Photoaffinity Labeling of *Crotalus atrox* Phospholipase A_2 by a Substrate Analogue†

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ABSTRACT: A photolabile analogue of phosphatidylethanolamine (photolabile PE analogue), 1,2-di-*O*-hexylglycero-3-(ethyl diazomalonamidoethyl phosphate), was synthesized in nonisotopic and ^{14}C-radiolabeled form and in both the L configuration (that of the naturally occurring phospholipids) and the racemic form. When the unlabeled racemic compound was photolyzed in the presence of phospholipase A_2 of *Crotalus atrox*, extensive enzyme inactivation was observed. The rate of inactivation was stimulated by Ca^{2+} and by formation of micelles of the photolabile compound. The dihexyl ether analogue of phosphatidylethanolamine protected the enzyme from inactivation. Phospholipase A_2 gave rise to a covalently labeled polypeptide when irradiated in the presence of either L or racemic ^{14}C-labeled photolabile PE analogue. The racemic compound labeled both the N-terminal region (residues 1–15) and the central region (residues 43–97) of the polypeptide while the L compound labeled only the N-terminal region. The lone methionine at position 10 of the *C. atrox* phospholipase A_2 permitted degradation by cyanogen bromide, which showed that labeling by the L compound was restricted to the first ten amino acid residues at the N-terminal end. Phospholipase A_2 has an absolute specificity for L-phospholipids, and D-phospholipids are competitive inhibitors. The results of these studies underscore the importance of the head-group region of the phospholipid in phospholipase–substrate interactions and suggest that the two optical isomers of the substrate may be rather differently oriented on the enzyme surface.

The interaction of lipolytic enzymes with their substrates poses a number of interesting and challenging problems. Because many of the enzymes that cleave lipid molecules are by all criteria normal globular, water-soluble proteins, while their substrates are usually insoluble, or, at most, very sparingly soluble in water, the reaction occurs at the interface between two separate phases. Frequently it can be demonstrated that the reaction rate is markedly enhanced when the substrate concentration reaches the point where soluble, hydrated molecules combine to form a micellar or bilayer phase (Sarda & Desnuelle, 1958; de Haas et al., 1971). A number of proposals have been advanced to explain the rate-enhancing effect of the interface. In the case of pancreatic lipase, for example, it is clear that the enzyme forms an association with the interface and that the association is promoted by the cofactor protein, colipase (Sémériva & Desnuelle, 1979).

In the case of phospholipase A_2, a similar rate enhancement in the presence of an aggregated substrate phase has been clearly demonstrated (Pieterson et al., 1974), but the explanation for the phenomenon remains unclear. One hypothesis suggests that the N-terminal region of the porcine pancreatic enzyme forms an "interfacial recognition site" that associates by virtue of hydrophobic effects with the lipid substrate phase (Verger et al., 1973; Pieterson et al., 1974). Acylation of the charged amino group at the N terminus destroys this association, and this has been explained by postulating the formation of a salt bridge between the N terminus and a buried carboxyl group. Wells (1974) has reported no evidence for penetration into the lipid interface by the *Crotalus adamanteus* venom enzyme, and the crystal structure of the bovine pancreatic enzyme shows no evidence of the putative salt bridge, but instead shows that the N terminus is directed into the active site region (Dijkstra et al., 1978).

Wells (1974) has attributed the rate enhancement, in the case of the *C. adamanteus* phospholipase A_2, to a lower entropy of activation as a result of the high degree of substrate orientation in a bilayer or micelle. Roberts et al. (1977) studied the interaction of the *Naja naja naja* venom phospholipase A_2 with micelles containing the detergent Triton X-100 or mixtures of the detergent and phospholipids. They showed that there was no association between enzyme and detergent micelles while association could be demonstrated when phospholipids were present in the micelle. As in all enzymic reactions involving phospholipase A_2, calcium ion was required for the interaction.

Results of phospholipase hydrolysis of phospholipids in intact erythrocyte membranes (Martin et al., 1975; Adamich & Dennis, 1978) stress the importance of head-group structure in enzymic specificity. Bonsen et al. (1972), using the porcine pancreatic enzyme, have shown that a negative charge in the head-group region is essential for enzymic activity and that the distance between the charged group and the susceptible bond is important for optimal reaction rates.

We felt that some new insights into understanding the interaction of enzyme and substrate might come from studies with photolabile substrate analogues that could, on photolysis, form covalent bonds with amino acids of the enzyme polypeptide chain (Chowdhry & Westheimer, 1979). We chose to examine analogues with such groups in the phospholipid head-group region, partly because this region is some distance from the enzymically cleaved bond and thus might allow definition of a larger area of the interaction between enzyme and substrate. We also wished to test the proposal that the interaction is primarily between enzyme and phospholipid head groups and the ester bonds lying at the interface and not with the methylene groups of the fatty acyl chains. Appropriate derivatives of phospholipids have been prepared by Chakrabarti & Khorana (1975), and it remained for us only to modify these in order to substitute ether for ester bonds to prevent enzymic destruction of the substrate analogue during photolabeling. A preliminary account of these experiments has been published (Huang & Law, 1978).

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Experimental Procedure

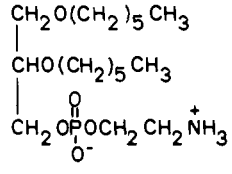
Materials

Silicar CC7 (Mallinckrodt) was used for column chromatography of lipids, while plates coated (0.25 mm) with Merck silica gel 60 or Uniplate silica gel GF (Analtech, Inc., Newark, DE) were used for TLC. Preparative TLC employed 20 × 20 cm plates coated to a thickness of 2 mm with Merck silica gel 60F-254.

Chemicals and solvents were the best available commercial grades. [1-^{14}C]Glycine was obtained from New England Nuclear Corp., Boston, MA. Lyophilized *C. atrox* venom was the product of the Miami Serpentarium, Miami, FL. Chymotrypsin and trypsin were from Worthington Biochemical Corp., Freehold, NJ, while thermolysin was a gift of Dr. Robert L. Heinrikson. Dioctanoyllecithin, used for monolayer rate assays, was from Supelco Co., Bellefonte, PA.

Synthetic Procedures. Infrared spectra were recorded in chloroform, using a Perkin-Elmer Model 137B instrument. NMR spectra were obtained in $CDCl_3$, using either the Varian A-60 or HR 270 instruments, and data are expressed as parts per million downfield from tetramethylsilane. Ultraviolet spectra were recorded with a Cary 15 instrument while optical rotations were obtained with a Rudolph MSP7 spectropolarimeter. Elemental analyses were performed by Micro-Tech Laboratories, Inc., Skokie, IL.

Racemic Photolabile PE Analogue. Synthesis of *rac*-1,2-di-*O*-hexylglycero-3-(ethyl diazomalonamidoethyl phosphate) (photolabile PE analogue, II) employed the procedure of



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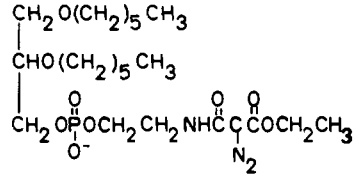

II

Chakrabarti & Khorana (1975), starting with *rac*-1,2-di-*O*-hexylglycero-3-(2-aminoethyl phosphate) (dihexyl-PE, I), prepared by the method of Thomas & Law (1966). This starting material was a white powder that gave a single ninhydrin- and iodine-positive spot, R_f 0.36 ($CHCl_3$:CH_3OH:H_2O, 65:25:4); R_f 0.52 (1-butanol:CH_3COOH:H_2O, 60:20:20); NMR δ 4.10 (t, 2 H, ethanolamine methylene adjacent to phosphate), 3.87 (t, 2 H, glycerol methylene adjacent to phosphate), ~3.50 (m, 9 H, methylenes adjacent to ethers or ethanolamine nitrogen, proton on C-2 of glycerol), 3.14 (broad, 3 H, $^+NH_3$), 1.53 (m, 4 H, methylenes on C-2 of hexyl chains), 1.29 (m, 12 H, remaining methylenes of hexyl chains), 0.98 (t×d, 6 H, CH_3–C). Anal. Calcd for $C_{17}H_{38}NO_6P$: C, 53.3; H, 10.0. Found: C, 52.0; H, 9.74.

After reaction of dihexyl-PE with ethyl diazomalonyl chloride, the product was purified by preparative TLC ($CHCl_3$:CH_3OH:H_2O, 65:25:4); yield 90%. The product gave a single spot on TLC, R_f 0.40 ($CHCl_3$:CH_3OH:H_2O, 65:25:4), R_f 0.61 (1-butanol:CH_3COOH:H_2O, 60:20:20), which was ninhydrin negative, but iodine and molybdic acid positive. It had strong absorbance in the UV [λ_{max} 254 nm (ϵ 4 × 10^3)] and typical amide absorption in the IR.

L *Enantiomer of Photolabile PE Analogue.* 1,2-Di-*O*-hexyl-3-*O*-benzyl-*sn*-glycerol was prepared by the method of Kates et al. (1963). The product was recovered by fractional distillation in vacuo [bp 154–157 °C (0.25 mm)] in 65% yield, based upon the 3-*O*-benzyl-*sn*-glycerol starting material: NMR δ 7.3 (s, 5 H, Ph), 4.55 (s, 2 H, benzyl protons), 3.50 (m, 8 H, methylenes adjacent to ethers), 3.30 (m, 1 H, proton on C-2 of glycerol), 1.50 (m, 4 H), 1.25 (m, 12 H), and 0.85 (t×d, 6 H), hexyl chains. Hydrogenolysis (10% Pd on charcoal) yielded 1,2-di-*O*-hexyl-*sn*-glycerol, which was purified by chromatography on a silicic acid column, from which it was eluted in 20–30% ether in hexane. IR and NMR spectra were identical with those of the racemic compound. The optical rotation was $[\alpha]^{25}_D$ −2.92° (neat).

The 1,2-di-*O*-hexyl-*sn*-glycerol was converted to the L-PE analogue by the method of Hirt & Berchtold (1957) in an overall yield of 50%. The IR and NMR spectra were identical with those of the racemic compound and the optical rotation was $[\alpha]^{25}_D$ +3.95° (benzene).

^{14}C Photolabile PE Analogues. Either the racemic or L-dihexyl analogues were converted to the photolabile PE analogues by the methods described for the unlabeled compounds, except that ethyl [1-^{14}C]diazoacetate was used. This was prepared from [1-^{14}C]glycine, which was converted to the ethyl ester by Fischer esterification. The ester was diazotized by treatment with $NaNO_2$ and the diazoacetate was recovered by extraction into dichloromethane. The crude product was mixed with phosgene and the resulting acid chloride was used to acylate the PE ether analogues. Final specific activities were, for the racemic compound, 0.118 mCi/mmol, and for the L compound, 0.445 mCi/mmol. The chromatographic properties and the UV spectra of the photolabile [^{14}C]PE analogues were identical with those of the unlabeled compounds.

Dibutyroylphosphatidylcholine. The method of Cubero Robles & van den Berg (1969) was used for the preparation of dibutyroyllecithin. The product, purified by chromatography on a silicic acid column (eluted with $CHCl_3$:CH_3OH, 1:4), gave a single spot on TLC, R_f 0.12 ($CHCl_3$:CH_3OH:H_2O, 95:35:6); NMR δ 4.2 (m, 4 H, protons on C-1 of glycerol, on choline methylene adjacent to phosphate), 3.9 (t, 2 H, 3-methylene of glycerol), 3.7 (m, 1 H, C-2 of glycerol), 3.5 (t, 2 H, methylene adjacent to choline nitrogen), 3.1 (s, 9 H, choline methyls), 2.3 (q, 4 H), 1.5 (m, 4 H), 0.9 (t×d, 6 H), butyl chains.

Enzyme. Phospholipase A_2 was prepared from lyophilized *C. atrox* venom by the method of Hachimori et al. (1971). The product was homogeneous as judged by polyacrylamide gel electrophoresis and had properties identical with those reported by Hachimori et al. (1971) and other samples prepared in our laboratory (Cohen et al., 1976). The enzyme was assayed by either the monolayer method (Shen et al., 1975) or the egg yolk emulsion method (Tsao et al., 1973). In some cases, dibutyroyllecithin was used as a substrate, and the evolution of protons was followed with a pH stat (Titrigraph SBR 2c, Radiometer, Copenhagen). Protein concentrations were measured spectrophotometrically; *C. atrox* phospholipase has an extinction coefficient, $\epsilon^{1\%}_{280}$, of 18.3.

Methods

Photolysis Experiments. An aliquot of the photolabile PE analogue in chloroform was transferred to a glass vial and the solvent was removed under a stream of nitrogen. Enzyme in buffer (0.01 M Tris-HCl, 0.1 M NaCl, 7 mM $CaCl_2$, pH 7.6) was added at 25 °C in dim light. The solution was deoxygenated by bubbling a stream of nitrogen through it for 5 min. The sample was then transferred to a 10-mm quartz cuvette or an 8 × 75 mm Pyrex tube maintained at 20–30 °C under N_2. A water-jacketed 450-W medium-pressure mercury lamp with a uranium glass filter was used as a light source. The filter effectively screens all wavelengths shorter than 340 nm. The progress of the photolysis was followed by monitoring the change in absorbance at 252 nm, and the remaining enzyme

Table I: Kinetic Constants for Phospholipase A_2 Catalyzed Hydrolysis of Dibutyroyllecithin at pH 8.0 and 25 °C[a]

K_M(DBL) = 0.035 M	V_m = 3.3 μequiv min/mg of protein
K_i(I) = 0.0021 M	turnover number = 95.7 min^{-1}/molecule
K_i(II) = 0.0015 M	V_m/K_M(DBL) = 49 s^{-1} mol^{-1}

[a] Abbreviations: I, *rac*-1,2-di-*O*-hexylglycero-3-phosphorylethanolamine; II, *rac*-1,2-di-*O*-hexylglycero-3-(ethyl diazomalonamidoethyl phosphate); DBL, dibutyroyllecithin.

activity was monitored by the egg yolk assay method. A control sample lacking photolabile PE analogue was also monitored in each case. In the case of inactivation experiments with nonradioactive photolabile compound, high ratios of ligand to protein were used (100–1000-fold), while radioactive ligands were used in only 10-fold excess, and the extent of enzyme derivitization was correspondingly lower.

Separation of the Photolysis Products. At the end of the photolysis period, the reaction mixture was lyophilized. The protein was then denatured with guanidinium chloride, reduced, S-alkylated with iodoacetamide, and citraconylated as described by Heinrikson et al. (1977). The reaction mixture was passed over a column of Sephadex G-50 (fine) and eluted with 0.1 M *N*-ethylmorpholine acetate, pH 8.8. The polypeptide fraction, which was well separated from the reagents and low molecular weight photolysis products (Huang & Law, 1978), was lyophilized.

Specific Polypeptide Cleavage and Peptide Separation. Tryptic, chymotryptic, and cyanogen bromide peptides were prepared from the derivatized polypeptide or its fragments as described by Heinrikson et al. (1977) or Tsao et al. (1975). Gel permeation chromatography in ammonium bicarbonate and high-voltage paper electrophoresis for peptide separations were carried out by the methods of Heinrikson et al. (1977), as were amino acid analyses and sequence analyses.

Peptide Nomenclature. The peptides derived by the cleavage of phospholipase A_2 are designated by the system proposed by Heinrikson et al. (1977), whereby Tc indicates a peptide derived by trypsin cleavage of the citraconylated protein and each peptide is given a number in the order of its alignment in the polypeptide chain, starting at the N terminus, e.g., Tc 1, Tc 2, etc. Reference to the sequence of the *C. atrox* phospholipase A_2 (Figure 3) shows that there are five such peptides, corresponding exactly to the five derived from the *C. adamanteus* enzyme (Heinrikson et al., 1977). Peptide Tc 3 was further degraded, after decitraconylation, by trypsin. The resulting peptides were given a designation such as Tc 3-T2, which would indicate the second tryptic peptide in the sequence of Tc 3. Cyanogen bromide peptides, of which there are only two, are similarly designated.

Results

Inhibition of Phospholipase A_2 by Substrate Analogues. The dihexyl ether analogue of phosphatidylethanolamine (I) and its *N*-(ethyldiazomalonyl) derivative (photolabile PE analogue, II) were tested, without photolysis, for inhibition of the *C. atrox* phospholipase A_2, using the water soluble substrate, dibutyroylphosphatidylcholine (Wells, 1972). Critical micellar concentrations for these two analogues were estimated to be about 1 mM and 2.6 mM, respectively, using the surface tension method (Lagocki et al., 1976). Therefore, we kept both substrate and inhibitor concentrations low to prevent formation of mixed micelles. A large extrapolation was therefore necessary in the double reciprocal plots (Figure 1), but the value obtained for K_M for dibutyroyllecithin agrees well with that obtained by Wells (1972) (Table I). The two

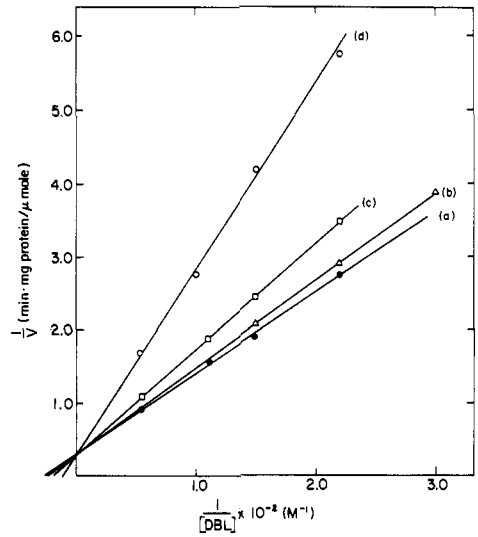

FIGURE 1: Competitive inhibition by substrate analogues. Double-reciprocal plots of velocity as a function of dibutyroyllecithin concentration for the reaction catalyzed by phospholipase A_2: (a) no additives; (b) dihexyl-PE, 0.40 mM; (c) dihexyl-PE, 0.80 mM; (d) racemic photolabile PE analogue, 1.86 mM.

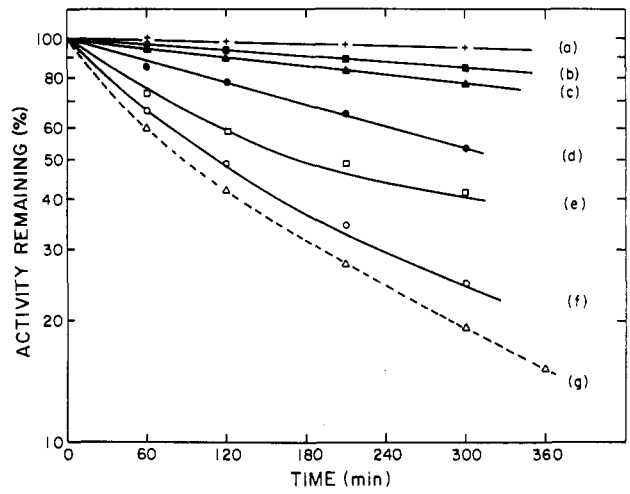

FIGURE 2: Inactivation of phospholipase A_2 by racemic photolabile PE analogue. All reactions were conducted in 0.01 M Tris-HCl, 0.1 M NaCl, 7 mM $CaCl_2$, pH 7.6, 2×10^{-6} M enzyme. (a) Enzyme alone under photolysis conditions; (b) 1.44 mM inhibitor; (d) 2.26 mM inhibitor; (e) 3 mM inhibitor; (f) 4 mM inhibitor; (g) % 252 nm absorbance remaining.

ether analogues appear to compete quite effectively for the active site of the enzyme.

Photoaffinity Labeling of Phospholipase A_2. Preliminary experiments showed that irradiation of the enzyme alone at 250 nm quickly destroyed activity. Therefore, it was necessary to use the longer wavelength absorption of the photolabile PE analogue in order to prevent photoinactivation of the enzyme. This proved to be an advantage, for the analogue photolyzed very slowly, allowing enzyme molecules to bind to substrate analogues many times during the photolysis and increasing the chances that they would react with carbenes generated in the photolysis reaction. Indeed, it was possible to achieve more than 75% inactivation of the enzyme before the reagent was completely destroyed (Figure 2). Enzyme in the absence of photolabile PE analogue was nearly unaffected by photolysis (Figure 2), and it was shown that if the PE analogue was photolyzed alone and then added to the enzyme, no inhibition resulted. From the initial portions of the curves shown in Figure 2, pseudo-first-order rate constants for inactivation could be calculated, and these were used to explore the pa-

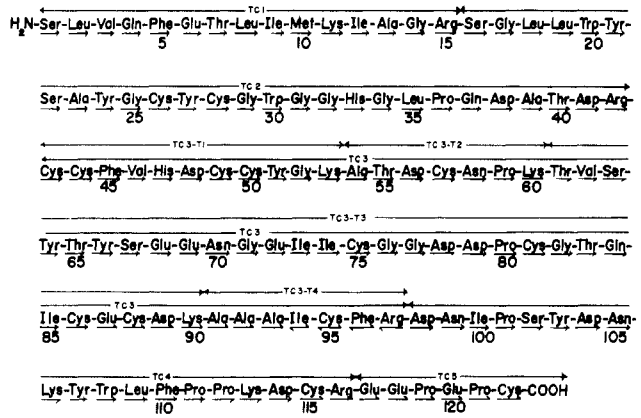

FIGURE 3: Primary sequence of *C. atrox* phospholipase A_2, showing peptides resulting from tryptic cleavage of the citraconylated enzyme (Tc peptides) as well as the tryptic peptides derived from decitraconylated Tc 3 (Tc 3-T peptides). Reprinted with permission from Randolph et al., 1980. Copyright 1980, Elsevier North-Holland.

rameters of the photolytic inhibition process.

As in the case of enzyme substrates, the photolytic inactivation of phospholipase A_2 by the photolabile PE analogue was dependent on calcium ion, but not absolutely so: addition of Ca^{2+} increased the rate 5-fold at the optimum of 8 mM. Also in accord with the enhanced rate observed when substrates pass from monomolecular solution to micelles or bilayers, the photoinactivation showed an anomalous concentration dependence, with an increase of 20-fold near the cmc.

Covalent Attachment of the Racemic ^{14}C Photolabile PE Analogue. *C. atrox* phospholipase A_2 was treated with a 10-fold molar excess of *rac*-[^{14}C]PE analogue and irradiated above 340 nm for 3 h. At the end of the irradiation, the protein was denatured with guanidinium chloride, reduced, carboxamidomethylated, and citraconylated. Gel permeation chromatography separated the polypeptides, both labeled and unlabeled, from other photolysis products and residual PE analogue. As reported earlier (Huang & Law, 1978), the amount of peptide modified during the photolysis was about 40% under these conditions, and the ratio between the loss of enzymatic activity and covalent attachment of the PE analogue was close to 1:1, indicating a high degree of specific labeling. Several attempts were made to separate the derivitized from underivitized polypeptides by gel permeation chromatography, ion-exchange chromatography, or electrophoresis, but these were not successful. Therefore, subsequent characterizations were made with a mixture containing approximately 40% covalently modified polypeptide.

In a control experiment, a mixture of labeled and unlabeled photolabile PE analogue was photolyzed under identical conditions, but in the absence of enzyme. At the end of the photolysis, the mixture was divided into two portions, and phospholipase A_2 was added to one of these. The reaction mixtures were processed as described above and chromatographed on Sephadex G-50. No radioactive compounds were found in the elution volume of the protein, indicating that the photolysis products alone neither are eluted from the column in the same place as the enzyme nor form stable complexes with the protein.

Tryptic Peptides. The amino acid sequence of the *C. atrox* phospholipase A_2 (Randolph et al., 1980) is shown in Figure 3. Trypsin cleavage of the carboxamidomethylated and citraconylated polypeptides recovered from the photolysis yielded a mixture of peptides that were separated by gel permeation chromatography on Sephadex G-50, according to Heinrikson et al. (1977). The elution pattern, shown in Figure 4, is similar

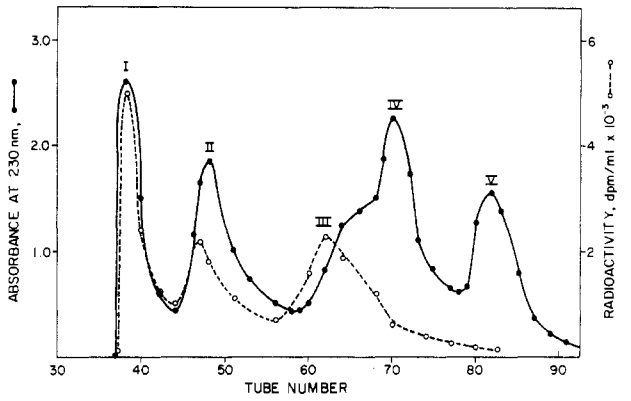

FIGURE 4: Gel permeation chromatography of peptides resulting from tryptic cleavage of reduced, carboxamidomethylated, citraconylated, ^{14}C-labeled phospholipase A_2 produced by photoinactivation with racemic ^{14}C-labeled photolabile PE analogue. The sample (10 mg in 1.5 mL) was digested with trypsin (1% by weight) in 0.2 M *N*-ethylmorpholine acetate buffer, pH 8.0, for 4 h at 37 °C. The digest was applied to a column of Sephadex G-50, superfine (1.0 × 180 cm), and eluted with 0.1 M NH_4HCO_2. Flow rate 12 mL/h; fractions, 1.8 mL each.

to that obtained in the course of the sequence determination of the *C. atrox* enzyme (R. L. Heinrikson, personal communication), and it is also similar to the elution pattern of tryptic peptides obtained from the *C. adamanteus* enzyme (Heinrikson et al., 1977), for the Arg–Pro bond, present in the Tc 3 peptide of the *C. adamanteus* protein, but replaced by a Leu–Pro bond in the *C. atrox* protein, is not cleaved by trypsin.

Fewer peaks were obtained in the case of the *C. atrox* phospholipase, in part because of poorer resolution on the gel permeation chromatography column. The order of elution was the same as that observed by Heinrikson et al. (1977), however, and therefore peak I represents a difficult-to-resolve complex of Tc 1 and Tc 2; peak II represents Tc 3; peak III, Tc 1 and a mixture of other peptides; peak IV, Tc 4 and Tc 5; and peak V, pure Tc 2. It was therefore concluded that Tc 2, Tc 4, and Tc 5 were not radioactive, while Tc 1 and Tc 3 were radioactive, and that the ratio of radioactivity in the two peptides was Tc 1/Tc 3 $\simeq$ 4.

The identities of the peptides were verified by further resolution by high-voltage electrophoresis at pH 2.1 (Heinrikson et al., 1977) and amino acid analysis (Table II). Resolution of peak I material was attempted by several methods but was unsuccessful or resulted in unacceptable losses of material. We considered that peak I might represent a single peptide comprising Tc 1 and Tc 2 resulting from failure to cleave the Arg-15–Ser-16 bond. However, when this material was subjected to automated Edman degradation, the amino acid derivatives corresponding to both peptides were obtained. It is clear, however, that no label is associated with the Tc 2 portion of this mixture, for peak V, which is devoid of radioactivity, corresponds reasonably well to Tc 2 in composition (Table II).

Covalent Incorporation of Label by Photolysis of the L-*PE Photolabile Analogue in the Presence of Phospholipase A_2.* Because of the relatively low incorporation of radioactivity into Tc 3 as compared to Tc 1, we postulated that Tc 1 was labeled by the L-substrate analogue which corresponds to the natural substrate isomer, while Tc 3 was labeled by the unnatural D-substrate analogue, which would be bound to the enzymes in a different orientation and perhaps less firmly. This was borne out by experiments with the L-substrate analogue. The Tc peptides produced from the polypeptide derivatized by photoaffinity labeling with the radioactive L analogue are shown in Figure 5. Now only two significant radioactive fractions were obtained, corresponding to peak I and peak III.

Table II: Amino Acid Composition of Tryptic Peptides of Photoaffinity Modified Protein Separated by Sephadex G-50 Chromatography[a] and by Paper Electrophoresis at pH 2.1[b]

amino acid	peak I,[a] spot a[b] exptl	peak I, spot a expected[c] (Tc 1 + Tc 2)	peak II, spot a exptl	peak II, spot a expected (Tc 3)	peak III, spot a exptl	peak III, spot a expected (Tc 1)
lysine	1.0 (1)	1	2.5 (2)	3	0.8 (1)	1
arginine	1.9 (2)	2	0.8 (1)	1	0.8 (1)	1
histidine	1.0 (1)	1	1.2 (1)	1		
asparatic acid + asparagine	2.2 (2)	2	>6.1 (>6)	7	0.8 (1)	
threonine	1.9 (2)	2	3.6 (4)	4	0.9 (1)	1
serine	2.8 (3)	3	2.0 (2)	2	1.0 (1)	1
glutamic acid + glutamine	3.0 (3)	3	5.0 (5)	5	2.1 (2)	2
proline	1.2 (1)	1	2.0 (2)	2		
glycine	>5.3 (>5)	7	5.4 (5)	5	1.4 (1)	1
alanine	3.0 (3)	3	3.3 (3)	4	1.0 (1)	1
carboxamidomethylated cysteine	1.7 (2)	2	2.3 (2)	10		
valine	0.7 (1)	1	1.8 (2)	2	0.6 (1)	1
methionine	0.9 (2)	1			0.6 (1)	1
isoleucine	1.9 (2)	2	2.8 (3)	4	1.5 (2)	2
leucine	>4.7 (5)	5			2.0 (2)	2
tyrosine	3.4 (3)	3	2.7 (3)	3		
phenylalanine	1.3 (1)	1	1.5 (2)	2	0.8 (1)	1
tryptophan	ND	2	ND			
sequence		1-15 16-42		43-97		1-15

amino acid	peak III, spot b exptl	peak III, spot b expected[d]	peak IV, spot a exptl	peak IV, spot a expected (Tc 5)	peak IV, spot b exptl	peak IV, spot b expected (Tc 4)	peak V, spot a exptl	peak V, spot a expected (Tc 2)
lysine	2.6 (3)				1.8 (2)	2	0.6 (1)	
arginine	1.2 (1)				0.4	1	1.3 (1)	1
histidine	1.0 (1)						1.0 (1)	1
asparatic acid + asparagine	>3.4 (>3)				5.1 (5)	5	2.1 (2)	2
threonine	2.4 (2)						0.7 (1)	1
serine	2.2 (2)				1.7 (2)	1	2.0 (2)	2
glutamic acid + glutamine	3.4 (3)		3.3 (3)	3			0.8 (1)	1
proline	3.0 (3)		1.8 (2)	2	2.9 (3)	3	1.1 (1)	1
glycine	3.5 (4)				1.6 (2)		4.6 (5)	6
alanine	2.4 (2)				0.7 (1)		2.5 (2)	2
carboxamidomethylated cysteine	0.3		0.6 (1)	1	0.4	1	2.3 (2)	2
valine	2.0 (2)							
methionine	0.8 (1)							
isoleucine	2.8 (3)				1.1 (1)	1		
leucine	2.9 (3)				1.2 (1)	1	2.3 (2)	3
tyrosine	2.3 (2)				1.9 (2)	2	2.0 (2)	3
phenylalanine	2.0 (2)				1.2 (1)	1		
tryptophan					ND	1	ND	2
sequence				117-122		98-116		16-42

[a] See the elution profile depicted in Figure 4. [b] Separated by the method of Heinrikson et al. (1977). [c] Based on sequence data. [d] This composition does not correspond to a pure tryptic peptide.

A small amount of label associated with peak II may represent some D compound formed as a result of racemization in the course of synthesis. It seems likely, therefore, that the L compound labels only Tc 1, the N-terminal peptide consisting of 15 amino acid residues, while the D compound of the racemate is responsible for the lesser amount of radioactivity in peptide Tc 3.

Further Degradation of Radioactive Tc 3 from the Reaction with Racemic Photolabile Analogue. Peptide from peak II (Tc 3), obtained by gel permeation chromatography of the Tc peptides resulting from trypsin digestion of the derivatized polypeptide reacted with racemic photolabile PE analogue (Figure 4), was decitraconylated and again cleaved by trypsin. Chromatography of the resulting peptides on Sephadex G-25 gave two radioactive peaks corresponding to Tc 3T3 (residues 61–90) and Tc 3T1 (residues 43–53). The identities were confirmed by amino acid analysis. Further cleavage of Tc 3T1 by chymotrypsin and Tc 3T3 by thermolysin showed that the radioactivity was located in peptides representing residues 43–45 and 65–84, respectively.

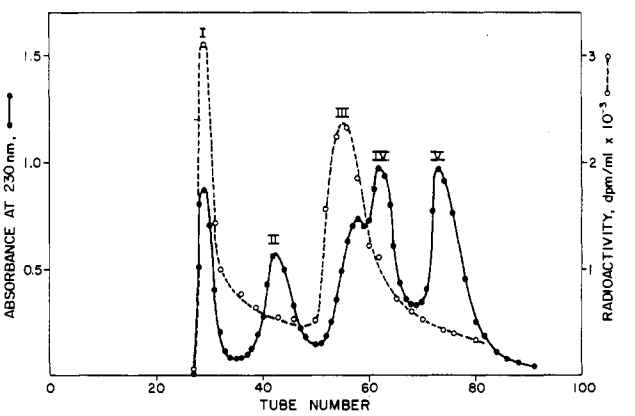

FIGURE 5: Gel permeation chromatography of peptides resulting from tryptic cleavage of reduced, carboxamidomethylated, citraconylated, ^{14}C-labeled phospholipase A_2 produced by photoinactivation with L-^{14}C-labeled PE analogue. Conditions as in Figure 4.

Further Characterization of the Product Obtained with the L Photolabile PE Analogue. Phospholipase A_2 reacted with

Table III: Amino Acid Composition of the Peptides Obtained after Cyanogen Bromide Fragmentation of *C. atrox* Phospholipase A_2 Modified by Photolysis of L Photolabile PE Analogue

	peptides			
	CB I		CB II	
amino acid	exptl	expected	exptl	expected
lysine			5.3 (5)	6
arginine			3.9 (4)	4
histidine			2.0 (2)	2
aspartic acid + asparagine	0.2		13.6 (14)	14
threonine	0.9 (1)	1	4.9 (5)	5
serine	0.9 (1)	1	4.6 (5)	5
glutamic acid + glutamine	2.1 (2)	2	9.0 (9)	9
proline			7.5 (8)	8
glycine			11.4 (11)	12
alanine			6.5 (6)	7
carboxamidomethylated cysteine			10.2 (10)	14
valine	0.67 (1)	1	2.0 (2)	2
methionine			0.2	0
isoleucine	1.0 (1)	1	5.7 (6)	6
leucine	2.1 (2)	2	4.6 (5)	4
tyrosine			8.0 (8)	8
phenylalanine	1.0 (1)	1	3.5 (4)	3
homoserine and homoserine lactone	0.6 (1)	1		
recovery of radioactivity	35%		35%	
sequence		1–10		11–122

L-^{14}C-labeled photolabile PE analogue was reduced and carboxamidomethylated and then subjected to cleavage by cyanogen bromide. The single methionine residue at position 10 resulted in a single large peptide and a decapeptide (Tsao et al., 1975). Radioactivity was equally distributed between the two peaks observed on gel permeation chromatography. The second peak represented CB I, corresponding to residues 1–9 plus homoserine, as shown by amino acid analysis (Table III). The first peak appeared to be a mixture of CB II plus intact polypeptide, the result of incomplete cleavage and the fact that some methionine residues had been oxidized during the photolysis and extensive derivatization. This was confirmed by trypsin cleavage after reduction, carboxamidomethylation, and citraconylation. All of the radioactive label was recovered in peptide Tc 1.

It is concluded from these results that the major site of labeling of phospholipase A_2 by the L-diazoethylmalonyl-PE analogue is within the first ten amino acid residues from the N-terminal end.

Discussion

Substrate analogues of phosphatidylethanolamine having ether groups and modified head groups appear to compete with substrate for the active site of the phospholipase A_2. The photolabile analogue can be covalently attached to the polypeptide in the course of photolysis. The rate of covalent modification is enhanced by calcium ion and by aggregation of the substrate analogue into a micellar form. This is in accord with the observations of Rock & Snyder (1975), who showed that calcium ion was necessary for the binding of *C. adamanteus* phospholipase A_2 to an affinity column with an ether analogue of a phospholipid as a bound ligand. It is also consistent with the rate enhancement seen as substrates form aggregated phases (Pieterson et al., 1974).

By use of a radioactive photolabile substrate analogue, covalent attachment yields a labeled polypeptide. Precise location of the site of covalent attachment has been hampered by a number of factors. The tendency for a portion of the tryptic peptides representing residues 1–15 and 16–42 to aggregate in some way (Heinrikson et al., 1977) led to the appearance of radioactivity in two main peptide fractions. It is clear, however, that only the N-terminal peptide, residues 1–15, had any significant radioactive label when the L photolabile analogue was used. Further specification of the location of the labeled ligand has resulted from the isolation of the short CNBr peptide, residues 1–10, which was labeled. Thus, the radioactive ligand was linked to one of the first ten amino acid residues.

We attempted to degrade either peptide Tc 1 (residues 1–15) or CB I (residues 1–10) by automated Edman degradation. Good yields of the phenylthiohydantoin derivative of serine and leucine were obtained in the first two cycles, but the yield of valine and subsequent residues was sharply diminished. However, appreciable amounts of ^{14}C were detected in every fraction, indicating a dissolution of the radioactive peptide, which should be rather hydrophobic in character, by the solvents during the run.

We also attempted to isolate a labeled amino acid after complete hydrolysis of the protein with 6 N HCl. A water-soluble product was obtained in low yield. On two-dimensional TLC it separated from the known amino acids in the area where dicarboxylic acids were found. We interpret the low yield in part to a loss of half the label in the decarboxylation of a malonic acid derivative in the course of hydrolysis. Insufficient product was obtained for further characterization.

Although no definitive evidence is available, it is tempting to suggest that the valine residue at position 3 is the site of modification. The analysis of the CB I peptide (Table III) shows good values for all residues except valine. If 40% of the polypeptides contained a modified valine, this result would be expected. Valine-3 lies sufficiently close to the charged N-terminal serine residue that the phospholipid head group could span the distance, so that the N-terminal serine would form an ion pair with the head-group phosphate charge. This idea is consistent with the recent demonstration that for the bovine pancreas enzyme the N-terminal portion of the polypeptide points into the active site (Dijkstra et al., 1978).

The fact that the racemic analogue labeles peptide Tc 3 to a much greater extent than does the L compound suggests a different mode of head-group binding for the D compound than for the L compound. It is known that D-phosphatides are competitive inhibitors of action on the L substrates in the case of the porcine pancreatic enzyme (Bonsen et al., 1972) and also that the enzyme binds to D-substrate micelles (Pieterson et al., 1974). We interpret the fact that significant labeling of Tc 3 was obtained only with the racemic analogue to indicate that the head-group region of the D compound lies across the active site in a very different way than does the analogue of the natural L substrate. If indeed the binding of the head group is markedly different for the two isomers, the role of the head group in the enzymic reaction is of more importance than previously believed.

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Dilatometry and Calorimetry of Saturated Phosphatidylethanolamine Dispersions†

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ABSTRACT: The specific volumes of a series of saturated phosphatidylethanolamine dispersions with 12, 14, and 16 carbon atoms per chain have been measured in the region of the chain melting transition, T_m. The change in specific volume at T_m for the 12 and 14 carbon compounds are 0.0160 and 0.0204 mL/g, respectively. Comparisons are drawn between this class of lipids and phosphatidylcholines. In both cases, T_m extrapolates with increasing chain length to the melting point of polyethylene. Both types of lipids appear to be packed in a similar way below T_m. One major difference is that dilaurylphosphatidylethanolamine undergoes a second transition above T_m.

In an earlier paper, we showed that the combination of calorimetry and density measurements with elementary theoretical considerations can lead to a more detailed molecular picture of the main phase transition of phosphatidylcholine bilayers (Nagle & Wilkinson, 1978). We now extend these measurements and this type of analysis to another major phospholipid group—the phosphatidylethanolamines (PE).[1] While highly sensitive calorimetry of saturated PE's has been done already (Mabrey & Sturtevant, 1978), no previous report of specific volume data has appeared.

The second aspect of this work involves additional polymorphism of the saturated phosphatidylethanolamines. It has been known for some time that well-defined reversible bilayer to hexagonal (H_{II}) phase transitions occur in certain unsaturated phosphatidylethanolamines (Cullis & de Kruijff, 1976). Such additional transitions, however, have not been found in saturated PE's (Cullis & de Kruijff, 1978). Since such transitions have a possible role of importance to play in membrane functions such as fusion (Cullis & Hope, 1978), it is worthwhile to see if there are conditions under which additional phases form. The stability of such phases in the presence of lipids which remain in the bilayer phase is also a question of importance to answer.

Materials and Methods

The phospholipids used in this study were obtained from Calbiochem and were not further purified. The best measure of purity is the narrowness of the phase transition. The widths

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[1] Abbreviations used: PE, phosphatidylethanolamines; DLPE, dilaurylphosphatidylethanolamine; DMPE, dimyristoylphosphatidylethanolamine; DPPE, dipalmitoylphosphatidylethanolamine; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine.

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