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The Membrane-embedded Segment of Cytochrome b_5 As Studied by Cross-linking with Photoactivatable Phospholipids

I. THE TRANSFERABLE FORM*

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Vesicles were prepared from a 9:1 (mole/mol) mixture of dipalmitoyl phosphatidylcholine and the radioactively labeled phospholipids, 1-palmitoyl-2-ω-(*m*-diazirinophenoxy)undecanoyl-*sn*-glycero-3-phosphocholine (PC-I) or 1-palmitoyl-2-ω-(2-diazo-3,3,3-trifluropropionyloxy)lauroyl-*sn*-glycero-3-phosphocholine (PC-II). Rabbit liver cytochrome b_5 was inserted into these vesicles spontaneously and the resulting vesicles containing the cytochrome b_5 in the transferable form were photolyzed. Cytochrome b_5 containing covalently cross-linked phospholipids was isolated by Sephadex LH-60 column chromatography using ethanol/formic acid as the solvent. Of the total radioactivity, 4.6% (PC-I) or 11.3% (PC-II) was linked to the protein; of the former, up to 51% was base-labile, while in the latter, 22% was base-labile. The sites of cross-linking of PC-I to the protein were investigated by fragmentation with trypsin, *Staphylococcus aureas* V8 protease, CNBr, and *o*-iodosobenzoic acid followed by Sephadex LH-60 chromatography and Edman sequencing (solid phase) of the appropriate fragments. The distribution of cross-linking was broad (Ser-104 to Met-130), showing a bell-shaped pattern with a significant peak at Ser-118. The labeling pattern is consistent with the previously proposed loop-back model for the membranous segment in the transferable form of cytochrome b_5.

Interactions between proteins and phospholipids in biological membranes are only poorly understood. Further studies of such interactions are necessary to develop an understanding of the functions of the membrane proteins at the molecular level. We have recently introduced a general chemical approach to such studies that aims at covalent cross-linking between phospholipids and membrane-embedded polypeptide chains (1–3). Photoactivatable groups capable of generating reactive carbene intermediates are incorporated by chemical synthesis (4, 5) into the ω-position of the fatty acid present at the *sn*-2 position of the glycerol backbone (PC-I,[1] PC-II, Fig. 1). These phospholipid analogues form sealed vesicles upon sonication in aqueous media and the latter on photolysis form carbene intermediates which have been demonstrated to insert into C-H bonds of neighboring fatty acyl chains resulting in lipid-lipid intermolecular cross-linking (6–9). Although a number of studies on membrane proteins using the above phospholipid analogues have been reported (3, 10–12), a closer investigation of several aspects was considered desirable for a detailed assessment of this approach. For example, 1) what are the preferred target sites in polypeptide chains for the insertion of generated carbenes?, 2) is there a light-induced damage to the system under investigation leading to artifactual results?, and 3) can one expect a correlation between the sites of cross-linking and the structures of the photoactivatable phospholipids as was previously found for phospholipid-phospholipid cross-linking? (8, 9).

Liver microsomal cytochrome b_5 is an integral membrane protein of low molecular weight (M_r = 15,223) (13, 14) which functions as a component of electron transfer chain. Its catalytic domain, comprising about 70% of the total protein, is present in the aqueous medium. This domain is anchored into the membrane by a hydrophobic segment comprising only about 30 amino acids proximal to the COOH terminus. The primary sequences of the hydrophobic segments are known for cytochrome b_5 in five different mammalian species (15–20). The mode of insertion of cytochrome b_5 into the membrane has been studied extensively (21–25), especially by Strittmatter and his colleagues. These studies revealed two different modes of insertion of cytochrome b_5 into the phospholipid bilayer, one which undergoes intermembrane transfer (transferable or loose binding form) and the second, a nontransferable or tight binding form, which does not show intermembrane transfer (22). The transferable form is obtained by the spontaneous insertion of cytochrome b_5 into phospholipid vesicles prepared from certain phospholipids. Because of its sensitivity to carboxypeptidase Y, this form has been concluded to have the COOH terminus protruding into the aqueous phase on the same side as the catalytic domain (22, 24).

Because of the above chemical and biochemical progress and the defined nature of the reconstituted cytochrome b_5 vesicles, this system is very attractive for studies of interactions between the membrane-embedded polypeptide chain and phospholipids. Therefore, we have investigated the use of the phospholipids containing photoactivatable groups in

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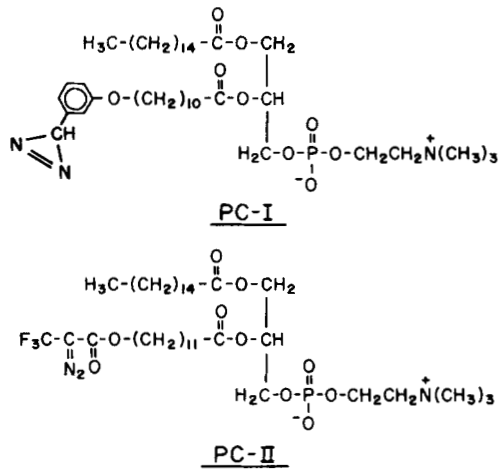
[1] The abbreviations used are: PC-I, 1-palmitoyl-2-ω-(*m*-diazirinophenoxy)undecanoyl-*sn*-glycero-3-phosphocholine; PC-II, 1-palmitoyl-2-ω-(2-diazo-3,3,3-trifluoropropionyloxy)lauroyl-*sn*-glycero-3-phosphocholine; DPL, dipalmitoyl-*sn*-glycero-3-phosphocholine; DMF, dimethylformamide; NMM, *N*-methyl morpholine; tBOC, *tert*-butyloxycarbonyl; PTH, phenylthiohydantoin; EDC, 1-ethyl-2-dimethylaminopropyl carbodiimide.



PC-I

PC-II

FIG. 1. Photolabeled phospholipids used in the present study.

cross-linking. In particular, it was hoped that a detailed analysis of the cross-linking sites in the two forms of cytochrome b_5 might provide additional insights into the topography of the hydrophobic segment embedded in the lipid bilayer. In the present report, cross-linking studies are described using cytochrome b_5 in the transferable form. The observed sites of cross-linking showed a broad distribution, falling between Ser-104 and Met-130 forming a bell-shaped pattern with a peak at Ser-118. Interpretations of these results are given. In an accompanying paper (26), we report on cross-linking experiments with the nontransferable form of cytochrome b_5.

EXPERIMENTAL PROCEDURES

Materials—Cytochrome b_5 was isolated from the livers of pregnant New Zealand white rabbits (27). Carboxypeptidase Y and diphenylcarbamoyl chloride-treated bovine trypsin were from Sigma, proteinase K was from Boehringer Mannheim, and *Staphylococcus aureus* V8 protease was from Miles. CNBr (97%), diisocyanatohexane, and tBOC azide were from Aldrich and *o*-iodosobenzoic acid and NMM were from Pierce. Distilled anhydrous trifluoroacetic acid was from Sequemat. Other sequencing reagents were from Pierce and solvents were from Burdick and Jackson. [^{35}S]Phenylisothiocyanate was from Amersham. [^{14}C]Phosgene was from New England Nuclear.

The preparation of PC-I (Fig. 1) with [1-^{14}C]undecanoyl chain or with [11-^{3}H]undecanoyl chain has been described (5). ^{14}C-labeled PC-I was used with a specific activity of 18.4 mCi/mmol while the ^{3}H-labeled PC-I used had a specific activity of 58.4 mCi/mmol. The preparation of PC-II was as described (4) except that [^{14}C]phosgene was used to introduce ^{14}C label into the propionyl group. This radioactive preparation had a specific activity of 2.2 mCi/mmol. DPL was from Calbiochem. ^{14}C-labeled DPL was a gift of Dr. W. Curatolo of this laboratory. ω-(*m*-Hydroxymethylphenoxy)undecanoic acid and 1-palmitoyl-2-ω-(*m*-hydroxymethylphenoxy)undecanoyl-*sn*-glycero-3-phosphocholine were gifts from Dr. A. H. Ross of this laboratory.

Preparation of Phospholipid Vesicles and Reconstitution of Cytochrome b_5 into Vesicles in the Transferable Form—A benzene solution of a mixture of 4.5 μmol of DPL and 0.5 μmol of PC-I or PC-II in a test tube was evaporated under N_2 and the residue was dried *in vacuo* overnight. After the addition of 0.375 ml of 50 mM Tris/acetate buffer, pH 8.1, containing 0.1 mM Na-EDTA and 0.1 M NaCl, the test tube was capped with a rubber septum, flushed with N_2, vortexed after warming up to 50 °C, and sonicated in a bath-type sonicator (80 watts, Laboratory Supplies Co., Hicksville, NY) at 45 °C, in the dark, for a total of 6 min with 30-s intervals between each 15-s sonication. The sonicated solution was optically clear indicating the formation of unilamellar phospholipid vesicles. To this preparation was added 0.1 μmol of purified cytochrome b_5 and the solution was incubated at 45 °C, in the dark, for 6 h.

Photolysis of Vesicles—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 two filters (WG 345 and WG 360, Schott Optical, Duryea, PA). Samples were irradiated under N_2. Under the conditions used for photoirradiation, photolysis of the vesicles containing PC-I was complete in 2 min. To the vesicles containing PC-II, an equal volume of 50 mM tryptophan in 10 mM Tris/acetate buffer, pH 8.1, containing 0.1 mM Na-EDTA and 0.1 M NaCl was added; half-life of the photolabel under this condition was 13 min.

Gel Permeation Chromatography—Gel permeation chromatography was performed on a Sephadex LH-60 (Pharmacia) column (1.5 × 47 cm) in ethanol, 88% formic acid (4:1, v/v). The molecular weight of the material eluted was estimated from a standard curve previously described (19).

Fragmentation of Cross-linked Cytochrome b_5—Tryptic digestion, *Staphylococcus aureus* V8 protease digestion, and CNBr cleavage were carried out as described previously (19). Cleavage with *o*-iodosobenzoic acid was performed according to Mahoney *et al.* (28). Proteinase K digestion was performed first at 25 °C in 0.2 M sodium phosphate buffer, pH 8.0, for 5 h using 6 μg of the enzyme per 10 μg of cytochrome b_5 and then for another 5 h after addition of sodium dodecyl sulfate to a final concentration of 0.2%.

Hydrolysis of Base-labile Cross-links—The alkaline treatment was performed in a mixture of 250 μl of ethanol and 200 μl of H_2O with NaOH added to the desired pH. All fatty acyl ester linkages of phospholipids were hydrolyzed completely within 30 min at pH 12.3. Base treatment was also performed in DMF (distilled over P_2O_5 and stored over molecular sieve 4A) containing 10% NMM and 15% tBOC azide at 50 °C for 5 h; this treatment did not hydrolyze ester bonds in phospholipids.

Automated Edman Degradation of the Peptide Fragments from Cross-linked Cytochrome b_5—The peptide fragments from phospholipid-cross-linked cytochrome b_5 were subjected to Edman degradation both by the automated liquid-phase sequencing method using the Beckman 890C Sequencer and automated solid-phase sequencing method using a modified Sequemat Mini 15. The sequencing program for liquid-phase sequencing was as previously described (19). We experienced extensive washout of the cross-linked peptide from the spinning cup because of hydrophobicity of the fragments. Also, extraction of the cross-linked amino acids by *n*-chlorobutane and ethyl acetate used in liquid-phase sequencing was inefficient. Automated solid-phase sequencing was performed at 47 °C with 0.2 M Quadrol/trifluoroacetic acid buffer, pH 8.2, in 1-propanol:H_2O (3:4, v/v) as the coupling buffer, and methanol and benzene as the washing solvents. Coupling reaction was performed for 16 min with 10% phenylisothiocyanate (35 μl/min) and coupling buffer (80 μl/min), washing was performed for 3 min each with coupling buffer (80 μl/min), methanol, and benzene (1 ml/min each), and trifluoroacetic acid cleavage was performed for 20 min (100 μl/min). Although the added advantage of the solid-phase method for the present analysis is in the use of strong protic solvent trifluoroacetic acid for the cleavage and extraction, prolonged incubation in trifluoroacetic acid caused some degradation of cross-links and slight leaching out of peptide fragments. The peptides were, therefore, pretreated with base to remove labile cross-links. After sequencing, an aliquot of the trifluoroacetic acid fraction was analyzed on silica gel TLC. After conversion reaction, PTH-amino acid derivatives were identified and quantitated by high performance liquid chromatography with a Zorbax CN column (DuPont) as described previously (29).

Activated glass beads were prepared by derivatizing controlled pore glass 240 (Electro-Nucleonic Inc., Fairfield, NJ) with 3-aminopropyltriethoxysilane. Arylamino-glass was prepared by derivatizing aminopropyl-glass with α-bromo-*p*-nitrotoluene followed by reduction with sodium dithionite. Peptides were coupled to these glass beads by EDC in 1 M pyridine-HCl, pH 5.0, for 2 h at 25 °C. Isothiocyanato-glass was prepared by derivatizing aminopropyl-glass with *p*-phenylene diisothiocyanate and the peptides were coupled according to Machleidt and Wachter (30). Triethylenetetramine-glass was prepared by first derivatizing aminopropyl-glass with diisocyanatohexane and then with triethylenetetramine. Homoserine lactone coupling of cyanogen bromide fragment to triethylenetetramine-glass was performed according to Horn and Laursen (31).

Silica Gel TLC—Silica gel TLC was performed with solvent I: $CHCl_3$:CH_3OH:H_2O (65:25:4, v/v/v) or solvent II: $CHCl_3$:CH_3OH:H_2O (50:35:10, v/v/v) on plastic-backed TLC plate (Merck No. 5775) or with solvent III: *sec*-butyl alcohol:H_2O:88% formic acid (70:21:18, v/v/v) on glass-backed TLC plate (Merck No. 5763) by ascending chromatography in a glass tank.

The position of the phospholipid-cross-linked PTH-amino acids on TLC plates used was separately determined using ^{35}S-labeled

phenylisothiocyanate for sequencing the peptide fragments cross-linked with nonradioactive PC-I. The radioactive spots on TLC after such sequencing, which were not PTH-amino acids, were regarded as the cross-linked PTH-amino acids. In NaOH-treated samples, these appeared in the fatty acid region (R_F = 0.8–1.0) above the PTH-amino acid region (R_F = 0.45–0.8) in silica gel TLC with solvent I. In the peptides treated with organic base, these appeared with the mobility the same as and higher than PC-I (R_F = 0.38–0.6) in the TLC solvent II.

Other Analyses—The radioactivity was measured with Hydrofluor (National Diagnostics, Somerville, NJ) liquid scintillation mixture. Peptides on TLC were visualized by spraying with fluorescamine (Roche, Cranbury, NJ) in acetone (0.1%, w/v) containing 1% triethylamine. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed using the procedure of Swank and Munkres (32) with 15% gel. Identification of the NH_2-terminal amino acid using dansyl-chloride was performed according to Gray (33).

RESULTS

Photolytic Cross-linking of the Transferable Form of Cytochrome b_5 with PC-I and PC-II

Following spontaneous insertion of cytochrome b_5 into sonicated phospholipid vesicles (see "Experimental Procedures"), photoirradiation was performed with monochromatic light (366 ± 10 nm) under nitrogen.[2] The photolyzed samples were lyophilized immediately and subjected to Sephadex LH-60 gel permeation column chromatography.

Gel Permeation Chromatography of Cross-linked Cytochrome b_5

Vesicles Containing PC-I—Fig. 2 shows the result of chromatography of products obtained by photolysis of cytochrome b_5 reconstituted into DPL:PC-I vesicles. After irradiation at 25 °C for 2 min, 4.6% of input radioactivity (0.23 mol of PC-I per mol of cytochrome b_5) remained with the cytochrome b_5 peak, whereas the reconstituted vesicles before irradiation retained only 0.05% of the radioactivity in the protein peak. In another control experiment, where cytochrome b_5 and the vesicles were irradiated separately and then mixed, 0.14% of the total radioactivity remained associated with cytochrome b_5 after Sephadex LH-60 chromatography. These results make it highly likely that the radioactivity due to PC-I which emerges with the protein is covalently linked with the latter.

In view of the observation by Curatolo *et al.* (34) that photocross-linking is sensitive to phase separation in phospholipids, further experiments were performed in which photoirradiation was performed at different temperatures. At 2 and 50 °C, 4.6% (0.23 mol of PC-I per mol of cytochrome b_5) and 3.8% (0.19 mol), respectively, of input radioactivity was associated with cytochrome b_5.

Vesicles Containing PC-II—Cytochrome b_5 reconstituted into vesicles containing PC-II was irradiated for a longer time (40 min) due to slower photolysis of the diazo group. Sephadex LH-60 chromatography of the photolyzed sample showed the radioactivity in PC-II to be associated with both monomeric cytochrome b_5 (5.6%) and also with a polymeric cytochrome b_5 species emerging in the void volume (5.7%) (Fig. 3). The addition of tryptophan in aqueous phase reduced this photopolymerization but did not abolish it. When cytochrome b_5 was reconstituted into vesicles containing only ^{14}C-labeled DPL and the vesicles were photoirradiated under identical conditions, only 0.15% of DPL was associated with cytochrome b_5, as determined by gel permeation chromatography. This result eliminates the possibility of photoexcitation of the protein and subsequent cross-linking to phospholipid.

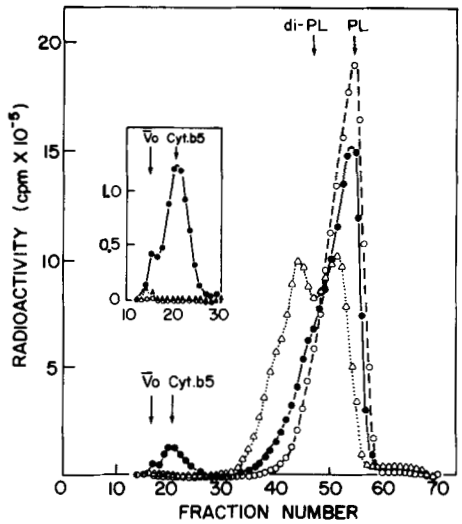


FIG. 2. **Sephadex LH-60 chromatography of cytochrome b_5 reconstituted into sonicated vesicles containing PC-I and DPL (see "Experimental Procedures.").** O– – –O, before photoirradiation; ●——●, after photoirradiation; Δ····Δ, cytochrome b_5 and phospholipid vesicles were photoirradiated separately and reconstituted. The elution positions of cytochrome b_5 (*Cyt. b5*), phospholipid (*PL*), and photocross-linked dimer phospholipid (*di-PL*) are indicated. *Inset* is an enlargement of the fractions immediately below.

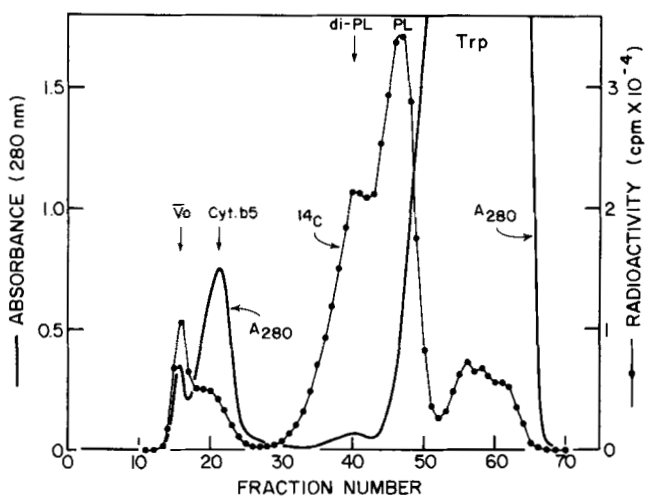


FIG. 3. **Sephadex LH-60 chromatography of cytochrome b_5 reconstituted into vesicles containing PC-II and DPL.** After reconstitution (see "Experimental Procedures"), the sample was photoirradiated for 40 min at 45 °C with the addition of tryptophan (see "Experimental Procedures"). The elution positions of cytochome b_5 (*Cyt. b5*), phospholipid (*PL*), photocross-linked dimer phospholipid (*di-PL*), and tryptophan (*Trp*) are indicated.

Characterization of Photocross-linked Products

Degradation by Protease—Cytochrome b_5 cross-linked with PC-I was purified by Sephadex LH-60 chromatography and then digested with proteinase K (see "Experimental Procedures"). The digest was analyzed by silica gel TLC using solvent I. The autoradiogram of TLC (Fig. 4) showed that several new radioactive bands traveling between the origin and PC-I were present. A small amount (3.8%) of the total radioactivity moved with the same mobility as PC-I (R_F 0.29). Since standard amino acids traveled in between PC-I and the origin in this TLC system, the newly formed bands were likely

[2] A small amount of photodimerization of dioleoyl-*sn*-glycero-3-phosphocholine was observed under normal atmosphere but not under nitrogen (G. E. Gerber and H. G. Khorana, unpublished results). Photoirradiation of gramidicin A with 366 nm light under atmosphere resulted in significant degradation of tryptophan, but not under nitrogen (A. Majumdar and H. G. Khorana, unpublished results).

to be amino acids and short peptides cross-linked with radioactive PC-I. The absence of free PC-I after extensive digestion further showed that PC-I was covalently bound to cytochrome b_5.

Digestion with Trypsin—As previously shown, trypsin digests the hydrophilic domain of denatured cytochrome b_5 into small fragments (35) and the membranous segments starting either from Leu-91 or Pro-94 to the COOH-terminal, Asp-133, are the only peptide fragments that are produced with more than 30 amino acids. Thus, examination of the tryptic digest should indicate whether cross-linking is located between Leu-91 and Asp-133 or within the hydrophilic domain (Fig. 5). Fig. 6*A* shows the gel permeation profile of tryptic digest of cytochrome b_5 cross-linked with PC-I. At least 85% of the radioactivity was present in a single peak which eluted slightly ahead of the major peptidic peak. Since only a minor proportion of cytochrome b_5 was cross-linked, the profile of absorbance at 280 nm is predominantly that of uncross-linked peptide fragments; the profile of radioactivity indicates the cross-linked peptides. As is evident from gel permeation chromatography, the peptides containing cross-linked phospholipids showed an increase in molecular weight relative to the uncross-linked peptides.

Solid-phase Edman sequencing of the pooled radioactive fractions (*a* in Fig. 6*A*) showed only two parallel sequences, one starting from Leu-91 and the second from Pro-94. This result indicates that the cross-links are mostly located in between Leu-91 and the COOH terminus.

Cleavage with CNBr—As expected, CNBr cleaves rabbit liver cytochrome b_5 to give four peptide fragments (NH_2 terminus to Met-95, Glu-96 to Met-125, Tyr-126 to Met-130, and Ala-131 to Asp-133, Fig. 5). These fragments separate on Sephadex LH-60 chromatography (19). Cytochrome b_5 cross-linked with PC-I gave three radioactive peaks (Fig. 6*B*). These were pooled as shown. *Pool a* contained 36% of total radioactivity and contained a mixture of the fragment (NH_2 terminus to Met-95) and uncleaved cytochrome b_5 (19, 36). That the radioactivity in this pool was due to uncleaved cytochrome b_5 was shown by further digestion with trypsin. A single radioactive peak corresponding to the cross-linked peptides, Leu-91 to Met-126 and Pro-94 to Met-126, was now obtained on Sephadex LH-60 chromatography. *Pool b* contained 33% of the total radioactivity and contained a single phospholipid-cross-linked peptide fragment, Glu-96 to Met-126, as judged by Edman degradation. *Pool c* with 31% of the total radioactivity was analyzed on silica gel TLC using solvent III. Of the two radioactive spots seen, one contained the peptide fragment, Tyr-126 to Met-130. The second product appeared to be free lipid, because it had the same mobility as PC-I and it did not stain with fluorescamine. Therefore, some of the cross-

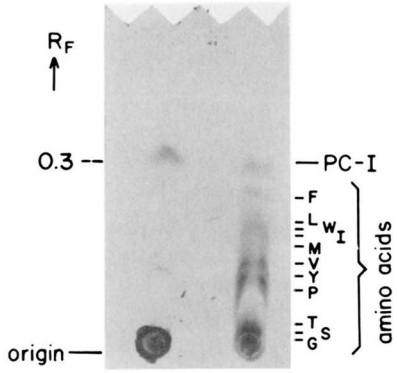


FIG. 4. **Silica gel TLC of proteinase K digest of cytochrome b_5 cross-linked with PC-I.** The digestion was performed as described under "Experimental Procedures" and samples spotted on Merck No. 5775 silica gel plate were developed with solvent I. The radioactivity in [^{14}C]PC-I is shown in autoradiography. The location of amino acids (*one-letter code*) obtained in separate TLC are indicated. *A*, control; *B*, proteinase K digest.

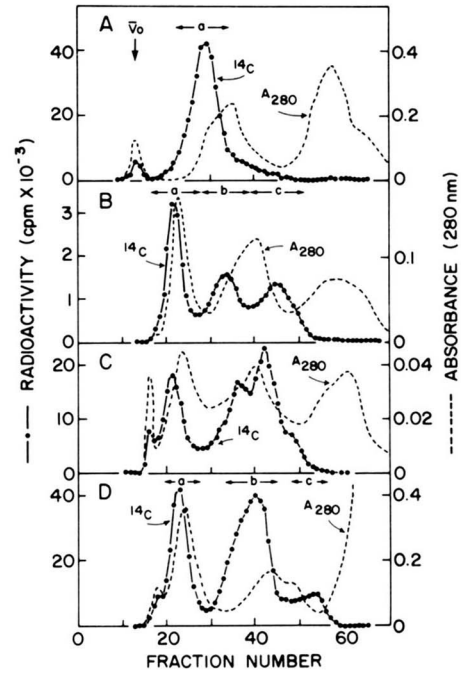


FIG. 6. **Sephadex LH-60 chromatography of cross-linked cytochrome b_5.** *A*, tryptic digest of cytochrome b_5 cross-linked with PC-I; input radioactivity, 370,000 cpm. *B*, CNBr cleavage product of cytochrome b_5 cross-linked with PC-I; input radioactivity, 39,600 cpm. *C*, CNBr cleavage product of cytochrome b_5 cross-linked with PC-II; input radioactivity, 393,500 cpm. *D*, *o*-iodosobenzoic acid cleavage product of cytochrome b_5 cross-linked with PC-I; input radioactivity, 685,000 cpm. High absorbance (280 nm) after fraction 60 is due to *o*-iodosobenzoic acid.

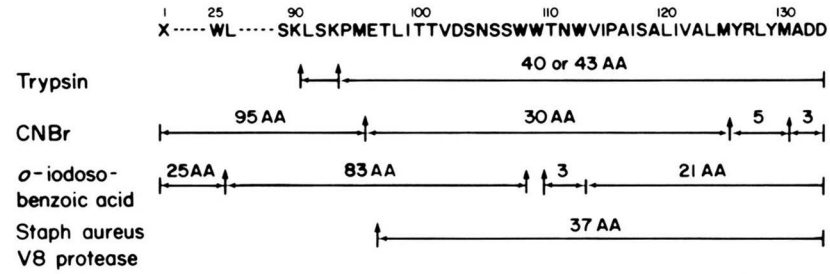


FIG. 5. **Peptide fragments of rabbit liver cytochrome b_5 generated by chemical degradation and proteolysis.** The amino acid sequence of the membranous segment (19) is shown and the cleavage sites of chemical degradation and proteolysis on the left column are shown by *arrows* pointing upwards. The *numbers* between these *arrows* indicate the length of peptide in number of amino acids.

linked phospholipid was cleaved off from the protein during the analytical procedures. The above results indicate that cross-linking of PC-I was confined to the hydrophobic peptide segment, Glu-96 to Met-130.

Cytochrome b_5 cross-linked to PC-II gave peaks corresponding to uncleaved cytochrome b_5 and a peptide Glu-96 to Met-125, and also a major radioactive peak at fraction 42 (Fig. 6*C*), which was not seen in cytochrome b_5 cross-linked to PC-I. Since the last peak gave dansyl-Glu and dansyl-Val after the reaction with dansyl-chloride, the peptide fragment, Glu-96 to Met-125, was presumed to be cleaved further in between Trp-112 and Val-113. It is likely that prolonged irradiation caused some damage to the tryptophan residues such that they become more susceptible to CNBr cleavage (36).

Cleavage with o-Iodosobenzoic Acid—*o*-Iodosobenzoic acid, which cleaves at the COOH-terminal side of tryptophan, gives the four expected peptide fragments from cytochrome b_5 (Fig. 5). These separated according to size on Sephadex LH-60. Cytochrome b_5 cross-linked with either PC-I or PC-II was treated with *o*-iodosobenzoic acid. The three fragments, Leu-27 to Trp-108, Val-113 to Asp-133, and Thr-110 to Trp-112 were pooled as shown (*a–c* in Fig. 6D). Since they all contained substantial amounts of radioactivity, they indicate a broad distribution of cross-linking, within the hydrophobic fragment.

Hydrolysis of Cross-linked Cytochrome b_5 with Base or Acid—Although most of the cross-linked phospholipids stayed with the peptides during the above degradation procedures, a substantial amount of PC-I was released from cross-linked cytochrome b_5 after hydrolysis with base or acid. A release of up to 51% of radioactivity was observed after treatment with NaOH (pH 12.5) at 25 °C for 30 min, a treatment which leads to complete hydrolysis of the ester bonds in the phospholipids (Table I). The cross-linked cytochrome b_5 was treated with NaOH and the products were separated on silica gel TLC (Fig. 7). The major product (about 30% of the total radioactivity) had the same mobility as ω-(*m*-hydroxymethylphenoxy)undecanoic acid, while the second released product had mobility identical with that of ω-(*m*-diazirinophenoxy)undecanoic acid. About 50% of the radioactivity in PC-I was also released under milder conditions (DMF, NMM, and t-BOC azide at 50 °C for 5 h) when the ester bonds in phospholipids were not hydrolyzed. Methanolic HCl released 43% while anhydrous trifluoroacetic acid released 13% of the radioactivity (Table I).

In the case of PC-II-cross-linked cytochrome b_5, NaOH treatment at pH 12.3 at 25 °C for 10 min released 22% of the radioactivity from the cross-linked product. Since this condition hydrolyzes the ester bond between trifluoropropionyl group and the lauroyl chain completely, the result indicated that at least 78% of the cross-links with PC-II were base-stable and, therefore, involved linkage of the radioactive trifluoropropionyl group to the protein.

TABLE I

Base lability of cross-links between cytochrome b_5 and photoactivatable phospholipids

Phospholipid used in cross-linking	Conditions of hydrolysis[a]	Radioactivity released[b]
		%
PC-I	NaOH, pH 12.5, 25 °C, 30 min	51
	DMF, 10% NMM, 15% tBOC-azide, 50 °C, 5 h	50
	1.5 M HCl/MeOH, 55 °C, 15 min	43
	Anhydrous trifluoroacetic acid, 50 °C, 8 h	13
PC-II	NaOH, pH 12.3, 25 °C, 10 min	22

[a] Condition of hydrolysis is described under "Experimental Procedures."

[b] After the hydrolysis, the samples were spotted on silica gel TLC, developed by solvent I, and the radioactivity migrated out of the origin was measured.

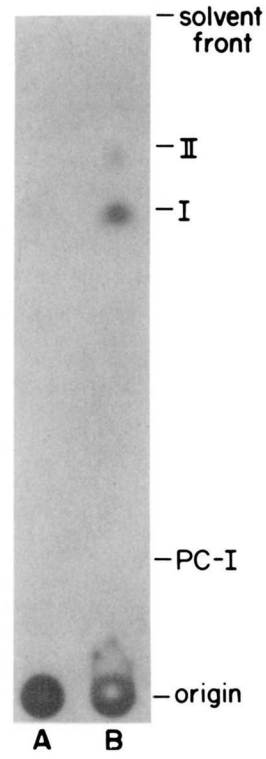


FIG. 7. **Autoradiogram of TLC of alkali-treated cytochrome b_5 cross-linked with PC-I.** The samples treated with NaOH, pH 12.5, at 25 °C for 30 min was spotted on Merck No. 5775 TLC plate and developed with Solvent I. The positions of standard ω-(*m*-hydroxymethylphenoxy)undecanoic acid and ω-(*m*-diazirinophenoxy)-undecanoic acid are marked as *I* and *II*, respectively. *A*, control; *B*, alkali-treated.

Analysis of Cross-linking Sites between Cytochrome b_5 and PC-I

Cytochrome b_5 cross-linked with PC-I was cleaved with *Staphylococcus aureus* V8 protease, *o*-iodosobenzoic acid, or CNBr and the fragments were separated by Sephadex LH-60 chromatography. The peptides obtained were treated with base to hydrolyze labile bonds and were then subjected to sequential degradation by the Edman method using the solid-phase method.

In Fig. 8, *A–D*, are shown the patterns of radioactivity observed at different cycles on stepwise degradation of three different peptides. Throughout, the radioactivity released at each cycle is plotted in *solid line* and the value corrected for the repetitive yield is plotted as a *broken line*. Although the latter plot should reflect more accurately the amount of cross-linking, there is also the amplification, with increasing cycles, of ambiguities resulting from accumulation of incompletely degraded peptides. Because of this limitation in Edman degradation, the accuracy of the data declined as the sequencing steps increased.

Fig. 8*A* shows the results of sequencing the fragment Thr-97 to Asp-133 obtained by *Staphylococcus aureus* V8 protease digestion. The radioactivity rises above the background level after the 7th cycle (Asp-103) and continues to increase thereafter. Fig. 8*B* shows the results of another experiment with the same peptide in which the pretreatment was varied. The results again show the increase of radioactivity above the

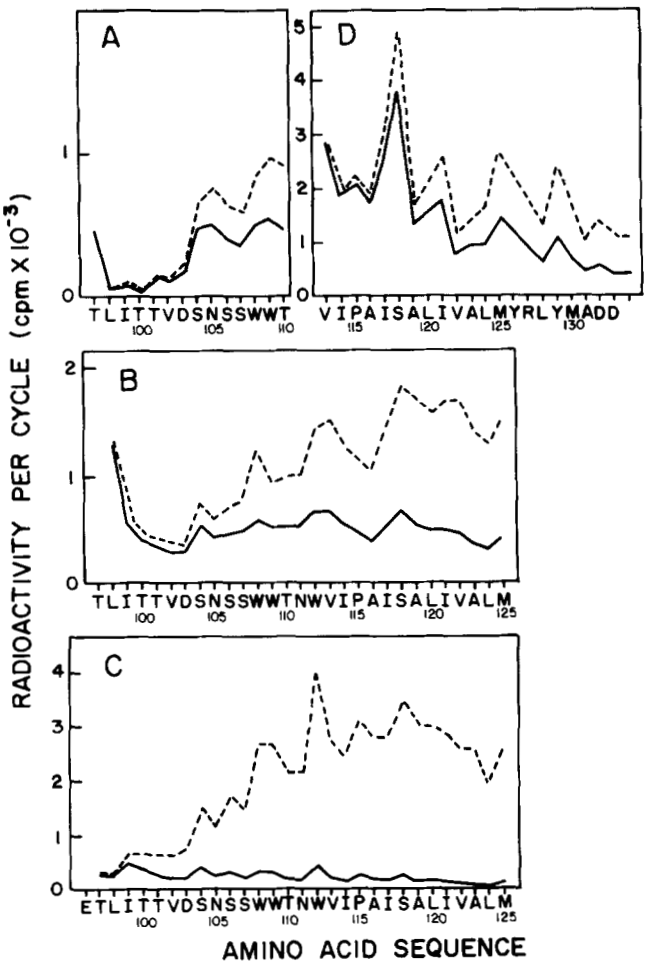


FIG. 8. **Edman degradation of peptide fragments obtained from cytochrome b_5 cross-linked with PC-I.** The radioactivity obtained in the cross-linked anilinothiazolinone and PTH-amino acid region of TLC (solvent I for B and solvent II for others) is plotted in *bold line*. The value corrected for repetitive yield (*A*, *B*, and *D* = 95%; *C* = 82%) is plotted in *broken line*. *A*, *Staphylococcus aureus* V8 protease fragment was pretreated with DMF, 10% NMM, 15% tBOC azide and coupled to arylamino-glass with EDC. Input radioactivity was 107,000 cpm. *B*, *Staphylococcus aureus* V8 protease fragment was derivatized with ethylenediamine by EDC at pH 8.0 and purified on Sephadex LH-20 column chromatography with ethanol:formic acid:H_2O (5:2:3, v/v). After NaOH treatment in 250 μl of ethanol and 300 μl of H_2O at pH 12.5, 25 °C, 20 min, the reaction was quenched by the addition of ethyl formate (50 μl) and the sample was purified on Sephadex LH-60 column chromatography with ethanol:formic acid (4:1, v/v). The sample was derivatized to isothiocyanate glass in 1 M dimethyl-*N*-allylamine buffer, pH 9.5 (Pierce). Input radioactivity was 235,850 cpm. *C*, CNBr fragment was derivatized to triethylenetetramine-glass by the homoserine lactone method (31). Input radioactivity was 94,110 cpm. *D*, *o*-iodosobenzoic acid fragment was pretreated with DMF, 10% NMM, 15% tBOC azide and coupled to arylamino-glass with EDC. Input radioactivity was 111,890 cpm. All sequencing results are aligned according to their amino acid sequences to make direct comparison easier.

background level after Asp-103. Further sequencing shows an increase of radioactivity with distinct peaks at Trp-108, Trp-112, Val-113, and finally Ser-118 after which follows a decline of radioactivity.

Fig. 8*C* shows the results obtained with the fragment (Glu-96 to Met-125) obtained by CNBr cleavage. This gave lower (82%) repetitive yield, while others (Fig. 8, *A*, *B*, and *D*) gave higher repetitive yields (95%). However, despite the poor repetitive yield of this CNBr fragment, the distribution of cross-linking was similar to that observed in Fig. 8*B*.

Fig. 8*D* shows the results of sequencing of the fragment (Val-113 to Asp-133) obtained by *o*-iodosobenzoic acid cleavage. In this sequence, a sharp peak of radioactivity at Ser-118 was obtained and this was followed by a decline of radioactivity to the background level at Ala-131 with a small peak at Ile-121, Met-125, and Tyr-129. The sharpness of the peak at Ser-118 in this sequence, compared with the more diffused peak at the position of the same amino acid in Fig. 8, *B* and *C*, shows that ambiguities increase with increasing sequencing steps.

The results of Fig. 8, all of which have been aligned according to the protein sequence, have the following three common features: 1) the radioactivity rises above background after Asp-103 and increases during the subsequent 10 cycles, 2) the radioactivity decreases after Ser-118 and seems to decline to background level after Met-130, and 3) there is a significant peak at Ser-118.

DISCUSSION

Cross-linking by photoaffinity labeling is being widely used in biochemical studies aimed at probing interactions between molecules. In particular, groups that generate the reactive carbenes on photolysis are proving useful. Previously, we have described an approach which utilizes phospholipids of the type PC-I and PC-II in studies of phospholipid-phospholipid and phospholipid-protein interactions (1–12). In photolytic studies of vesicles prepared from these phospholipids, we showed that the desired carbene insertion into C-H bonds of neighboring fatty acyl chains was indeed the major reaction (8, 9). However, in the limited studies reported on the application of this approach to phospholipid-protein interactions in model membranes, reactions alternative to the straightforward insertion reactions have been observed. Thus, in a study of gramicidin A reconstituted into vesicles containing photoactivatable phospholipids, the tryptophan residues were the major sites of cross-linking[3] (37) and, in a similar study of glycophorin A, the carboxylic group of glutamic acid residue 70 was the main acceptor of the photoactivated phospholipid (12).

The nature of the reactions between photogenerated carbenes and the different structural components of the proteins are likely to be varied and complex and further studies of the competing reactions that can occur are highly desirable. In this and the following paper, we have reported on a closer study of the reactions between irradiated phospholipids PC-I and PC-II and the two forms of membrane-embedded cytochrome b_5. The main features of the results are as follows: 1) sequencing analysis shows that the expected normal C-H insertions do occur, 2) amino acids with functional groups that are particularly nucleophilic, such as serine-118, are preferred sites of cross-linking reactions (*cf.* Smith and Knowles (38)), 3) substantial amounts of base-labile cross-links can be formed, these are likely to be the benzyl esters of carboxylic groups or *N*-substituted imidates formed by the electropholic attack of the carbene on the carbonyl oxygen of the groups of peptide bonds. Additional evidence for imidate formation was obtained by the observed exchangeable incorporation of ^{14}C-labeled glycine ethylester·HCl which can only be explained by the transimidation reaction (39). The formation of imidates by the attack of phenylcarbene on the carboxyl group of amide bond has been demonstrated previously by White *et al.* (40). Despite the above reactions and others that may occur with the membrane proteins, useful topological information can be obtained by this approach.

Previous work from the laboratories of Strittmatter (21, 22)

[3] A. Majumdar, unpublished work.

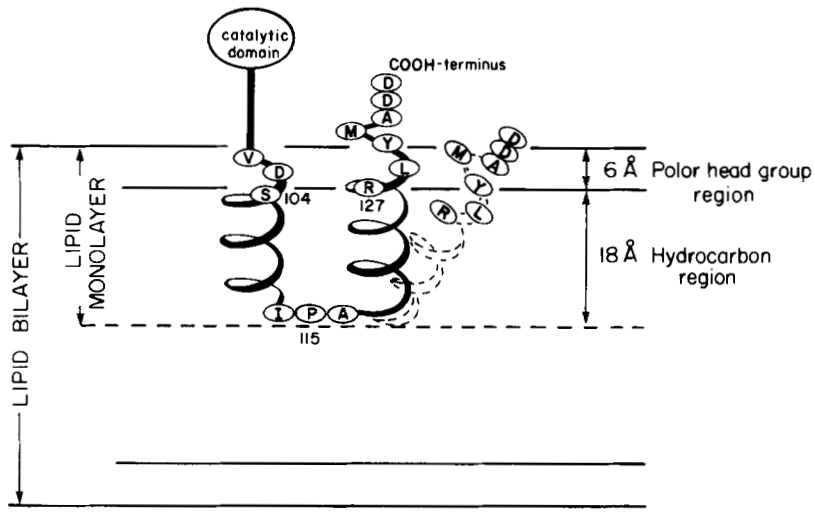


FIG. 9. **Model of tertiary structure of the membranous segment of rabbit liver cytochrome b_5 in transferable form, depicted from the distribution of photocross-linking with PC-I.** Eleven-amino acid stretch of α-helix from Ser-104 to Ile-114 and Ala-116 to Tyr-126 is joined by Pro-115 at the middle of lipid bilayer. A possible explanation of the cross-linking of PC-I to Tyr-129 is envisioned by occasional tilting of the α-helix from Ala-116 to Met-130.

and Sato (24, 41, 42) and their respective colleagues has led to the following conclusions regarding the structure of the transformable form of cytochrome b_5 in vesicles. 1) The hydrophilic domain, which comprises from the NH_2 terminus to amino acid-90, must be in the aqueous phase outside of the vesicles. 2) The carboxyl end is susceptible to proteolysis (22, 24) and Tyr-129 seems to be labeled by lactoperoxidase-catalyzed iodination (24). Therefore, a few amino acids from the COOH terminus must protrude into the aqueous phase outside of the vesicles (hence loop-back form). 3) CD measurements on derivatives of cytochrome b_5 that have shortened COOH terminus indicate the region between Ser-107 to Tyr-125 to be in helical conformation (21). If the polypeptide segment that can possibly enter the bilayer were to do so as an α-helix in loop-back form, then it is likely to insert only into the outer leaflet as is assumed from its length. Additional evidence from the work of Sato and colleagues (41, 42) supports this conclusion.

Our results show that the cross-linking indeed involves only the hydrophobic portion of cytochrome b_5. The distribution of cross-linking is broad. It extends from Ser-104 to Met-130 to give a bell-shaped pattern with a peak at Ser-118. There is also a distinctive peak at around Trp-108, Trp-112, Ile-121, and Met-125. Although Tyr-129 has been concluded to be outside of lipid bilayer (24), we obtained a small but appreciable cross-linking at Tyr-129 (see also the significantly higher amount of cross-linking at Tyr-129 in nontransferable form (26)). This might be interpreted to mean that Tyr-129 is partially embedded (Fig. 9), a conclusion that is supported by the prediction algorithm of von Heijne (43) and Argos *et al.* (44). The broad cross-linking distribution, which could be at least partly due to the dynamic property of the loosely embedded transferable form of the hydrophobic segment (25), can be reconciled with either the peptide traversing both leaflets or making a loop within one leaflet. This is because the photoactivatable groups are located at the ω-positions of the *sn*-2-fatty acyl groups in phospholipids with their densities peaking at the interior of the bilayer (9).

Assuming the loop-back structure to be correct for the transferable form, our cross-linking data provide a refinement (Fig. 9) for the model proposed by Tajima and Sato (24). Starting with the hydrophilic domain, the amino acids Val-102 and Asp-103 are in the polar head group region and Ser-104 has entered the hydrophobic region. The 11 amino acid residues, Ser-104 to Ile-114, form an α-helical stretch, which is nicely accommodated within the monolayer width of a palmitoyl chain (45). This helical segment is connected by Pro-115 to the second α-helical stretch containing 11 amino acid residues, Ala-116 to Tyr-126. Finally, Arg-127 is in the polar head group region. The minor cross-linking observed at Tyr-129 could be explained by postulating that the second helical segment exists partially in the tilted form as shown.

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