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-\( \omega \)-(m-diazirinophenoxy)undecanoyl-sn-glycero-3-phosphocholine (PC-I) or 1-palmitoyl-2-\( \omega \)-(2-diazo-3,3,3-trifluoropropionyloxy)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 aureus V8 protease, CNBr, and \( \varepsilon \)-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 segmental domain 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 \( \omega \)-position of the fatty acid present at the sn-2 position of the glycero backbone (PC-I, PC-II).

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The abbreviations used are: PC-I, 1-palmitoyl-2-\( \omega \)-(m-diazirino-

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 carbene?, 2) is there a light-induced damage to the system under investigation leading to artificial 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 (\( M_1 = 15,223 \) (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 the study of the interactions of the different forms of cytochrome \( b_5 \) with phospholipids.
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 topology 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). Carbamoylphosphilidase \(Y\) and diphenyl

**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 \(\mu\)mol of DPL and 0.5 \(\mu\)mol 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 \(^\circ\)C, and sonicated in a bath-type sonicator (80 watts, Laboratory Supplies Co., Hicksville, NY) at 45 \(^\circ\)C, in the dark, for 6 min with 30-s intervals between each 15-s sonication.

**Fig. 1.** Photolabeled phospholipids used in the present study.

Schott Optical, Duryea, PA). Samples were irradiated under \(N_2\). Under the conditions used for photoradiation, 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 15 min.

**Gel Permeation Chromatography**—Gel permeation chromatography was performed on a Sephadex LH-60 (Pharmacia) column (1.5 \(\times\) 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-iodosobenzonic acid was performed according to Mahoney et al. (28). Proteinase K digestion was performed first at 25 \(^\circ\)C in 0.2 M sodium phosphate buffer, pH 8.0, for 5 h using 6 \(\mu\)g of the enzyme per 10 \(\mu\)g 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 350 \(\mu\)l of ethanol and 200 \(\mu\)l of \(H_2\)O 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 DMP (distilled over molecular sieve 4A) containing 10% NMM and 15% tBOC azide at 50 \(^\circ\)C for 5 h; this treatment did not hydrolyze ester bonds in phospholipids.

**Automated Edman Degradation of the Peptide Fragments from Cross-linked Cytochrome-b5**—Cross-linked 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 860C Sequencer and automated solid-phase sequencing method using \(\epsilon\) 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 \(^\circ\)C with 0.2 M Quadrrol/trifluoroacetic acid buffer, pH 8.2, in 1-propanol:\(H_2\)O (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 \(\mu\)l/min) and coupling buffer (80 \(\mu\)l/min), washing was performed for 3 min each with coupling buffer (80 \(\mu\)l/min), methanol, and benzene (1 ml/min each), and trifluoroacetic acid cleavage was performed for 15 min (100 \(\mu\)l, v/v). Ammonium hydroxide was used as the elution buffer in both sequencing methods. The 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-Nucleonics Inc., Fairfield, NJ) with 3-aminopropyltrietioxyxysilane. Arylaminio-glass was derived by derivatizing am-inopropyl-glass with \(a\)-bromo-\(p\)-nitrotoluene followed by reduction with sodium dithionite. Peptides were coupled to these glass beads by \(C\)-shows 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**

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**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 \(\mu\)mol of DPL and 0.5 \(\mu\)mol 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 \(^\circ\)C, and sonicated in a bath-type sonicator (80 watts, Laboratory Supplies Co., Hicksville, NY) at 45 \(^\circ\)C, in the dark, for 6 min with 30-s intervals between each 15-s sonication. The sonicated solution was optically clear indicating the formation of the Vesicles—Oriel 1000-watt Hg-Xe arc lamp (Model 8640 equipped with 9775-lamp, Oriel Corp., Stamford, CT) was used as the light source. The light beam was passed through a monochro-

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RESULTS

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

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

Gel Permeation Chromatography of Cross-linked Cytochrome bs

Vesicles Containing PC-I—Fig. 2 shows the result of chromatography of products obtained by photolysis of cytochrome bs 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 bs) remained with the cytochrome bs peak, whereas the reconstituted vesicles before irradiation retained only 0.05% of the radioactivity in the protein peak. In another control experiment, where cytochrome bs and the vesicles were irradiated separately and then mixed, 0.14% of the total radioactivity remained associated with cytochrome bs 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 bs) and 3.8% (0.19 mol), respectively, of input radioactivity was associated with cytochrome bs.

Vesicles Containing PC-II—Cytochrome bs, reconstituted into vesicles containing PC-II was irradiated for a longer time (40 min) due to slower photolysis of the dioleoyl ethanolamine group. Sephadex LH-60 chromatography of the photolyzed sample showed the radioactivity in PC-II to be associated with both monomeric cytochrome bs (5.6%) and also with a polymeric cytochrome bs 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 bs was reconstituted into vesicles containing only 14C-labeled DPL and the vesicles were photoirradiated under identical conditions, only 0.15% of DPL was associated with cytochrome bs, as determined by gel permeation chromatography. This result eliminates the possibility of photoexcitation of the protein and subsequent cross-linking to phospholipid.

Characterization of Photocross-linked Products

Degradation by Protease—Cytochrome bs, 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 (Rf 0.29). Since standard amino acids traveled in between PC-I and the origin in this TLC system, the newly formed bands were likely...
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 bs.

Digestion with Trypsin—As previously shown, trypsin digests the hydrophilic domain of denatured cytochrome bs 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 trypsin digest should indicate whether cross-linking is located between Leu-91 and Asp-133 or within the hydrophilic domain (Fig. 5). Fig. 6A shows the gel permeation profile of trypsic digest of cytochrome bs 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 bs was cross-linked, the profile of absorbance at 280 nm is predominantly that of uncross-linked peptides; 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. 6A) 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 between Leu-91 and Pro-94.

Cleavage with CNBr—As expected, CNBr cleaves rabbit liver cytochrome bs to give four peptide fragments (NH2 terminus to Met-95, Glu-96 to Met-126, Tyr-126 to Met-130, and Ala-131 to Asp-133, Fig. 5). These fragments separate on Sephadex LH-60 chromatography (19). Cytochrome bs cross-linked with PC-I gave three radioactive peaks (Fig. 6B). These were pooled as shown. Pool a contained 36% of total radioactivity and contained a mixture of the fragment (NH2 terminus to Met-95) and uncleaved cytochrome bs (19, 36). That the radioactivity in this pool was due to uncleaved cytochrome bs 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-linked peptide fragment, Glu-96 to Met-126, as judged by Edman degradation.

FIG. 4. Silica gel TLC of proteinase K digest of cytochrome bs 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 [3H]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. 5. Peptide fragments of rabbit liver cytochrome bs 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.

FIG. 6. Sephadex LH-60 chromatography of cross-linked cytochrome bs. A, trypsic digest of cytochrome bs cross-linked with PC-I; input radioactivity, 370,000 cpm. B, CNBr cleavage product of cytochrome bs cross-linked with PC-I; input radioactivity, 39,600 cpm. C, CNBr cleavage product of cytochrome bs cross-linked with PC-II; input radioactivity, 393,500 cpm. D, a-iodosobenzoic acid cleavage product of cytochrome bs cross-linked with PC-I; input radioactivity, 685,000 cpm. High absorbance (280 nm) after fraction 60 is due to a-iodosobenzoic acid.
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 bs cross-linked to PC-II gave peaks corresponding to uncleaved cytochrome bs, and a peptide Glu-96 to Met-125, and also a major radioactive peak at fraction 42 (Fig. 6C), which was not seen in cytochrome bs 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 bs (Fig. 5). These separated according to size on Sephadex LH-60. Cytochrome bs 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 (a–c in Fig. 6D). Since they all contained substantial amounts of radioactivity, they indicate a broad distribution of cross-linking, within the hydrophilic fragment.

**Hydrolysis of Cross-linked Cytochrome bs 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 bs 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 bs 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-diazinophenoxy)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 bs, 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.

**Analysis of Cross-linking Sites between Cytochrome bs and PC-I**

Cytochrome bs, 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.

**Table I**

<table>
<thead>
<tr>
<th>Phospholipid used in cross-linking</th>
<th>Conditions of hydrolysis*</th>
<th>Radioactivity released*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC-I NaOH, pH 12.5, 25 °C, 30 min</td>
<td>51</td>
<td>%</td>
</tr>
<tr>
<td>DMF, 10% NMM, 15% tBOC azide, 50 °C, 5 h</td>
<td>50</td>
<td>45</td>
</tr>
<tr>
<td>1.5 M HCl/MeOH, 55 °C, 15 min</td>
<td>43</td>
<td>Anhydrous trifluoroacetic acid, 50 °C, 8 h</td>
</tr>
<tr>
<td>PC-II NaOH, pH 12.3, 25 °C, 10 min</td>
<td>22</td>
<td>PC-I</td>
</tr>
</tbody>
</table>

*Condition of hydrolysis is described under "Experimental Procedures."
*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 bs cross-linked with PC-I.** The samples treated with NaOH, pH 12.5, at 25 °C for 30 min were spotted on Merck No. 5775 TLC plate and developed with Solvent I. The positions of standard ω-(m-hydroxymethylphenoxy)undecanoic acid and ω-(m-diazinophenoxy)undecanoic acid are marked as I and II, respectively. A, control; B, alkali-treated.
activity was 111,890 cpm. All sequencing results are aligned according to their amino acid sequences to make direct comparison easier.

Cross-linking was similar to that observed in Fig. 8B.

Fig. 8D 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 carbones 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 (8,9). 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 photolabile phospholipids, the tryptophan residues were the major sites of cross-linking (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. 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 (35)), 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 electrophilic 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 14C-labeled glycine ethylester-HCl which can only be explained by the transimidation reaction (39). The formation of imidates by the attack of phenytarben 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 topographical information can be obtained by this approach.

Previous work from the laboratories of Strittmatter (21, 22)
and Sato (24, 41, 42) and their respective colleagues has led to the following conclusions regarding the structure of the transformable form of cytochrome b₅ in vesicles. 1) The hydrophilic domain, which comprises from the NH₂ terminus to amino acid-90, must be in the aqueous phase outside of the vesicles. 2) The carbonyl side 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 b₅ 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 b₅. The distribution of cross-linking is broad. It extends from Ser-104 to Met-130. Although Tyr-129 has been concluded to be transferable form, our cross-linking data provide a refinement of cross-linking is broad. It extends from Ser-104 to Met-130. This might be interpreted to mean that Tyr-129 is transferable form of the hydrophobic segment (25), and Tyr-129 is envisioned by occasional tilting of the α-helix from Ala-116 to Met-130.

Assuming the loop-back structure to be correct for the transformable 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.

REFERENCES

Cross-linking of Cytochrome b₅ to Photoactivatable Phospholipids