *et al.*, 1972b). The fundamental role of certain interfacial parameters (in phospholipase A catalyzed hydrolysis) has been suggested also by monolayer studies (Zografi *et al.*, 1971; Verger *et al.*, 1973), which allow a continuous change of surface concentration and orientation of the substrate at the air–water interface. In this latter study a kinetic model was proposed which describes the action of soluble enzymes on interfaces. As shown in Figure 1, two successive equilibria were supposed to exist, first a rate-limiting, reversible penetration of the enzyme into the interface, followed by the formation of the Michaelis complex.

In this concept, a lipolytic enzyme is presumed to differ from other enzymes by the presence of an additional hydrophobic region, which has been called penetration site[1] which is functionally distinct from the active site. During penetration, the protein is thought to undergo a conformational change (E → E*) in which the optimal alignment of the active site amino acid residues is reached.

Pancreatic phospholipase A 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. The zymogen consists of one single polypeptide chain of 130 amino acids, cross-linked by six disulfide bridges (de Haas *et al.*, 1970a,b). Limited proteolysis by trypsin of the Arg-7·Ala-8 linkage transforms the zymogen molecule into the active enzyme through the release of the N-terminal heptapeptide of the precursor chain.

In recent reports of this laboratory (Pieterson *et al.*, 1974; Volwerk *et al.*, 1974) it is shown that several features of the active site of pancreatic phospholipase A are present also in the zymogen molecule: calcium ions, which are specifically required for catalytic activity, bind in a 1:1 molar ratio to the active enzyme and to the zymogen, producing in both proteins a very similar conformational change. In addition, the affinity of the metal ion to both proteins appeared to be the same. Subsequently, it was found that a single histidine residue in phospholipase A (His-53) could be specifically modified by *p*-bromophenacyl bromide with a stoichiometric loss of enzyme activity. All criteria for active-site modification appeared to be fulfilled. The same histidine residue in the zymogen molecule reacted with a similar rate with this halo ketone producing a not-activable modified zymogen. These results suggest that an important part of the active site of phospholipase A preexists already in the zymogen molecule

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[1] Because the mode of interaction between the enzyme and the lipid–water interface is still unknown, we prefer to call this region in the present study the "recognition site for interfaces."

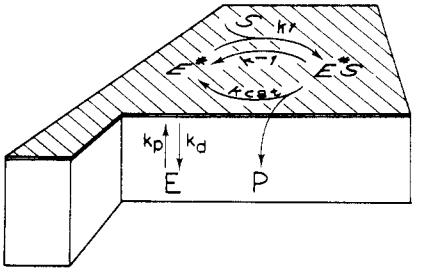

FIGURE 1: Model for the action of a soluble enzyme at an interface.

and the question arises: Is the zymogen of phospholipase A completely unable to degrade phospholipids?

In the present study we will see that the precursor is indeed able to catalyze the hydrolysis of lecithin provided the substrate is in monomeric form. Furthermore, it will be shown that the inertness of the precursor to attack organized lipid–water interfaces can be explained by the absence of a recognition site for interfaces in the zymogen.

Experimental Section

Materials. Porcine pancreatic phospholipase A and the zymogen were obtained as described in the accompanying papers (Pieterson *et al.*, 1974; Volwerk *et al.*, 1974). 3-*sn*-Phosphatidylcholines (L-lecithins) and 1-*sn*-phosphatidylcholines (D-lecithins) containing two identical fatty acids varying in chain length between 6 and 14 carbon atoms were prepared as described previously (Bonsen *et al.*, 1972a). 1-Myristoyl- and 1-octanoyl-*sn*-3-phosphatidylcholines (L-lysolecithins) were prepared from the corresponding diacyl compounds by phospholipase A hydrolysis.

Methods. The assay procedures of the enzyme are given in the accompanying paper (Pieterson *et al.*, 1974). Determination of the critical micelle concentrations was done as described previously (Bonsen *et al.*, 1972b). The equilibrium gel filtration technique on Sephadex G-25 (Hummel and Dreyer, 1962) to measure the dissociation constants between proteins and small ligands was used to determine the affinity of phospholipase A (or the zymogen) for monomeric substrate molecules (Pieterson *et al.*, 1974). A similar technique using Sephadex G-100 allowed the determination of affinity between phospholipase A and micellar lipids which have a Stoke radius much larger than that of the enzyme. Radioactivity measurements were done as described in the accompanying paper (Volwerk *et al.*, 1974). Affinity constants between the proteins and water-soluble short chain substrates were also determined by ultraviolet difference spectroscopy. Spectroscopic data were treated as described in the previous paper (Pieterson *et al.*, 1974). As all lecithin analogs used in this study possess optical densities in the wavelength region of interest, two-compartment, so-called "tandem cells" were used to correct for the contribution of the lipids to the optical density of the protein solution. Phospholipid concentrations were determined by phosphorus analysis according to the Fiske and Subbarow (1925) method, as modified by Bartlett (1945).

Results

Kinetic Studies. The hydrolysis of 1,2-diheptanoyl-*sn*-glycero-3-phosphorylcholine by pancreatic phospholipase A_2 is shown in Figure 2, curve a. In the concentration range where the substrate is present as monomers, normal Michaelis kinetics are found. However, when the substrate concentration reaches the critical micellar concentration (cmc) and aggregation into spherical micelles starts, there is a dramatic increase in enzyme activity. On the other hand, the zymogen (Figure 2, curve b) shows a normal hyperbolic dependence of the hydrolysis rate on the lecithin concentration over the entire substrate concentration range. K_m values for both proteins, acting on monomeric L-diheptanoyllecithin, derived from a double reciprocal plot of the date of Figure 2[2] are included in Table I. V_{max} values are 5 and 2.8 (U/mg) for phospholipase and prophospholipase, respectively. Control experiments were performed in order to investigate the possibility that the zymogen activity was caused by contamination with phospholipase A. On starch gel and polyacrylamide disc electrophoresis at different pH values both proteins

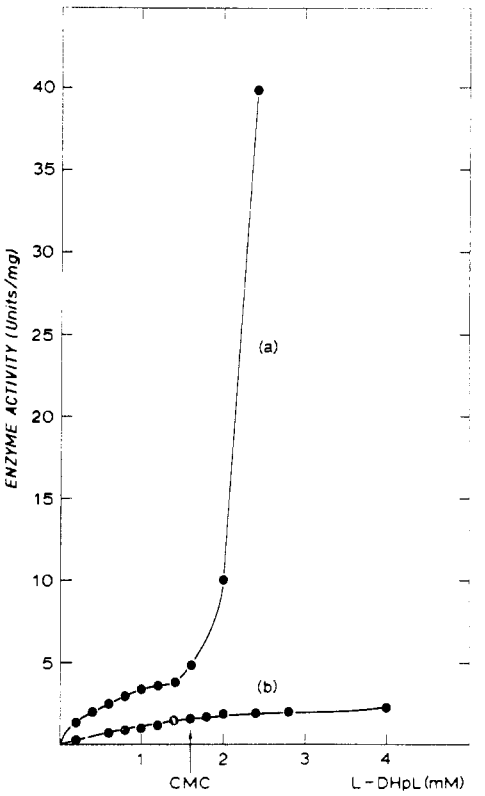

FIGURE 2: Michaelis curves showing the activity of phospholipase A (curve a) and its zymogen (curve b) as a function of diheptanoyllecithin concentration. Assay conditions: 0.5 mM NaAc, 0.1 M NaCl, pH 6.0, 40°. Cmc stands for the critical micelle concentration range.

TABLE I: Dissociation Constants between (Pro-) Phospholipase A and D-Diheptanoyllecithin at pH 6.0.

Measured by	K_s (mM)	
	Prophospholipase A	Phospholipase A
Kinetics[a]	0.9	0.8
Gel filtration	0.7	0.4
Difference spectroscopy	1.5	0.9

[a] In this case an apparent K_m instead of K_s is measured.

[2] Only sub-cmc values were included in this plot.

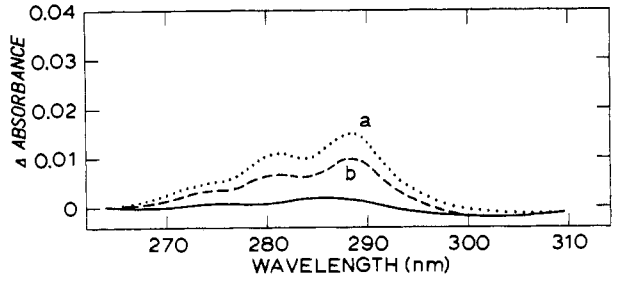

FIGURE 3: Uv absorption difference spectrum of phospholipase A (curve a) and the zymogen (curve b) induced by D-diheptanoyllecithin monomers. Buffer, 0.05 M NaAc–0.1 M NaCl–10^{-3} M EDTA (pH 6.0); protein concentration, 60 μM; D-diheptanoyllecithin concentration, 1.2 mM.

separate completely and no mutual contamination could be detected. An even more sensitive criterion is the enzymatic assay of both proteins using long-chain lecithin dispersions. The specific activity of phospholipase A measured in the egg-yolk lipoprotein system amounts to 1270 whereas the zymogen preparation used in this study possessed a specific activity of only 1.6 (0.13%) under these assay conditions.

Further investigations of the kinetic properties of the zymogen showed that it has an absolute requirement for Ca^{2+}, a pH optimum around 6.0, and that it is unable to hydrolyze the stereoisomeric D-diheptanoyllecithin in agreement with previous reports on the properties of phospholipase A (de Haas *et al.*, 1968, 1971).

Direct Binding Studies with Monomeric Substrate Analogs. The affinity of both proteins for substrate monomers in the absence of Ca^{2+} was studied using ultraviolet difference spectroscopy and equilibrium gel filtration.

As shown in Figure 3, both proteins display a similar difference spectrum with peaks at 282 and 289 nm in the presence of monomeric D-diheptanoyllecithin.[3] From measurements of ΔA at 289 nm as a function of the lecithin concentration, the number of binding sites (n) and the dissociation constants were determined. The spectroscopic data were treated as described in the preceding paper (Pieterson *et al.*, 1974). For both proteins n values close to one were observed. The values for the dissociation constants are included in Table I.

Figure 4 gives a typical elution pattern of phospholipase A from a Sephadex G-25 column equilibrated with a monomeric D-diheptanoyllecithin solution. A similar pattern was obtained with the zymogen. From the equal peak and trough areas in the lecithin elution pattern and the total amount of protein eluted in the break-through volume the dissociation constant was calculated (Pieterson *et al.*, 1974).

Table I summarizes the dissociation constants at pH 6.0 obtained by the different techniques.

Direct Binding Studies with Micellar Substrate Analogs. Direct binding studies were performed in order to test the possibility that the inertness of the zymogen toward micellar substrates is due to the inability of this protein to interact with organized lipid–water interfaces as was suggested in the preceding paper. For this purpose the equilibrium gel filtration technique was chosen. Figure 5 shows the typical elution pattern of a micellar system, containing equimolar amounts of D-didecanoyllecithin and 1-myristoyl-L-lysolecithin.[4] A Sephadex G-100 column was used previously equilibrated with an

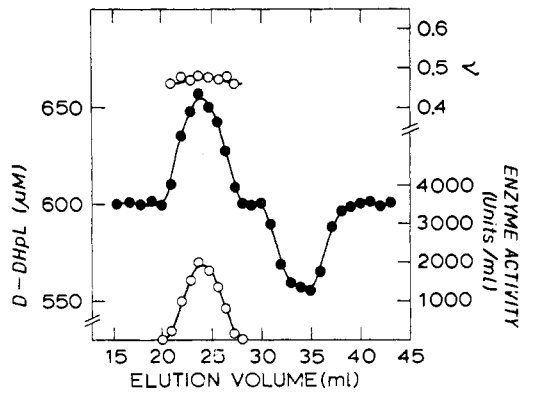

FIGURE 4: Typical elution pattern of 0.3 μmol of phospholipase A on a Sephadex G-25 fine column (50 × 0.6 cm) previously equilibrated with 0.6 mM D-diheptanoyllecithin. Buffer, 0.05 M NaAc–0.1 M NaCl–10^{-4} M EDTA (pH 6.0); (○) D-diheptanoyllecithin; (●) phospholipase A activity. ν represents the number of lecithin molecules bound per molecule of phospholipase A.

equimolar mixture of phospholipase A and prophospholipase A. Apparently an interaction occurs between the lipid micelles and phospholipase A whereas the zymogen has no affinity for the lipid–water interface. From the equal peak and trough areas as well as from the individual fractions in the lipid–protein peak the number of phospholipase A molecules bound per mole of lipid (ν) was calculated. Similar experiments were performed with different phospholipase concentrations in the absence of the zymogen. ν values determined in this manner are plotted according to Scatchard (1949) as shown in Figure 6. A straight line is obtained the slope of which corresponds to a "dissociation constant" K_D of 2.1 (± 0.1) × 10^{-5} M.

In a similar way the dependence of K_D on pH was studied. The results are represented in Figure 7 where K_D is plotted *vs.* pH according to Dixon (1953). Two straight lines with slopes 0 and −1 are obtained intersecting at pH 8.1.

The possibility that a site functionally distinct from the

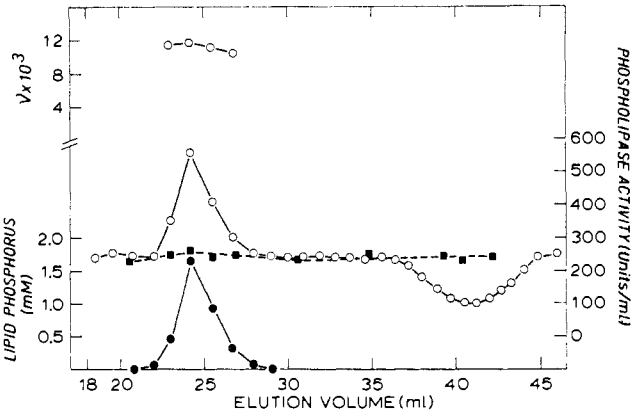

FIGURE 5: Elution pattern of a 3.0-μmol sample of mixed micelles of D-didecanoyllecithin + 1-myristoyl-L-lysolecithin (molar ratio 1:1) on a Sephadex G-100 column (50 × 1.3 cm) previously equilibrated with phospholipase A (1.4 × 10^{-5} M) + prophospholipase A (1.4 × 10^{-5} M): (○—○) phospholipase A; (■- -■) prophospholipase A; (●—●) lipid phosphorus. ν represents the number of phospholipase A molecules bound per molecule of lipid.

[3] This optical antipode had to be used in order to avoid enzymatic breakdown. Previous studies (Bonsen *et al.*, 1972b) have demonstrated that the D compounds are pure competitive inhibitors of the enzyme, characterized by identical affinity constants as the L isomers.

[4] The fact that the physicochemically similar mixture consisting of L-didecanoyllecithin + 1-myristoyl-L-lysolecithin is rapidly hydrolyzed by the pancreatic phospholipase A indicates that these mixed micelles produce a favorable lipid–water interface for interactions with the enzyme.

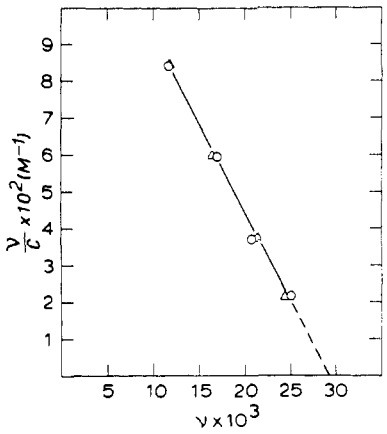

FIGURE 6: Scatchard plot for binding data between phospholipase A and the mixed micellar lipid system consisting of equimolar amounts of D-didecanoyllecithin + 1-myristoyl-L-lysolecithin. ν values were obtained from peak and trough areas of elution profiles as shown in Figure 5 for different free enzyme concentrations (C) ranging from 1.4×10^{-5} to 9.8×10^{-5} M. Buffer conditions, 0.05 M NaAc–0.1 M NaCl–10^{-4} M EDTA (pH 6.0); (Δ—Δ) points derived from peak areas; (O—O) points derived from trough areas.

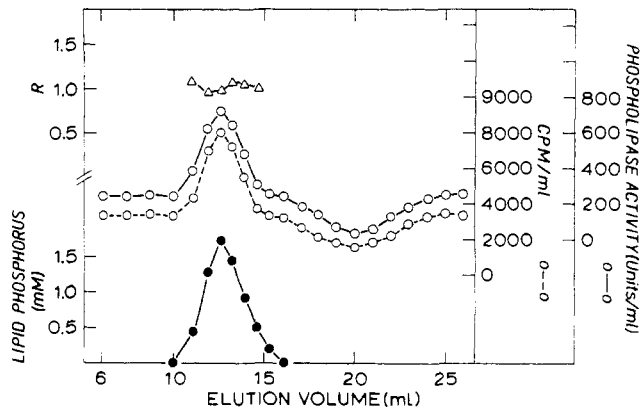

FIGURE 8: Elution pattern of a sample of mixed micelles of D-didecanoyllecithin + 1-myristoyl-L-lysolecithin (molar ratio 1:1) on a Sephadex G-100 column (24 × 1.3 cm) previously equilibrated with phospholipase A (1.4×10^{-6} M) + [^{14}C]-*p*-bromophenacyl bromide inhibited phospholipase A (1.4×10^{-5} M): buffer, 0.05 M NaAc–0.1 M NaCl–10^{-4} M EDTA (pH 6.0); (●—●) lipid phosphorus; (O—O) phospholipase A, measured by enzyme activity; (O- - - - -O) [^{14}C]-*p*-bromophenacyl–phospholipase A measured by radioactivity. R represents the molar ratio of both proteins in the lipid–protein complex eluting at the break-through volume of the column.

active site is involved in the interaction of phospholipase A with lipid–water interfaces was further investigated with the Sephadex equilibrium techniques. An equilibrium gel filtration experiment similar to that described in Figure 5 was performed on a G-100 column equilibrated with an equimolar protein mixture of [^{14}C]-*p*-bromophenacyl bromide inhibited enzyme (compare preceding paper, Volwerk *et al.* (1974)) and native phospholipase A. Analysis of the proteins eluting with the micellar lipid at the break-through volume of the column was performed by measuring radioactivity (^{14}C-labeled inactive enzyme) and by phospholipase A assay. As is evident from Figure 8, both the ^{14}C-labeled inactive protein and the native phospholipase A are able to interact with the micellar lipid–water interface. Moreover, the insert shows that the molar ratio of the complexed proteins $R = 1.0$ (± 0.05) is the same as in the equilibrating protein mixture. Notwithstanding the fact that in the [^{14}C]bromophenacyl-labeled protein the active site is completely destroyed, this experiment shows that the modified and native proteins have a similar capacity to interact with lipid–water interfaces.

Discussion

Roholt and Schlamowitz (1961) and Wells (1972) have demonstrated that phospholipase A from *Crotalus adamanteus* slowly hydrolyzes monomeric short-chain lecithins and that a dramatic increase in enzyme activity occurs when the substrate is present as an organized lipid–water interface. Earlier

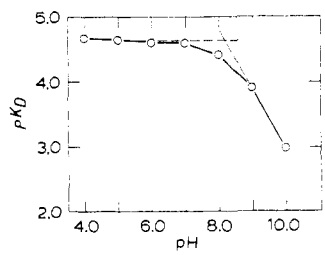

FIGURE 7: Dixon plot of the effect of pH on the interaction between phospholipase A and the mixed micellar lipid system consisting of equimolar amounts of D-didecanoyllecithin + 1-myristoyl-L-lysolecithin. K_D values were calculated from elution patterns as in Figure 5.

reports from our laboratory (de Haas *et al.*, 1971) as well as the results of the present study are in agreement with the findings of these authors: diheptanoyllecithin in molecularly dispersed form is hydrolyzed also by the pancreatic enzyme following Michaelis kinetics.

The zymogen activity on monomeric lecithin substrates is clearly of the phospholipase A_2 type as far as metal ion requirement and stereospecificity are concerned and is in agreement with recent reports on enzymatic activity of the proteolytic precursors (Kassell and Kay, 1973). It has to be remarked, however, that in general the proteolytic zymogens possess activities which are several orders of magnitude lower than those of the corresponding enzymes, whereas the phospholipase A precursor attacks monomeric substrates at a rate comparable to that of the enzyme itself. Moreover, the zymogen and the active enzyme possess a similar affinity to monomeric substrates analogs as was demonstrated by ultraviolet difference spectroscopy and by equilibrium gel filtrations (Table I).

In the preceding papers it was already demonstrated that both proteins bind calcium ions with an identical affinity (Pieterson *et al.*, 1974) and that they are inhibited by *p*-bromophenacyl bromide with similar rates (Volwerk *et al.*, 1974). These results strongly suggest that phospholipase A and its zymogen possess a rather similar architecture of the ligand binding sites and catalytic center. Therefore one might wonder why phospholipase A is secreted by the pancreas in a zymogen form taking into account their similar catalytic activity *vs.* monomeric substrates.

However, the natural substrates of phospholipase A are long-chain phospholipids which are insoluble in water. *In vivo*, they are found either in membranes, in "solubilized" form in the serum as lipoproteins, or as mixed micelles in the intestine together with bile salts; that means they always occur in organized lipid–water interfaces. Taking into account the high specific activity of lipolytic enzymes working on interfaces, it is clear that effective protection against uncontrolled breakdown of interfaces is possible only if the zymogen is unable to interact with organized lipid–water interfaces. Notwithstanding its correctly constructed catalytic

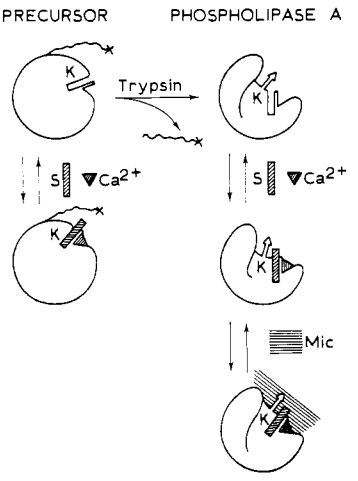

FIGURE 9: Schematic representation of pancreatic phospholipase A and its zymogen, showing the monomer binding site (⊔) and calcium binding site (▽), which together constitute the catalytic region (K). During tryptic activation, the zymogen loses its activation heptapeptide (〰×) and undergoes a conformational change in which an ion pair is formed between the α-NH_3^+ group of the N-terminal alanine and a buried carboxylate. Linked to the formation of this salt bridge is the creation of the recognition site (⌀), for interfaces.

apparatus and binding site for free substrate molecules, the zymogen remains inactive toward its natural substrates because in contrast to phospholipase A it lacks a site required for interaction with lipid–water interfaces. In order to obtain information on amino acid residues involved in this recognition site, the pH dependence of the enzyme–interface interaction was studied. The experiments described in this paper reveal that the interaction with interfaces is controlled by a single amino acid residue with a pK = 8.1 (Figure 7). As was shown before (Abita *et al.*, 1972), during tryptic hydrolysis of the Arg-7·Ala-8 bond in the zymogen, a limited conformational change takes place in which the newly formed protonated α-amino group of the N-terminal alanine forms a salt bridge with a carboxylate. This salt bridge was thought to stabilize the adequate geometry of the active site. Differential proton titration studies (Janssen *et al.*, 1972) and fluorescence measurements (Abita *et al.*, 1972) yielded a pK value of the N-terminal amino function of 8.3 at 25°. Therefore it is highly tempting to suppose that the formation of the salt bridge in phospholipase A triggers the induction of the recognition site for interfaces whereas no direct relation with the active site exists.

The correlation between the salt bridge formation and the presence of a site for interfacial binding is supported by preliminary experiments in which the N-terminal amino group of phospholipase A has been specifically modified. Upon selective transformation of this function into either positive (amidination), negative (citraconylation), or neutral (acetylation) derivatives (Abita *et al.*, 1972), salt bridge formation is no longer possible and the modified proteins completely lose their enzymic activity toward substrates present in an organized lipid–water interface. However, these modifications do not involve the active site and the N-terminal blocked phospholipase A derivatives still possess activities toward monomeric substrates similar to that of the native enzyme (A. J. Slotboom, J. C. Vidal, J. J. Volwerk, and G. H. de Haas, manuscript in preparation). On the other hand, the *p*-bromophenacyl bromide inhibited enzyme, which has lost its capacity to bind Ca^{2+} as well as monomeric substrate analogs, shows an unaltered affinity for lipid–water interfaces in spite of its nonfunctional active site. Therefore, we must conclude that the recognition site for interfaces is not only functionally but also topographically distinct from the monomeric binding region and catalytic site.

The question if part of the apolar, N-terminal region of the enzyme is directly involved in the recognition process or that the creation of the salt bridge and the concomitant conformational change in the enzyme induces the formation of the recognition site in another region of the enzyme molecule remains to be answered. Figure 9 tries to visualize in a schematic way our interpretation of the experimental results discussed in this paper.

In this concept, the enzymatic activity of a lipolytic enzyme such as phospholipase A is largely determined by the physicochemical properties of the interface. Small differences in chemical structure of a substrate are known to produce a different lipid–water interface, interacting in a different way with the recognition site. It is tempting to use this system as a model for the mechanism of action of membrane-bound enzymes. Metabolic activity induces slight alterations in lipid structure and therefore could exert a powerful control of the activity of enzymes linked to the membrane.

Acknowledgments

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A Fluorescence Study of Hybrid Hemoglobins Containing Free Base and Zinc Protoporphyrin IX†

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ABSTRACT: We have studied the fluorescence emission and excitation spectra and fluorescence lifetimes of the following hybrid hemoglobins containing protoporphyrin IX (P) or zinc protoporphyrin IX (ZnP): $\alpha(+)_2\beta(P)_2$, $\alpha(P_2)\beta(+)_2$, $\alpha(ZnP)_2\beta(+)_2$, $\alpha(+)_2\beta(ZnP)_2$, $\alpha(P)_2\beta(ZnP)_2$, and $\alpha(ZnP)_2\beta(P)_2$. The results clearly demonstrate energy transfer between unlike chains with α to β transfer more pronounced than β to α. Changes in the energy transfer rate constant observed on converting the hybrid hemoglobins from oxy to deoxy indicate a change in heme–heme orientation with the change in protein conformation. The change is more pronounced when the oxy to deoxy transition occurs in the β chains.

It has long-been known that free base porphyrins and metalloporphyrins other than heme may be incorporated into the heme binding sites of apohemoglobin (Hill and Holden, 1962; Gibson, 1964; Sebring and Steinhardt, 1970). Similar results have been reported for other heme proteins like myoglobin (Breslow and Koehler, 1965) and cytochrome *c* peroxidase (Yonetani and Asakura, 1968). 8-Anilino-1-naphthalenesulfonate and structurally similar molecules have been the preferred choice of fluorescent dyes for studying dye–protein conjugates because they have a very small fluorescence quantum yield in aqueous solution and quantum yields approaching unity when bound to a hydrophobic protein site. Their emission maxima are usually red shifted from the longest wavelength absorption maxima so there is usually little problem with self-absorption. This is particularly important in fluorescence depolarization studies.

Free base and closed shell metalloporphyrins have a distinct red fluorescence when excited at the Soret or other absorption maxima (Becker and Allison, 1963; Allison and Becker, 1960). The shortest wavelength emission maximum is approximately at the same wavelength as the longest wavelength absorption maximum. Quantum yields are typically 0.05–0.2. The lower quantum yield and approximate coincidence of emission and absorption maxima are disadvantages to the use of fluorescent porphyrins as dye labels for proteins. However, with those heme proteins in which the heme group may be replaced by a fluorescent porphyrin, the above disadvantages are offset by the greatly increased structural similarity between the porphyrin-labeled protein and the naturally occurring heme protein. We report here the results of our fluorescence study of protoporphyrin globin (PHb), zinc protoporphyrin globin (ZnPHb), and hemoglobin hybrids in which the α chains contain heme while the β chains contain protoporphyrin IX (P), or zinc protoporphyrin IX (ZnP). These will be referred to as $\alpha(+)_2\beta(P)_2$ and $\alpha(+)_2\beta(ZnP)_2$, respectively. We have also studied the corresponding hybrids $\alpha(P)_2\beta(+)_2$ and $\alpha(ZnP)_2\beta(+)_2$ in which substitution has occurred in the α chain. The hybrids $\alpha(ZnP)_2\beta(P)_2$ and $\alpha(P)_2\beta(ZnP)_2$ were also prepared and studied.

Experimental Section

Materials and Methods. Protoporphyrin IX dimethyl ester was obtained from Sigma. Free protoporphyrin IX was prepared from the dimethyl ester by acid hydrolysis in 6 N HCl (Falk, 1964). Zinc protoporphyrin IX was prepared from the free base by refluxing 100 mg of free base protoporphyrin and 456 mg of hydrated zinc acetate in 50 ml of dimethylformamide. The reaction was complete within 10 min. The reaction may be monitored by recording visible absorption spectra of aliquots withdrawn from the reaction flask. The materials were chromatographed on $CaCO_3$ and recrystallized.

Aqueous solutions of both porphyrins were prepared by dissolving crystalline material in a minimum volume of 0.1 N NaOH and diluting with water or buffer.

Crystalline human hemoglobin was prepared from citrated whole blood as described previously (Waterman and Yonetani, 1970). Apohemoglobin was prepared from hemoglobin by the acid–butanone method. Hemoglobin subunits ($\alpha(+)$ and $\beta(+)$), apo subunits ($\alpha(-)$ and $\beta(-)$) and semi-hemoglobins ($\alpha(+)_2\beta(-)_2$ and $\alpha(-)_2\beta(+)_2$) were all prepared as described previously (Waterman *et al.*, 1971).

Protoporphyrin IX or zinc protoporphyrin IX was incorporated into apoprotein or semi-hemoglobin by mixing the porphyrin and protein (porphyrin in slight excess) in 50 mM potassium phosphate buffer (pH 7.0) and allowing the solution to stand overnight at 0°.

The hybrids $\alpha(ZnP)_2\beta(P)_2$ and $\alpha(P)_2\beta(ZnP)_2$ were prepared by combining the porphyrins with the apo subunits as de-

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