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Synthesis of phospholipids containing photoactivatable carbene precursors in the headgroups and their crosslinking with membrane proteins

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Many integral membrane enzymes require for their activity interactions with the polar headgroups of phospholipids, in addition to the hydrophobic interactions within the lipid bilayer. The interactions with the polar headgroups may have preferential or absolute specificity. To study such interactions, phospholipids have been synthesized which carry photoactivable moieties in their headgroups. Three types of phospholipids, PL-I, PL-II and PL-III, were synthesized. The synthetic phospholipids, PL-I and PL-II were able to reconstitute enzymatic activity of the membrane proteins which were studied. Covalent crosslinking between these phospholipids and the membrane proteins was demonstrated after photolysis of the reconstituted phospholipid-protein complexes.

Introduction

Studies of protein-lipid interactions are important to the understanding of the structural and functional properties of biological membranes. To study such interactions and how they modulate membrane function, a number of physical and chemical approaches has been proposed. One general chemical approach to this problem involves labeling of membrane proteins through the use of diffusable molecules that carry photoactivatable groups [1]. This allows mapping of hydrophobic domains of membrane-bound proteins. A more specific approach to the study of phospholipid-protein interactions is to attach photoactivatable groups to the fatty acyl chains of phospholipids [2]. Membrane proteins are reconstituted with these phospholipids into vesicles and upon photolysis, highly reactive intermediates are formed which crosslink with the polypeptide chains. Investigation of the sites of these crosslinks has provided insights into the structures of the membrane-embedded portions of certain membrane proteins. This unique approach has also allowed the localization of polypeptide chains to one or both leaflets of the lipid bilayer [3–5]. Thus, photoactivatable phospholipid analogues can provide information on the structure of membrane proteins within the hydrocarbon region of the membrane.

Frequently, membrane proteins display, in addition, interactions with the polar headgroups of phospholipids. Such interactions show specificity

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Abbreviations: DCC, *N*,*N*′-dicyclohexylcarbodiimide; DMAP, 4-(*N*,*N*-dimethylamino)pyridine; DOPC, dioleoylphosphatidylcholine.

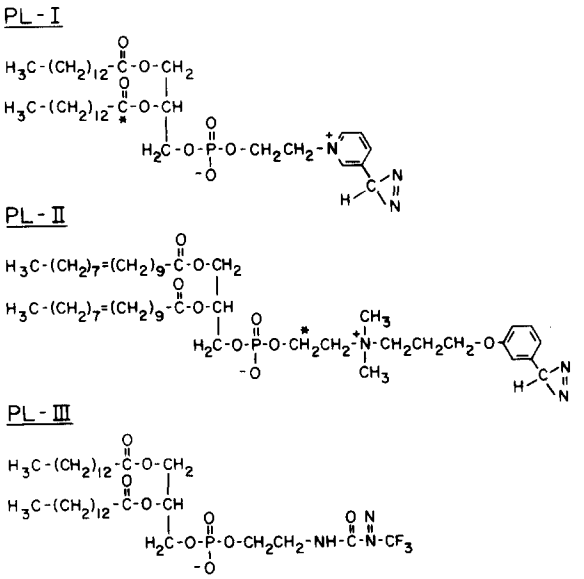

Fig. 1. The structures of the three phospholipid analogues synthesized. The asterisk (*) denotes the position of the radioactive tracer in PL-I and PL-II.

which may be absolute or preferential [6]. In order to study such interactions, we undertook to prepare a series of phospholipids that carry photoactivatable carbene precursors in the polar headgroups (Fig. 1). In designing such syntheses, it was clearly important to bear in mind the steric requirements of the active sites of the enzymes for the polar headgroup. In this paper, we report on the synthesis of the three types of phospholipids that are shown in Figs. 2–4. The diazirine-containing phospholipids PL-I and PL-II, allowed completely successful reconstitution of enzymatic functions that we studied. We also report on initial experiments on photoinduced crosslinking between these synthetic phosphatidylcholine analogues and three different membrane proteins.

Procedures

Materials

L-α-Glycerophosphorylcholine was obtained from Calbiochem. 4-(*N*,*N*-Dimethylamino)pyridine (DMAP, recrystallized from chloroform/diethyl ether, m.p. 112°C), pyridine-3-carboxaldehyde, *N*,*N'*-dicyclohexylcarbodiimide (DCC), ethylene oxide, dibromopropane, dibromoethane, *m*-hydroxybenzaldehyde and 2,4,6-triisopropylbenzenesulfonyl chloride (recrystallized from pentane, m.p., 95°C) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Myristic acid (recrystallized from acetone) was obtained from J.T. Baker Chemical Co. (Phillipsberg, NJ) and *tert*-butyl hypochloride was from Frinton Laboratories (South Vineland, NJ). Hydroxylamine-*O*-sulfonic acid was from Ventron. Monochlorodimethyl ether was obtained from Pfaltz and Bauer, Inc. Crude rattlesnake venom (*Crotalus adamanteus*), from Ross Allen Reptile Farm (Florida) was used as the source of phospholipase A_2. Phospholipase D (cabbage), 120 units/mg were obtained from Sigma Chemical Co. (St. Louis, MO). [^{14}C]Palmitic acid was obtained from Amersham (Arlington Heights, IL). Sephadex LH-20 and LH-60 were obtained from Pharmacia and Rexyn I-300 resin was obtained from Fisher Scientific.

Delipidated bacteriorhodopsin prepared as described [7] was the gift of Dr. K.S. Huang. Delipidated β-hydroxybutyrate dehydrogenase was obtained from bovine heart mitochondria according to the procedure of Burnett and Khorana [8]. Cytochrome b_5 was isolated from the livers of New Zealand white rabbits [9]. Synthetic phospholipids were either synthesized (see below) or obtained from Avanti Polar Lipids (Birmingham, AL). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphorylcholine (POPC) was obtained from Calbiochem. Cholic acid was recrystallized from aqueous acetone and then dried under vacuum.

Methods

Reconstitution of delipidated bacteriorhodopsin into phospholipid vesicles containing reactive phosphatidylcholine analogues. The reconstitution of bacteriorhodopsin into phospholipid vesicles followed the procedure of Huang et al. [7]. Phospholipids (3.5 mg) in CH_2Cl_2 were dried under a stream of N_2, dissolved in ether, and dried once more under N_2. A high vacuum pump (0.2 mmHg) removed any residual solvent. To the dried lipid was added 0.4 ml of 2% sodium cholate in 0.15 M KCl at pH 8.0. The mixture was sonicated until clear, and then rapidly mixed with delipidated bacteriorhodopsin (0.4 ml of 0.01 M Tris-HCl/0.15 M NaCl/0.25% deoxycholic acid/0.025% NaN_3

at pH 8.0 containing 0.19 mg of protein poer ml) at 4 C. The clear, purple solution was immediately dialyzed against 0.01 M Tris-HCl(pH 8.0)/0.15 M NaCl/0.025% NaN_3 at room temperature for two days (two 1-liter changes per day) and then dialyzed for one more day against 0.15 M NaCl/ 0.025% NaN_3 (two 1-liter changes).

Proton translocation. Bacteriorhodopsin-phospholipid reconstituted vesicle systems were assyed for proton pumping activity using the procedure detailed by Racker and Stockenius [10]. Pumping experiments employed 2 ml of 2 M NaCl as the assay solution, and proton pumping rates and extents were measured after calibration with 1 M HCl.

Reconstitution of delipidated β-hydroxybutyrate dehydrogenase with phospholipids. Reconstitution of β-hydroxybutyrate dehydrogenase was done as described [8].

Reconstitution of cytochrome b_5 into phospholipid vesicles. Cytochrome b_5 was reconstituted into phospholipid vesicles by the cholate-gel filtration method [4].

Photolysis of vesicles. An Oriel 1000 watt Hg-Xe arc lamp (Model 8540 equipped with 977B-lamp, Oriel Corp., Stamford, CT) was used as the light source. The light beam was passed through a monochromator (Model 7240, Oriel) and for photolysis at 366 nm, the light beam was also passed through two filters (WG345 and WG360, Schott Optical, Duryea, PA). Samples were irradiated under N_2.

Analytical methods. Gel permeation chromatography was performed on Sephadex LH-60 columns as described in figure legends. Soduum dodecyl sulfate-polyacrylamide gel electrophoresis was performed using the procedure of O'Farrell [12].

Results

Synthesis of the phospholipids (PL-I, PL-II and PL-III) containing photoactivatable carbene precursors in the headgroup

General method for the synthesis of phospholipids

Fatty acid anhydrides were made by the method of Selinger and Lapidot [13]. The cadmium chloride adduct of *sn*-glycero-3-phosphorylcholine was acylated with fatty acid anhydrides in freshly distilled chloroform using 4-(*N*,*N*-dimethylamino)pyridine as a catalyst [14]. After removal of the cadmium chloride and the catalyst using a column of Rexyn I-300 resin, the product was purified by either silica gel plates using methylene chloride/methanol/water (65 : 25 : 4, v/v) or Sephadex LH-20 column chromatography using methylene chloride/methanol (1 : 1, v/v), followed by filtration as a solution in methylene chloride through a sintered-glass funnel. Glycero-3-phosphoric acid was prepared by phospholipase D catalyzed hydrolysis of *sn*-glycero-3-phosphorylcholine. Five hundred mg of *sn*-glycero-3-phosphorylcholine were dissolved in 25 ml of ether. A solution of cabbage phospholipase D (50 mg) in 90 ml of 17 mM sodium acetate (pH 5.6) and 20 ml of 25 mM calcium chloride were then added. The flask was sealed and stirred at 25–30°C for 8 h under nitrogen. The organic layer was removed, the aqueous layer extracted three times with 20 ml of diethyl ether, and the organic layers were combined. The organic layer was then extracted with an equal volume of 1M HCl and the solvent removed. The phosphatidic acid was purified by silica gel column chromatography.

Synthesis of 3-pyridyl-3H-diazirine (IV), (Fig. 2). The procedure for the synthesis of 3-pyridyl-3H-diazirine (IV) is basically that of Smith and Knowles [15], with some modifications in the methods of purifications. Freshly distilled pyridine-3-carboxaldehyde (I) (17.2 g, 0.16 mol) was dissolved in *tert*-butylamine (30 ml, 0.29 mol) and allowed to stand at room temperature for 3–4 h. It is important to have the pyridine-3-carboxaldehyde (I) free of contaminating 3-cyanopyridine. Addition of anhydrous benzene followed by solvent removal at moderate vacuum gave a light yellow oil. This was dissolved in absolute ethanol (100 ml), triethylamine (40 ml), and water (40 ml). After cooling to −10°C, hydroxylamine-*O*-sulfonic acid (24 g, 0.21 mol) was added over 30 min with vigorous stirring, and stirring continued for an additional 2 h. *tert*-Butyl hypochlorite (24 ml, 0.21 mol) was added over 30 min and the solution stirred at 4°C overnight. After the addition of water (100 ml), sodium bicarbonate was added to bring the pH to 7. The product was extracted into methylene chloride (4 × 100 ml),

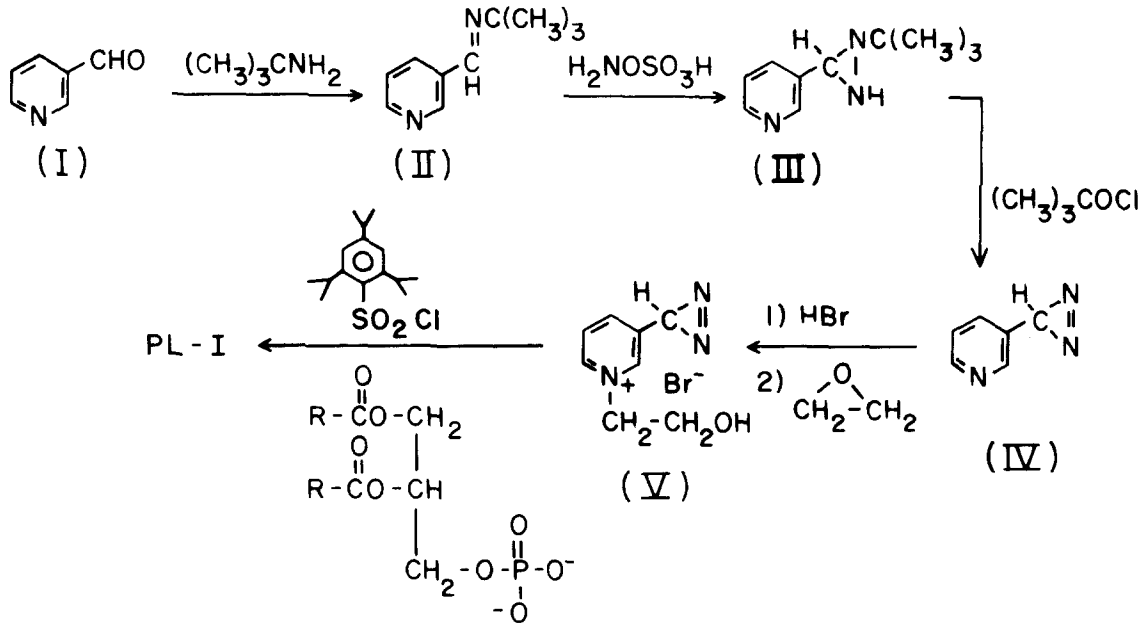

Fig. 2. Synthesis of PL-I. Details of the synthesis are presented in the text.

and the product purified on silicic acid columns using gradients of acetone in petroleum ether, followed by a second column using ethyl ether in methylene chloride. This was then purified by HPLC on a Waters microporasil column (0.7 × 30 cm) using methylene chloride/acetonitrile (7 : 3, v/v). Infrared spectroscopy (neat): 1595 cm^{-1} (N=N); ultraviolet (cyclohexane): $\lambda_{max} = 344$ nm (ϵ-220); NMR (CCl_4): 2.07 (1H,s, diazirine), 6.75 (2H, d, 3-H), 8.43 (2H, d, 2-H). The yield was 15–20% after purification. Caution should be exercised as these compounds are rather volatile and can cause skin irritations.

N-β-Hydroxyethyl-3-diazirinoidinium bromide (V). For the quaternization of the 3-diazirinopyridine (IV), equimolar amounts of 48% HBr and the diazirinopyridine (350 mg) were mixed at −20°C and the solvent removed at room temperature under high vacuum. Water (2 ml) was added, followed by ethylene oxide (4 mol excess, 0.65 ml). A solid CO_2/acetone cold finger was attached and the solution stirred at room temperature 4–5 h. After the solvent was removed, the product was crystallized from ethanol/ether. NMR (D_2O): 2.8 (1H, s, diazirine), 4.1 (2H, d), 4.7 (2H, d), 7.7 (2H, d), 8.8 (2H, d). Ultraviolet (water): $\lambda_{max} = 331$ nm. Alternatively and with lower yields, alkylation was performed with iodothanol in acetonitrile or acetone.

Synthesis of N,N-dimethyl-N-propyl-3-phenoxy(m-diazirino)choline (IX) (Fig. 3)

m-(Hydroxy)phenyldiazirine (VII). The procedure of the synthesis of *m*-(hydroxy)phenyldiazirine is described in detail elsewhere [14].

(m-Diazirino)phenoxypropyl bromide (VIII). *m*-(Hydroxy)phenyldiazirine (VII, 110 mg, 0.8 mmol) was dissolved in 5 ml of anhydrous methanol and then dried. The material was redissolved in 5 ml of anhydrous methanol and was then treated with a solution of sodium methoxide (24 mg, 1.0 mmol) in 2 ml of methanol (2 ml). After standing for 5 min, the methanol was removed by rotary evaporation and the residue dried under a vacuum. The yellow solid was dissolved in 2 ml absolute ethanol, to which was added dibromopropane (0.5 g, 2.5 mmol). The reaction mixture was sealed in a test tube and heated at 37 C for 16 h. Diethyl ether (1 ml) was added to precipitate the NaBr formed in the reaction. The tube was centrifuged and the supernate was then evaporated under vacuum. The crude

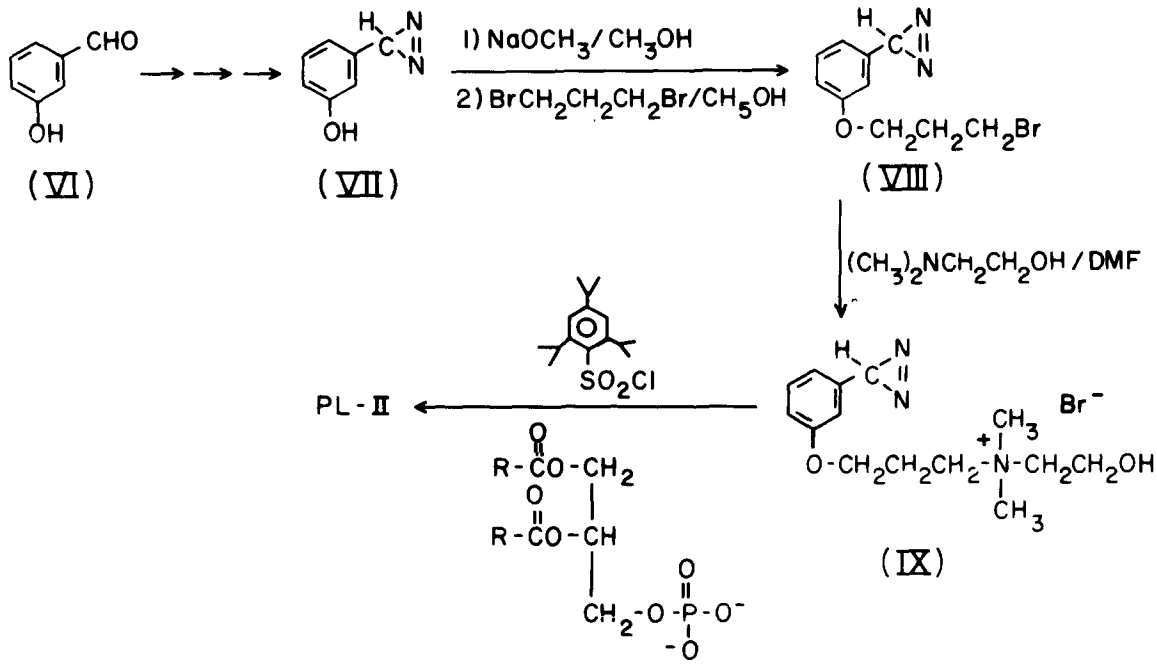

Fig. 3. Synthesis of PL-II. Details of the synthesis are presented in the text.

product was dissolved in 0.5 ml ethanol, and purified by HPLC on silica gel column (DuPont semi-prep) equilibrated in 2.5% diethyl ether/hexane. The yield was 60% (0.5 mmol). Infrared spectroscopy: 1590 cm^{-1} (N=N); ultraviolet (cyclohexane): λ_{max} 358 ($\epsilon = 270$).

N,N-Dimethyl-N-propyl-3-phenoxy(m-diazirino) choline (IX). To the diazirinophenoxypropyl bromide (VIII, 0.5 mmol, 100 mg) in anhydrous dimethylforamide (0.5 ml), *N,N*-diisopropylethylamine (0.5 mmol, 60μl), and dimethylethanolamine (0.6 mmol, 50 μl) were added. The reaction was allowed to proceed for 4 h at room temperature. The reaction was monitored by paper chromatography on Whatman No. 1 (*n*-butanol/2-chloroethanol/ammonia/water (50 : 15 : 5 : 22.5, v/v)), with the desired product having an R_F of 0.8. The compound can be visualized with ultraviolet light. Since it also contains a quaternary ammonium ion, it can also be visualized by spraying with Dragendorf's reagent. The solvents were removed, the solid washed with diethyl ether, and the choline analogue crystallized in ethanol with a trace of diethyl ether. The yield was 80% (0.4 mmol, 130 mg). Ultraviolet: λ_{max} 360 ($\epsilon = 268$); infrared spectroscopy: 1590 cm^{-1} (N=N), 3500 cm^{-1} (-OH).

Condensation of N-β-hydroxyethyl-3-diazirinopyridinium bromide (V) or N,N-dimethyl-N-propyl-3-phenoxy(m-diazirino)choline (IX) with sn-glycero-3-phosphoric acid (Figs. 2 and 3)

sn-Glycero-3-phosphoric acid (0.2 mmol) was dried by the repeated addition and evaporation of dry pyridine after mixing with *N*-β-hydroxyethyl-3-diazirinopyridinium bromide (0.2 mmol) or *N,N*-dimethyl-*N*-propyl-3-phenoxy(*m*-diazirino) choline (0.2 mmol). The solid was dissolved in anydrous pyridine (1 ml) and 2,4,6-triisopropylbenzene sulfonylchloride (0.4 mmol) was added. The solution was flushed with nitrogen and then stirred for 5–6 h in a dry box. The reaction was monitored by silica gel TLC in chloroform/methanol/ammonia (75 : 25 : 4, v/v), with the PC analogue having an R_F of 0.3 and phosphatidic acid an R_F of 0. The reaction was stopped by first cooling the reaction on ice and then adding an equal volume of 50% pyridine in water. The solvent

was removed and the residue dissolved in a minimal amount of diethyl ether (1 ml), and the solution filtered. The filtrate was applied to a silica gel column equilibrated with 5% methanol in methylene chloride, and the PC analogue was eluted with increasing concentrations of methanol in methylene chloride. The yields were approx. 50–70% in both cases. The material was positive for phosphate, had the correct mobility on silica gel TLC, could be visualized under ultraviolet light, and was positive for quaternary amines as determined with Dragendorf's reagent.

Synthesis of 1,2-dimyristoyl-sn-glyvero-3-(N-(3,3,3-trifluoro-2-diazopropionyl) aminoethyl phosphate (PL-III, Fig. 4)

Dimyristoyl phosphatidylethanolamine (30 mg, 0.47 mmol) was dissolved in 2 ml of freshly distilled chloroform. After drying, 1 ml of chloroform 41 μl of 3,3,3-trifluoro-2-diazopropionyl chloride (2-fold excess) and 31 μl of diisopropylethylamine (5-fold excess) were added. After 1 h, the reaction was complete and the product purified on a LH-20 gel permeation chromatography in methylene chloride/methanol (1 : 1, v/v). TLC showed one product. An infrared spectrum of the compound showed the expected band at 2140 cm^{-1}.

Radiolabeled phospholipids with the modified headgroups

Radiolabeled PL-I. PL-I was synthesized containing a [^{14}C]palmitoyl chain by two different routes. One method, PL-I was first hydrolyzed with phospholipase A_2, followed by reacylation with [^{14}C]palmitic anhydride. PL-I (0.05 mmol) was added to a solution of ether/methanol (8 ml, 99 : 1, v/v) followed by 4.5 ml of an aqueous solution containing 40 mM $CaCl_2$, 20 mM Tris-HCl (pH 8.0) and 1 mg of *Crotalus adamanteus* rattlesnake venom. The reaction flask was sealed and stirred vigorously for 5 h, or until the reaction was complete as determined by TLC (methylene chloride/methanol/water (65 : 25 : 4, v/v)) to give 2-lyso PL-I. The organic solvents were removed by a gentle stream of nitrogen, and the residue was extracted by the Bligh-Dyer procedure [16]. The residue was rendered anhydrous by evaporation under vacuum in the presence of anhydrous benzene. To the dry lyso compound thus obtained was added [^{14}C]palmitic anhydride (0.06 mmol) and *N,N*-dimethylaminopyridine (0.05 mmol) followed by 1 ml of freshly distilled anhydrous chloroform. The flask was flushed with nitrogen, sealed, and stirred for two days. The solvent was removed under reduced pressure and the residue extracted by the method of Bligh and Dyer, using 1 M HCl in place of the water. The radiolabeled [^{14}C]PL-I was easily purified either on a silica gel or Sephadex LH-20 column, the yield being approx. 70%.

Alternatively, [^{14}C]dipalmitoyl phosphatidylcholine was converted to radiolabeled PL-I using the transesterification reaction catalyzed by phospholipase D. [^{14}C]Dipalmitoylphosphatidylcholine was dissolved in methylene chloride (0.5 ml). An aqueous solution (0.55 ml) containing 3-diazirino-pyridinium-*N*-ethanol bromide (110 mg), 0.1 M $CaCl_2$, 0.1 M Tris-HCl (pH 8.0) and cabbage phospholipase D (19 mg) was added. After 6 h, the reaction was terminated by the addition of EDTA. A Bligh-Dyer extraction fol-

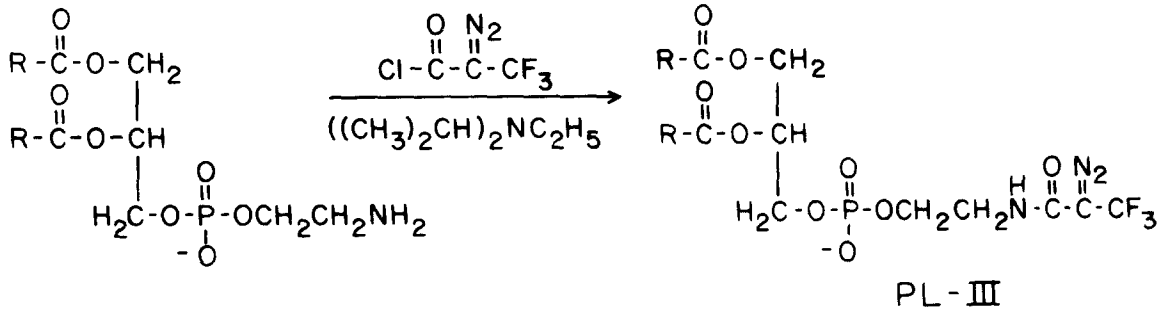

Fig. 4. Synthesis of PL-III. Details of the synthesis are presented in the text.

lowed by preparative TLC (methylene chloride/methanol/water (65 : 25 : 4, v/v)) purified the [^{14}C]PL-I from [^{14}C]dipalmitoylphosphatidylcholine and the hydrolysis product [^{14}C]dipalmitoylphosphatidic acid.

Radiolabeled PL-II. PL-II containing a tritium label was synthesized by using tritiated dimethylethanolamine. Tritiated dimethylethanolamine was formed from tritiated ethanolamine (165 mCi/mmol, New England Nuclear) by reductive methylatuon using formaldehyde and sodium cyanoborohydride [17]. The ratio of ethanolamine: formaldehyde : sodium cyanoborohydride was 1 : 20 : 2. The formaldehyde was added in four aliquots and the sodium cyanoborohydride in two aliquots over several hours. After the final addition, the reaction was allowed to stand overnight at room temperature. The total reaction volume was 50 μl. The dimethylethanolamine was extracted with 5 × 50 μl aliquots of diethyl ether. The pooled ether extracts were diluted with an equal volume of dimethylformamide, for a volume of approximately 400 μl. One equivalent each of diazirinophenoxypropyl bromide (V) and *N*,*N*-diisopropylethylamine were then added and the mixture allowed to stand at room temperature for 24 h. The solvents was removed under high vacuum and the residue twice dissolved in and dried down from anhydrous pyridine (0.5 ml). The sample was dissolved in 100 μl anhydrous pyridine and two equivalents of 2,4,6-triisopropylbenzenesulfonyl chloride and 1.2 equivalents of dioleoylphosphatidic acid were added. The reaction was allowed to stir under nitrogen in a dry box overnight. The reaction was quenched by the addition of an equal volume of 50% pyridine in water. The mixture was then dried and dissolved in a minimal amount of 10% methanol in methylene chloride. The product could then be purified by silica gel chromatography or by silica gel TLC.

Photolysis of vesicles formed from phospholipids containing photoactivatable groups

Photogenerated carbenes, due to their high reactivity, are capable of both insertion into unreactive C-H bonds, as well as insertion into O-H bonds. The latter process is favored about 50-times over the rate C-H insertion [15]. To test if the carbenes generated by photolysis are totally scavenged by water or some desired crosslinks also occur, phospholipid vesicles containing PL-I, a diazirine containing phospholipid and [^{14}C] DMPC, or PL-III, a diazo containing phospholipid and [^{14}C]DMPC were prepared. These vesicles were then irradiated for 10–15 min at 344 nm for PL-I, and at 366 nm for PL-III. The products of the photolysis were then examined by either silica gel TLC (solvent, methylene chloride/methanol/water (65 : 25 : 4, v/v) or by Sephadex LH-20 column chromatography (2.5 × 90 cm, in methylene chloride/methanol (1 : 1, v/v)). When analyzed by TLC, the percentage crosslinking with PL-I was found to be 4%. With Sephadex LH-20, the percentage crosslinking was determined to be 3%. Vesicles which had not been irradiated showed that only 0.04–0.1% of the radioactivity migrated as crosslinked material.

However with PL-III, a diazo containing PL, there was a very high background, probably due to the instability of the compound under the experimental conditions. Photolysis of these vesicles produced 1.3% of the radioactivity migrating as crosslinked material on TLC, but the nonphotolyzed material showed 1.0% of the radioactivity migrating as crosslinked material.

III. Reconstitution of enzymatic activity with synthetic phospholipids containing photoactivatable groups

A. Bacteriorhodopsin

Bacteriorhodopsin is a transmembrane protein which catalyzes light-driven proton translocation across the membrane. Complete delipidation of this protein and subsequent reconstitution with exogenous phospholipids in vesicles that were capable of translocating protons has been reported previously [7]. The same reconstitution procedure was used with delipidated bacteriorhopsin and the phospholipid analogue, PL-I (Table I). When mixtures of PL-I and DOPC in the ratio of 1 : 9, 1 : 1 or 9 : 1 (w/w) were used, the observed proton pumping activity was about the same as observed with DOPC alone. These results show that PL-I can support vectorial proton pumping activity of bacteriorhodopsin, and must also be able to form sealed vesicles.

TABLE I

PROTON PUMPING ACTIVITIES OF BACTERIORHODOPSIN (BR) RECONSTITUTED WITH VARIOUS RATIOS OF DIOLEOYL-PHOSPHATIDYLCHOLINE (DOPC) AND PL-I

PL-I/DOPC (mol/mol)	Initial rate (H^+/Br mol/s)	Total (H^+ pumped/BR mol)
0:1	1.5	3.3
1:9	1.9	5.6
1:1	1.4	3.6
9:1	0.8	3.6

B. β-Hydroxybutyrate dehydrogenase

β-Hydroxybutyrate dehydrogenase from bovine heart mitochondria is known to exhibit an absolute requirement for phosphatidylcholine for its enzymatic activity [18]. We have previously demonstrated that detergent delipidated β-hydroxybutyrate dehydrogenase can be reconstituted with preformed sonicated vesicles [8]. As shown in Table II, the enzymatic activity can be reconstituted with dioleoylphosphatidylcholine (DOPC), but not with dioleoyl phosphatidyethanolamine (DOPE) or dioleoylphosphatidylglycerol (DOPG). DOPE and DOPG, however, are helpful in maximizing the reconstituted activity. PL-II can satisfy this phosphatidylcholine requirement in mixtures with DOPG and DOPE. 1,2-dioleoyl-*sn*-glycero-3-phosphorylcholine-*N*-(phenylpropane) (Phenyl-PC), which is an analogue of PL-II devoid of deazirine group, can also reconstitute the activity. In addition, PL-II is capable of forming sealed vesicles as shown by glucose trapping.

TABLE II

RECONSTITUTION OF β-HYDROXYBUTYRATE DEHYDROGENASE.

% activity: Asolectin set at 100%.

Phospholipid	% Activity
Asolectin	100
DOPC	70
DOPE	0
DOPG	0
DOPC/DOPE/DOPG (5:4:1, mol/mol)	85
PL-II/DOPE/DOPG (5:4:1, mol/mol)	65
Phenyl-PC DOPE/DOPG (5:4:1, mol/mol)	62

IV. Crosslinking between membrane proteins and phosphatidylcholine analogues

A. Bacteriorhodopsin

Delipidated bacteriorhodopsin was reconstituted with the mixture of ^{14}C-labeled PL-I and DOPC (1:9 w/w) by detergent dialysis method as described. This reconstituted vesicles were photolysed at 344 nm, lyophilized and dissolved in a mixture of formic acid and ethanol (3:7, v/v). The sample was then applied on Sephadex LH-60 gel permeation chromatography in formic acid/ethanol (30:70, v/v) to remove non-covalently associated lipids from the protein. As shown in Fig. 5, 1.5% of the radioactive lipid coeluted with the protein. When rerunning the same column, all radioactivity ran with the protein as expected from covalent attachement. A control experiment of an unphotolyzed sample showed less than 0.1% of radioactivity co-eluting with the protein.

B. β-Hydroxybutyrate dehydrogenase

Delipidated β-hydroxybutyrate dehydrogenase was reconstituted with ^3H-labeled PL-II, DOPE and DOPG (5:4:1, mol/mol). After photolysis at 366 nm for 10 min, the protein-lipid mixture was

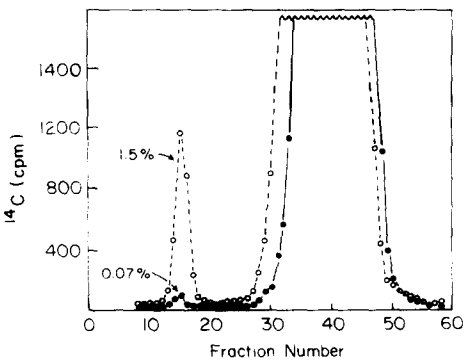

Fig. 5. A Sephadex LH-60 column elution profile (0.6×20 cm) showing the separation of noncovalently attached [^{14}C]PL-I from bacteriorhodopsin using formic acid/ethanol (7:3, v/v). The sample was photolysed for 15 min at 344 nm. The open circles represent the photolysed samples, closed circles represent the nonphotolysed sample.

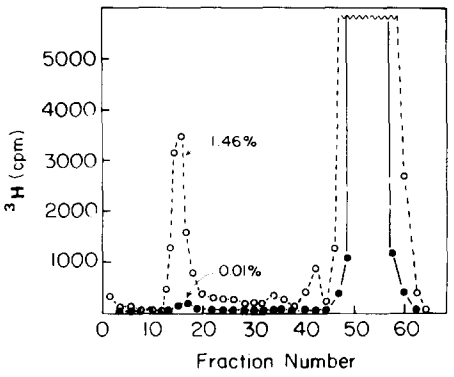

Fig. 6. A Sephadex LH-60 column elution profile (0.7×60 cm) showing the sepration of noncovalently attached [^{3}H]PL-II from β-hydroxybutyrate dehydrogenase using formic acid/ethanol (7:3, v/v). The sample was photolysed at 366 nm for 10 min. Open circles represent the photolysed sample, closed circles represent the nonphotolysed sample.

chromatographed over a Sephadex LH-60 column in ethanol/formic acid (7:3, v/v) as shown in Fig. 6. The radioactivity coeluting with the protein accounted for 1.46% of the total, and represented approx. 0.15 phospholipid molecules per protein. The small peaks of radioactivity as fractions 34 and 42 probably represent trimers and dimers of crosslinked lipid, respectively. Reconstituted enzyme without photolysis was also subjected to gel permeation chromatography, which showed less than 0.1% of the radioactivity eluted with protein. The photolysed sample was also subjected to SDS-polyacrylamide gel electrophoresis, and the autoradiogram shown in Fig. 7, which further shows the covalent association of the radioactive phospholipid with the protein.

C. Cytochrome b_5

The cholate-filtration method was used to reconstitute rabbit liver cytochrome b_5 into phospholipid vesicles containing radioactive PL-I (10 mol%) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphorylcholine (POPC, 90 mol%). Previously, the cholate-filtration method was shown to reconstitute cytochrome b_5 into 'non-transferable form' or 'tight binding' form, in which membranous segment has been concluded to span the lipid-bilayer [5]. After photolysis at 366 nm, the sample was lyophilized and applied on a Sephadex LH-60 column with ethanol/formic acid (4:1, v/v) as shown in Fig. 8. The amount of crosslinking was determined to be 0.2 mol of PL-I per mol of cytochrome b_5. The sample without photolysis showed less than 0.003 mol of PL-I per mol of cyotchrome b_5 co-eluting. Previously the phospholipid with diazirinophenoxy group or trifluorodiazopropionyl group at ω-position of fatty acyl chain gave the amount of crosslinking of 0.23 or 0.56 mol of phospholipid per mol of cytochrome b_5, respectively [4].

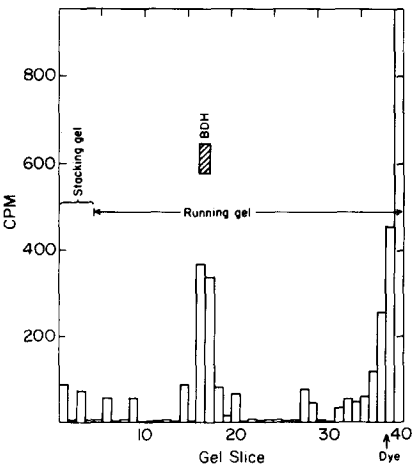

Fig. 7. A 12% SDS-polyacrylamide gel was used to separate noncovalently attached [^{3}H]PL-II from β-hydroxybutyrate dehydrogenase (BDH).

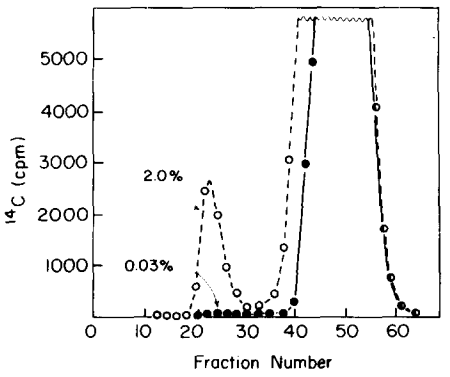

Fig. 8. A Sephadex LH-60 column elution profile (1.5×45 cm) showing the separation of noncovalently attached [^{14}C]PL-I from cytochrome b_5 using formic acid ethanol (7:3, v/v). The sample was photolysed at 366 nm for 2 min. Open circles represent the photolysed sample, closed circles represent the nonphotolysed sample.

Discussion

Photoaffinity labels that form, upon photolysis, highly reactive intermediates such as carbenes are being currently used to study lipid-protein interactions in biological membranes. One approach involves the use of hydrophobic photoactivatable molecules that are freely diffusable within the lipid bilayer [1]. A second approach that has been designed for the study of specific interactions between phospholipids and membrane proteins involves the incorporation of photoactivatable groups as integral parts of the acyl chains of phospholipids [19]. A potentially important third possibility, which has so far not been investigated, is the study of interactions between the polar headgroups of phospholipids and the domains of membrane proteins that presumably protrude out of the hydrophobic regions of the lipid bilayer. Such an approach would be useful because a number of membrane-embedded enzymes have been shown to possess either absolute or preferential requirement for specific polar headgroups of phospholipids.

This paper has reported on the synthesis of phospholipids that contain two types of photoactivatable carbene precursors, aromatic diazerine and diazo groups, in their polar headgroups. These photoactivatable groups have been shown previously to be useful in crosslinking reactions in the hydrocarbon region of lipid bilayers [19]. In the polar headgroups region where scavenging by water becomes a serious side reaction, only aromatic diazirines gave acceptable levels of crosslinking in phospholipid model systems. In addition to the low yields obtained in crosslinking reactions with the diazo groups, the latter are also known to display undesirable side reactions in the dark with the thiol groups of such as dithiothreitol and mercaptoethanol [20].

In the case of PL-I, the pyridinium diazirine moiety provided the positive charge in the headgroup. With PL-II, because of the steric requirements of certain enzymes, in particular β-hydroxybutyrate dehydrogenase, the approach involved the synthesis of a substituted quarternary amine. The strict requirement of a quarternary amine by β-hydroxybutyrate dehydrogenase was met as shown by the reconstitution of enzymatic activity. The synthetic method described for PL-II can easily be adapted to the synthesis of phosphatidylethanolamine derivatives for use with enzymes that prefer this class of phospholipids.

In order for the synthetic phospholipids to be useful they must be able to form sealed vesicles. This was demonstrated for the lipids reported here as shown by the ability to support proton pumping as well as the ability of both PL-I and PL-II to trap glucose. Furthermore, they have been shown to reconstitute and restore activity of two delipidated enzymes, bacteriorhodopsin and β-hydroxybutyrate dehydrogenase. The second requirement is the ability of the phospholipids to form sufficient amount of crosslinks with membrane proteins. The experiments with bacteriorhodopsin, β-hydroxybutyrate dehydrogenase and cytochrome b_5 showed the formation of acceptable levels of crosslinks although less than that observed previously with photoactivatable groups in ω-position of fatty acyl chains. This is undoubtedly due to the presence of water at the membrane interface.

Although the present experiments were performed in artificial membrane systems, these phospholipid analogues could be introduced into natural membrane systems by fusion with phospholipid-containing vesicles or by using phospholipid exchange proteins. In one experiment, PL-I was successfully incorporated into erythrocyte ghosts by bovine phosphatidylcholine exchange protein (Robson, R., Ross, A., Wirtz, K.W.A. and Khorana, H.G. unpublished data). The use of phospholipid exchange proteins would also enable one to create lipid-bilayers with the phospholipid analogue asymmetrically distributed within a single leaflet of the bilayer. This method was successfully used with phospholipids containing photoactivatable groups in the ω-position of fatty acyl chain [5].

Our results on determining the sites of crosslinking between photoactivated phospholipids and the protein are preliminary and further peptide sequenching is clearly necessary. However, we have been able to successfully fragment β-hydroxybutyrate dehydrogenase crosslinked with PL-II with cyanogen bromide and separate the resulting labeled peptides. Chemical fragmentation and analysis after crosslinking with PL-I has been more difficult due to reaction at the pyridium nitrogen or the ester bond of the phospholipid resulting in some loss of the radioactive label. However, this

problem could be overcome by either using enzymatic cleavages or introducing the radioactive label into the pyridinium ring itself.

Finally, in addition to its use for analysis of the interactions between proteins and the headgroups of phospholipids, the present approach offers the only method to define the peptide regions of the integral membrane proteins that are at the membrane interface. This analysis would complement other labeling and proteolysis studies, allowing a more detailed positioning of a polypeptide chain within the bilayer.

Acknowledgments

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