The Transmembrane Domain of Glycophorin A as Studied by Cross-linking Using Photoactivatable Phospholipids*

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Glycophorin A, the major sialoglycoprotein of the human erythrocyte, consists of an NH₂-terminal carbohydrate-rich region which forms a transmembrane bridge, and a COOH-terminal hydrophilic region extending into the cytoplasm. With the aim of further defining the membrane-embedded region, the protein has been reconstituted into vesicles formed from dimyristoylphosphatidylcholine and phospholipids containing photosensitive carbene precursors. The photosensitive groups were incorporated either at the ω-position of sn-2 fatty acyl chain or the polar head group of lecithins. Following photolysis, covalent cross-linking (1-2%) of the photoactivatable phospholipids to the protein was demonstrated. Degradation and sequence analysis showed that in the case of phospholipids containing photoactivatable groups in the fatty acyl chains most of the covalent cross-linking involved the carboxyl group of Glu-70. Therefore, the latter residue must be within the bilayer. This conclusion was supported by the reaction of the membrane-permeant [14C]dicyclohexylcarbodiimide with glycophorin reconstituted into vesicles. The same residue was labeled. Photolysis of glycophorin vesicles containing phospholipids with photolabels in the polar head group gave products in which the cross-links were present in peptide fragments (residues 52-81 and 82-96). These results define the probable boundaries of the membrane-embedded segment of glycophorin A. Corresponding experiments with erythrocyte ghosts gave similar results.

Glycophorin A, the major sialoglycoprotein of the human erythrocytes, is a widely studied and well characterized membrane protein (1). The protein, which consists of a single polypeptide chain of 131 amino acids, has been shown to have a tripartite structure (2). The NH₂ terminus of this protein is exposed to the outside of the erythrocyte membrane and carries all of the carbohydrate (3). The protein then traverses the membrane through a predominantly hydrophobic region of about 20 amino acids and, finally, there is a hydrophilic segment at the COOH terminus which is on the cytoplasmic face of the membrane (4). Further, the total amino acid sequence of the protein has been determined (2). Because of this chemical progress, glycophorin A is especially attractive for further structural studies.

A general chemical approach that aims at covalent cross-linking between membrane proteins and phospholipids is being investigated in this laboratory (5). Photoactivatable groups capable of generating reactive carbene intermediates are incorporated into phospholipids as structural components in two types of phospholipids. In one type, the photoactivatable groups are introduced by chemical synthesis (6, 7) into the ω-position of the fatty acid present at the sn-2 position of the glycerol backbone (phospholipids I-III). The aim in the use of these phospholipids is to achieve covalent cross-linking between the intramembranous regions of the membrane proteins and phospholipids (8, 9). In the second type of photoactivatable phospholipids, the label is introduced in the polar head groups of the phospholipids (e.g. phospholipids IV and V) (7). Proteins reconstituted into vesicles containing such phospholipids should form cross-links with segments of the polypeptide chains immediately exposed to the outside of the membrane.

In this paper, we report on the use of the photoactivatable phospholipids (I-V, Fig. 1) in a study of the membrane-embedded segment and its flanking regions in glycophorin A. The results using phospholipids I-III (Fig. 1) showed, in particular, that Glu-70 is embedded in the membrane and this conclusion was further supported by the reaction of [14C]dicyclohexylcarbodiimide with glycophorin reconstituted into vesicles. The use of phospholipids IV and V (Fig. 1) provided additional insight into the regions flanking the membrane in the reconstituted glycophorin vesicles. Based on these studies and on previous physical and biochemical data, a model is proposed for the transmembrane structure of glycophorin A. The model places residues 63-94 within the membrane in an α-helical conformation. Several polar residues which are embedded within the bilayer may form salt bridges or hydrogen bonds to mask their hydrophilicity.

**EXPERIMENTAL PROCEDURES**

**Materials**

Mercaptopapain and trypsin were from Worthington Biochemical Corp. Proteinase K was purchased from Boehringer Mannheim Biochemicals. Affinity-purified neuraminidase (Type IX) from Clostridium perfringens and cholic acid were obtained from Sigma Chemical Co. The cholic acid was decolorized with activated charcoal and then recrystallized twice from acetone. DMPC was from Calbiochem-Behring Corp. Sephadex LH-20 and LH-60 were obtained from Pharm...
macia Fine Chemicals. Bio-Gel A-1.5m, Bio-Gel A-15m, and Bio-Beads SM-2 were from Bio-Rad Corp. Wheat germ agglutinin-Sepharose was purchased from Vector Laboratories. Plastic-backed silica gel plates (Silica Gel 60) was purchased from E. M. Laboratories. Hydofluor was purchased from National Diagnost Corp. (°C)

Methyl iodide (10.0 M Ci/mmol) and [2,4-3H]choli acid (14 Ci/mmol) was then mixed with the protein solution; the solution was kept at room temperature for 1 h.

Hydrofluor was purchased from National Diagnostic Chemical Co. as a 30% aqueous solution under the name Ammonyx LO. The detergent was extracted with organic solvents with Kodak X-5 film.

Phospholipids II (Fig. 1) and ["CIDMPC were 48 and 4.4 mCi/mmol. Glycophorin A showed a weak ultraviolet absorbance consistent with the composition calculated from the amino acid

The detergent was then dried from ethanol to remove traces of water. The detergent was further purified as described (11).

Methods
Thin layer chromatography was performed using the following solvent systems: Solvent 1, chloroform/methanol/water (65:25:4, v/v/v); Solvent 2, hexane/isoproplther/isopropanol/acetic acid (2:1:1, v/v/v/v); Solvent 3, hexane/acetic acid/1.2-dichloroethane (30:22:25, v/v/v/v); Solvent 4, hexane/acetic acid/1.2-dichloroethane (90:20:25, v/v/v). Autoradiography was done at room temperature with Kodak XAR-5 film. For fluorography, silica gel plates (Silica Gel 60) that had been tritiated, the sample was sprayed with 2.5-diphenyloxazole in tolane and Kodak XR-5 film was exposed at -80°C. Liquid scintillation counting was performed using Hydofluor.

Synthesis of Phospholipids, Fatty Acids, and ["CIDicyclohexylcarbodiimide was used to couple hydrophobic containing m-diazonaphthyl groups in the fatty acyl chain and pyridyl diazirine in the polar head group were synthesized as described (7). ["CIDMPC labeled in the N,CH3 group was synthesized by published procedures (12).

Isolation of Glycophorin A—Human blood (not outdated) was obtained from the Boston Red Cross. A mixture of glycophorin A, B, and C from the erythrocytes was prepared according to Marchesi and Andrews (14). Glycophorin A was purified by passage through a column (2.9 x 94 cm) of Bio-Gel A-1.5m equilibrated with 0.1% (w/v) dodecylmethylammonium oxide, 25 mM NaCl, 5 mM sodium phosphate, pH 5.0, 0.01% Nα-sucinylsphingosine (15). Finally the glycophorin A preparation was delipidated by successive extractions with chloroform/methanol (1:1, v/v) and chloroform/methanol/concentrated HCl (150:150:1, v/v/v).

Amino acid analysis of glycophorin A prepared as above was consistent with the composition calculated from the amino acid sequence previously reported (2). The sialic acid content of the preparation was 21% (w/w), and the phosphate content was 0.42 ± 0.05 mol/mol. Glycophorin A showed a weak ultraviolet absorbance (E280 = 3.4 in 100 mM Tris, pH 7.5).

Incorporation of Glycophorin A into Artificial Vesicles—Single bilayer vesicles containing glycophorin A and phospholipids were prepared by the cholate dialysis method (16). In a typical experiment, 1.0 mg of DMPC or a mixture of DMPC and radioactive diazirine-containing phospholipid (e.g., III, Fig. 1) (usually at a ratio of 9:1 by weight) in chloroform solution was dried under nitrogen and the residue was suspended in 1.25 ml of 10 mM sodium cholate, pH 7.5, by vigorous Vortex mixing. The solution was sonicated for about 15 min in a bath sonicator (Laboratory Supplies Co., Hicksville, NY). A solution of glycophorin A (0.2 mg in 0.1 ml of water) was mixed with 10 mM sodium cholate (0.1 ml), pH 7.5. The sonicated lipid solution was then mixed with the protein solution; the solution was kept at room temperature for 1 h and then dialyzed at room temperature against 1 liter of 100 mM Tris, pH 7.5, containing 10 ml of SM-2 Bio-Beads that had been extensively washed with water. Following a change of the buffer after 24 h, the dialysis was continued for another 24 h. Vesicles were collected by centrifugation (100,000 x g) for 0.5 h, resuspended in the same buffer (0.25 ml), and stored at 4°C.

Analysis of Residual Cholic Acid in Vesicles—The amount of cholic acid that remained in the dialysed vesicle preparation following the above dialysis procedure was monitored by including some [3H] cholic acid. Following extraction of the lipids (10), the cholic acid was determined by thin layer chromatography (Solvent 1) and fluorography.

Sucrose Density Gradient Centrifugation—Samples of vesicles containing glycophorin A, DMPC, and a diazirine-carrying phospholipid (phospholipid II, Fig. 1) were layered on a linear sucrose density gradient (5-30% w/w). They were centrifuged at 149,000 x g for 15-24 h. The phospholipid distribution was measured by including either ["CIDMPC or radioactive phospholipid I (Fig. 1) during the preparation of the vesicles. Glycophorin A was measured by analysis of sialic acid after removal of sucrose by dialysis.

Orientation of Glycophorin A in Reconstituted Vesicles—Vesicles containing 12 μg of glycophorin A were incubated at room temperature with 0.019 unit of C. perfringens neuraminidase. The release of sialic acid was measured by one of two methods. In the first method, the reaction was terminated by adding SDS to 1% (w/v) concentration and sialic acid was assayed directly (17). No release of sialic acid was observed when neuraminidase was added to vesicles predissolved in 1% SDS. In the second method, 1.9 ml of 100 mM triethylamine bicarbonate (pH 8) at 0°C were added to the reaction mixture. The vesicles were pelleted by centrifugation (105,000 x g for 30 min), and after total solubilization, the acid contents of the pellet and of the supernatant solution were determined.

Papain was also used to determine the orientation of glycophorin A in the vesicles. After activation with 0.5 mM EDTA and 0.5 mM cysteine, mercapto-papain was incubated at room temperature with the vesicles at a protease/glycophorin A ratio of 1:40 (w/w). The solution was analyzed by the addition of 70 mM iodoacetate, and the sample was centrifuged as described above. Sialic acid was assayed after hydrolysis with 0.1 M H2SO4 at 80°C for 1 h.

Photolyses—Light from a 1000 W arc lamp from Oriel Corp. was passed successively through a 0.7 primary focusing lens, a water heat filter, an 820 secondary focusing lens, and a monochromator set at 366 nm, and a 345 nm cut-off filter from Schott Optical Glass, Inc. Unless noted otherwise, the sample was irradiated in a quartz cuvette held in a thermostated metal block. Water-saturated nitrogen was blown into the cuvette for 20 min prior to and during the photolysis. Under these conditions, the ultraviolet spectra of both bovine serum albumin and free tryptophan were unchanged after 10 min of photolysis. Unless stated otherwise, samples containing phospholipids I, II, and III (Fig. 1) were irradiated at 30°C for 4 min, and samples containing phospholipids IV and V for 10 min. The length of time between the termination of photolysis and solubilization of the vesicles (organic solvents) or detergent was at least 30 min unless stated otherwise.

Separation of Cross-linked Glycophorin A from Free Phospholipids—After photolysis, the solution containing the vesicles was lyophilized and the residue was dissolved in 88% formic acid/ethanol/water (20:60:30, v/v/v) or in some cases 88% formic acid and ethanol were added directly to form a solution with the same final concentration. The cross-linked glycophorin A was separated from the phospholipids on a Sephadex LH-60 column (10 x 45 cm) equilibrated with the same solvent. Fractions of 0.8 ml were collected every 5 min.

Reaction of ["CIDCC with Glycophorin A in Reconstituted Vesicles—Vesicles containing 0.21 mmol of glycophorin A and 46 mmol of DMPC were suspended in 0.2 ml of 100 mM Tris, pH 7.5. ["CIDCC (2-78 nm) dissolved in ethanol was added to the above suspension and then kept at room temperature for 16-18 h. The final ethanol content was 1% or less. Then formic acid was added to 25% (v/v) and the final solution was applied to form a solution with the same final concentration. The cross-linked glycophorin A was separated from the phospholipids on a Sephadex LH-60 column (10 x 45 cm) equilibrated with 88% formic acid/ethanol/water (20:50:30, v/v/v).

Fragmentation of Glycophorin A—CNBr cleavages of small amounts (50 μg) of cross-linked glycophorin A were carried out in 25 μl of a stock solution of CNBr in 70% formic acid (25 mg/ml) for 48 h at room temperature.

Digestion of the cross-linked glycophorin A with trypsin was carried out for 16 h at 37°C in 100 mM Tris, pH 7.5 (18). The weight ratio of the enzyme to the cross-linked protein was 1:30. The solution was adjusted to pH 4 with acetic acid and then filtered through a 0.45-μm Millipore filter. After rinsing the filter three times with 0.1 M HCl, an aliquot of the filtrate was counted for radioactivity.

Proteolysis of Glycophorin A in Photoalyzed Vesicles and the Isolation of Cross-linked Peptides—Mercapto-papain, which had been added with 0.5 mM EDTA, 0.5 mM cysteine, was added to photoalyzed glycophorin A containing vesicles at a ratio of 1 mg of papain/2 S. Kirchhaken, unpublished work.
25 mg of glycophorin A. The proteolysis was carried out at room temperature for 1 to 3 h and terminated by incubation with 10 mM iodoacetate for 10-15 min at room temperature. The proteolysed sample was then applied to a Bio-Gel A-15m column (1.5 x 41 cm) equilibrated with 100 mM Tris, pH 7.5, at 4°C. The vesicle-containing fractions were at the excluded volume of the column; these were pooled, dialyzed briefly to remove salt, and lyophilized.

**Analytical Methods—**Automated Edman degradation was performed in a Beckman 890C Sequencer. Polybrene (2 mg) was added to the cup to minimize washout of the sample (19). The program was modeled after the standard Beckman fast 1.0 M Quadrol program, but using 0.2 M Quadrol and reduced separate benzyl bromide and ethyl acetate washes. The thioldiones were converted to thiodythionoains with a Sequemat P-6 autoconverter in 1.5 M HCl in methanol. Phenylthiodythonoains were identified by high pressure liquid chromatography on a µBondapak C₈ column in a methanol gradient (14-55%, v/v) in 0.01 M sodium acetate, pH 4.1 (20) or by amino acid analysis after hydrolysis (24 h at 110°C in 55% HCl).

Amino acid analyses were performed with a Beckman 119C amino acid analyzer. Samples were hydrolyzed for 24 h at 110°C in sealed evacuated tubes containing either 6 N HCl or 3 M mercaptoethane-sulfonic acid. Depending upon the age of the resin and the particular amino acid analysis program, phenylalanine and tyrosine were sometimes derivatized by amino sugars.

**Base-catalyzed Hydrolysis of Radioactive Fractions from the Protein Sequencer, Phospholipids, and Cross-linked Peptides—**Hydrolysis of the radioactive fractions from the Sequencer was performed in 0.02 M NaOH in 50% ethanol at room temperature for 1 h. The sample was then neutralized with HCl and extracted (10), and the extract was dried in vacuo.

Phospholipids were hydrolyzed in 0.01 N NaOH in 50% ethanol at room temperature overnight. The mixture was then neutralized and extracted with an equal volume of diethyl ether, and the organic phase was dried in vacuo. Cross-linked glycophorin A and cross-linked proteolytic fragments rich in eteobhydrox were hydrolyzed at room temperature overnight in 7.2 M urea, 0.1 M aqueous NaOH, or 0.1 M NaOH in 50% ethanol (v/v). Cross-linked polypeptides lacking sugars were treated with vigorous Vortex mixing followed by bath sonication for 5 min. Glycophorin A was eluted with 0.5 M tetrabutylammonium hydroxide in dimethyl sulfoxide for 8000 min. Glycophorin A was analyzed by freeze-fracture electron microscopy. Single lamellar vesicles were seen with a diameter of 640 ± 220 Å (data not shown). Intramembranous particles analogous to those seen by previous workers (31, 32) were observed.

In some preparations, radioactively labeled cholate was

\[
\text{H}_2\text{C}_2\text{O}_3\text{O}_R \text{R}_1
\]

A. \[ \text{R}_2 = \text{C}_3 \text{H}_7 \text{O} - \text{C}_3 \text{H}_7 \text{N} - \text{CH}_3 \]

B. \[ \text{R}_2 = \text{O} - \text{C}_3 \text{H}_2 \text{CH}_3 \]

IV. \[ \text{R}_3 = \text{N} \]

V. \[ \text{R}_4 = \text{H} \]

FIG. 1. **Structures of the synthetic photoreactive phospholipids.** A, phospholipids I, II, and III contain aryl diazirines as an integral part of the fatty acid chain and are designed to form cross-links within the bilayer. B, phospholipids IV and V contain a diazirine-nepinoid moiety in the polar head group. They are designed to react with groups at or near the surface of the bilayer. *C* is indicated by *.
Vesicles containing glycophorin A, DMPC, and phospholipid above were used to study phospholipid-protein cross-linking. The large fraction of glycophorin A molecules is oriented with the NH2 terminus exposed to the outside of the vesicles. The remaining sialic acid was extremely stable, being un-photolyzed at 2000°C for 30 min. The bound phospholipid included in the reconstitution mixture to be dialyzed. With two lots of commercial preparations of [3H]cholic acid, a substantial fraction (0.3%) of the radioactivity remained in the dialysate. However, when [3H]cholic acid purified as described under "Methods" was used, only 0.015% of the radioactivity was retained by the vesicles, an amount corresponding to a ratio of 1 detergent molecule to 800 phospholipid molecules. This result was independent of the presence of glycophorin A in the reconstitution mixture.

**Orientation of Glycophorin A in Reconstituted Vesicles**—The orientation of glycophorin A in reconstituted vesicles was determined by the addition of the membrane-impermeable enzymes neuraminidase and papain. Glycophorin A-containing vesicles were incubated with neuraminidase (1.5 units/ng glycophorin A) at room temperature. A loss of 95 ± 5% of the total sialic acid was observed from vesicles containing 90% DMPC and 10% phospholipid I (Fig. 1); or 87% DMPC and 13% phospholipid IV. The neuraminidase removed 75 ± 5% of the total sialic acid from the glycophorin A in vesicles containing 90% DMPC and 10% phospholipid I. In a control, the neuraminidase removed 100% of the sialic acid from soluble glycophorin. About 75% of the sialic acid was released by papain from vesicles containing glycophorin A, 90% DMPC, and 10% phospholipid I. This proteolytic enzyme has been shown to effectively cleave glycophorin A in red blood cell ghosts (33). With both neuraminidase and papain, the reaction reached a plateau in 15 min. The remaining sialic acid was extremely stable, being uncleaved even after 3 h, demonstrating that neither neuraminidase nor papain penetrates the vesicles. The large fraction of sialic acid which was released shows that the majority of glycophorin A molecules is oriented with the NH2 terminus exposed to the outside of the vesicles.

**Cross-linking of Glycophorin A to Phospholipids on Photolysis of Vesicles**—The glycophorin A vesicles described above were used to study phospholipid-protein cross-linking. Vesicles containing glycophorin A, DMPC, and phospholipid I were photolyzed at 30°C for 4 min. The bound phospholipid (2.6%) was well separated from the free phospholipid by column chromatography on Sephadex LH-60 (Fig. 3). Little radioactivity chromatographed with glycophorin A in control experiments (Table I) in which the vesicles were not photolyzed or in which lipid and protein were photolyzed separately or in which [14C]DMPC was used instead of one of the photolabeled lipids shown in Fig. 1.

The stability of the cross-linked protein-lipid conjugate was tested by two methods. The cross-linked protein-lipid adduct was isolated as shown in Fig. 3. The fractions (10-12, Fig. 3) containing the conjugate were pooled and then dried. The residue was redissolved and analyzed by SDS-PAGE or again passed through the Sephadex LH-60 column. In both cases, at least 85%, and usually greater than 90%, of the total radioactivity remained with the glycophorin A. Hence, by two independent methods the radioactive phospholipids associated with glycophorin A appeared to be covalently cross-linked to the protein. Glycophorin A vesicles containing the phospholipids III-V shown in Fig. 1 were also photolyzed as described under "Methods" was used, only 0.015% of the radioactive phospholipid was isolated and analyzed for sialic acid and radioactive phospholipid.

**Fig. 2.** Sucrose density gradient profiles of glycophorin A mixed with preformed dialyzed phospholipid vesicles (A) and of glycophorin A reconstituted into vesicles containing radioactive phospholipid I and DMPC (1:9) as under "Experimental Procedures" (B). After centrifugation, 0.36-ml fractions were collected and analyzed for sialic acid and radioactive phospholipid.

**Table I**

| Radioactive phospholipid | Irradiation | Extent of cross-linking |
|--------------------------|------------|------------------------|
| DMPC^a                   | +          | 0.007                  |
| I                        | +          | 0.21                   |
| IV                       | +          | 1.8^b                  |
| III                      | +          | 0.13                   |
| IV                       | +          | 0.26                   |
| V                        | +          | 0.74^c                 |
| V                        | +          | 1.06                   |

^a The photolabel-containing phospholipid was nonradioactive phospholipid.

^b The extent of cross-linking was not altered by the addition of 15 mM glutathione, indicating that glutathione was not able to scavenge the reactive intermediate formed from either phospholipid.

^c Phospholipid vesicles and glycophorin A were photolyzed separately and then mixed prior to analysis.
described under "Methods." Phospholipid III which differs from phospholipid I only in the length of the photoreactive fatty acyl chain reacted in a similar manner (Table I). The phospholipids shown in Fig. 1B which contain a pyridyl diazirine in the polar head group reacted to a lesser extent (Table I).

Kinetics of Cross-linking of Phospholipids to Glycophorin A—The kinetics of lipid-lipid and lipid-protein cross-linking was similar as a function of the photolysis time (Fig. 4A). Even though cross-linking occurred mainly between phospholipid molecules, 2-3% of the photolabeled phospholipid (a substantial amount considering the ratios of the three different components in the vesicles) cross-linked to glycophorin A. The kinetic data suggested that the two cross-linking reactions occurred by a similar mechanism, the rate-determining step being the photolytic formation of the carbene intermediate (the half-life of such intermediates may be 10⁻⁶-10⁻⁷ s or even less). However, it should be noted that, in the experiment of Fig. 4A, the aliquots from photolysis mixture were kept for about 30 min before analysis (see "Experimental Procedures"). In the next experiment, vesicles were photolyzed for 4 min and aliquots were removed at different time intervals after photolysis, with the denaturation buffer (see "Experimental Procedures"), and the extent of cross-linking was measured by SDS-PAGE. As seen in Fig. 4B, the cross-linking of phospholipid I to glycophorin A in samples kept for 1 h or more prior to the addition of denaturation buffer was 2- to 3-fold greater than in samples immediately mixed with denaturation buffer. Thus, at least 50-75% of the cross-linking to glycophorin A seemed to occur by a long lived intermediate and not by a carbene.

In contrast to the above results with phospholipids containing membrane-embedded photosensitive groups, no significant postphotolysis reaction was observed in experiments with vesicles prepared from phospholipid IV (data not shown).

Identification of Cross-linked Glycophorin A Peptides—Glycophorin A cross-linked to radioactive phospholipids was isolated (Fig. 3) and cleaved using trypsin or CNBr. The insoluble tryptic fragment, T6 (residues 62-96), which has previously been isolated and characterized by Furthmayr et al. (18) was retained on a Millipore filter. In experiments with phospholipids I, III, IV, and V, 96-98% of the radioactivity in the cross-linked glycophorin A after tryptic digestion was retained on the filter. In a control experiment in which trypsin was omitted, only 15-20% of the radioactivity was retained on the filter. In some experiments, the radioactive compounds retained on the filter were eluted and electrophoresed (24). Their electrophoretic mobility was similar to that of fragment T6.

The CNBr fragments of the glycophorin A were also analyzed by gel electrophoresis (24). CNBr cleavage of glycophorin A results in four fragments previously designated by Tomita et al. (2) as follows: CB-1 (residues 9-81), CB-2 (residues 82-131), CB-3 (residues 1-8), and a partially cleaved fragment (residues 1-81). Fragment CB-3 does not appear in the SDS-PAGE analysis since it was too small to be stained. CNBr cleavage of glycophorin A cross-linked to phospholipid I gave the distribution of radioactivity shown in Fig. 5A. Thus, the bulk of radioactivity traveled with the mobility characteristic of CB-1. The radioactivity near the front (slices 15-22) was presumably nonpeptidic. Neither in this experiment nor in that performed using phospholipid III, was any significant radioactivity found in the region of CB-2.

In experiments using phospholipids IV and V, the distribution of radioactivity in CNBr peptides was markedly different (Fig. 5B) from those described above. There was a distinct peak of radioactivity associated with CB-2.

Isolation of Cross-linked Peptidic Fragments—In order to further characterize the site of phospholipid cross-linking, a cross-linked peptide fragment suitable for sequencing was isolated and characterized by amino acid analysis and gel electrophoresis.

![Fig. 4. Time course for lipid-lipid and lipid-protein cross-linking on photolysis of vesicles containing glycophorin A, DMPC, and phospholipid I, A, the vesicles were photolyzed and then incubated for 30 min before analysis. The cross-linking of photoreactive phospholipid I to glycophorin A was measured by chromatography on Sephadex LH-60 (Fig. 3). Lipid-lipid cross-linking was measured by thin layer chromatography (inset) on silica gel with Solvent 1. The phospholipid-phospholipid dimer is marked by a square in the inset. The arrow indicates the direction of solvent flow. B, shows the effect of varying the length of the incubation after irradiation. Vesicles containing radioactive phospholipid I and glycophorin A were photolyzed for 4 min. This longer time was given to provide for any variation in lamp intensity and to allow for decomposition of the diazo intermediate (see text). The sample was immediately mixed with denaturation buffer or incubated for different lengths of time as shown and then mixed. The extent of cross-linking was determined by SDS-PAGE.](http://www.jbc.org/)
Glycophorin A was reconstituted into vesicles containing phospholipid II and DMPC (1:9 by weight). The vesicles were photolyzed and then digested with papain as under “Experimental Procedures.” About 70% of the sialic acid was removed by papain, a value comparable to that observed with vesicles which had not been photolyzed. The photolyzed vesicles were then isolated on a Bio-Gel A-15m column (see “Experimental Procedures”) with an 85% recovery of the radioactivity. The possibility that protein in the vesicles was loosely bound, rather than being present in a transmembranous arrangement, was tested by amino acid analysis. Phenylalanine, an amino acid present exclusively in the protected hydrophobic segment, was recovered in 75% yield.

The isolated vesicles were lyophilized and dissolved in 88% formic acid/ethanol/water (20:50:30, v/v/v) and chromatographed on a Sephadex LH-60 column (Fig. 6). The protein-containing fractions were labeled A, B, and C. The peaks labeled D and E had insignificant amino acid content. Peak A stained with Coomassie blue and periodic acid-Schiff reagent which had not been photolyzed. The photolyzed vesicles were stained with Coomassie blue and periodic acid-Schiff reagent. The photolyzed vesicles were subjected to 10 cycles in the Sequencer (data not shown). Intact glycophorin A that had been cross-linked with phospholipid I was subjected to 10 cycles in the Sequencer; 12% of the radioactivity was released. Since proteolytic fragmentation studies discussed earlier showed no cross-linking in the NH₂-terminal region, this small amount of released radioactivity was probably due to cleavage of cross-links that were labile under sequencing conditions.

Peptide B (Fig. 6) was chosen for sequence analysis because the site of photophospholipid cross-linking appeared to be near its NH₂ terminus and, further, it has a polar COOH terminus to retain the peptide in the Sequencer cup. The results obtained on stepwise degradation are shown in Fig. 7A. The phenylthiolydantoin amino acids identified by high performance liquid chromatography and amino acid analysis were consistent with a homogeneous NH₂ terminus, Leu-64. No other phenylthiolydantoin amino acids above background were observed. The repetitive yield for sequence analysis of peptide B (residues 64–131) was 90%, a significantly higher yield than that observed (68%) for fragment T6 (residues 62–96). A large fraction (69 ± 16%) of the radioactivity was released during the stepwise degradation. As seen in Fig. 7A, the radioactivity in the cross-linked phospholipid II was released most strongly in the benzene-ethyl acetate wash of cycle 8. Evidently, the thiazolinone corresponding to the cross-linked glutamic acid was not effectively extracted by n-chlorobutane and, instead, was extracted by ethyl acetate wash during the following cycle. Lifter et al. (34) noted a similar phenomenon during the sequencing of photoaffinity-labeled immunoglobulin and Bayley et al. (35) encountered this in the sequencing of a modified bacteriophodospin peptide.

The relative molar amounts of fractions A, B, and C were 20–30% A, 55–70% B, and 0–15% C in vesicles prepared with 90% DMPC and 10% phospholipid II and 10–20% A, 60–80% B, and 0–15% C in vesicles prepared with 90% DMPC and 10% phospholipid III. The proportions of the fractions obtained in one of the above vesicle preparations (90% DMPC + 10% phospholipid II) was also checked by calculating the amino acid composition of the papain-cleaved vesicles from these relative mole fractions. The predicted values (Table II, second column in parentheses) were in good agreement with the values observed.

### Table II

Amino acid analyses of glycophorin A and papain-generated peptides from Fig. 6

| Glycophorin A | Papain-cleaved vesicles | Peptide A | Peptide B | Peptide C |
|--------------|-------------------------|-----------|-----------|-----------|
| Asx          | 7.8 (8)                 | 6.0 (4.4) | 5.9 (4)   | 4.0 (5)   | 0.8 (1)   |
| Thr          | 14.6 (15)               | 7.2 (6.0) | 12.6 (13) | 3.6 (4)   | 3.0 (2)   |
| Ser          | 17.4 (18)               | 10.4 (9.1)| 14.1 (14)| 6.4 (8)   | 3.3 (4)   |
| Gln          | 14.4 (15)               | 7.5 (6.8)| 11.0 (11)| 4.6 (6)   | 3.1 (2)   |
| Pro          | 9.9 (10)                | 7.7 (6.2)| 8.2 (6)   | 5.7 (7)   | 2.1 (2)   |
| Gly          | 6.0 (5)                 | 4.3 (4.2)| 7.0 (5)   | 4.1 (4)   | 5.2 (4)   |
| Ala          | 6.0 (6)                 | 4.1 (3.0)| 6.7 (6)   | 2.1 (2)   | 2.6 (2)   |
| Cys          | 0.0 (0)                 | 0.0 (0)   | 0.0 (0)   | 0.0 (0)   | 0.0 (0)   |
| Phe          | 2.1 (2)                 | 2.0 (2.0)| 2.0 (2)   | 0.0 (0)   | 0.0 (0)   |
| Leu          | 9.1 (11)                | 5.9 (9.2)| 9.3 (10)| 6.5 (9)   | 7.5 (8)   |
| Ile          | 8.0 (8)                 | 6.6 (6.6)| 8.5 (6)   | 6.2 (7)   | 6.2 (5)   |
| Val          | 5.1 (4)                 | 4.0 (4.1)| 4.0 (4)   | 4.0 (4)   | 4.0 (4)   |
| Thr          | 1.9 (2)                 | 1.0 (1.2)| 3.1 (2)   | 1.1 (1)   | 1.2 (1)   |
| Met          | 1.9 (2)                 | 0.0 (0)   | 0.0 (0)   | 0.0 (0)   | 0.0 (0)   |
| Ile          | 2.1 (2)                 | 2.0 (2.0)| 2.0 (2)   | 0.0 (0)   | 0.0 (0)   |
| Phe          | 4.9 (5)                 | 2.8 (2.8)| 4.3 (5)   | 1.9 (2)   | 1.9 (2)   |
| Ser          | 4.4 (5)                 | 3.5 (3.2)| 5.0 (4)   | 2.8 (3)   | 1.7 (2)   |
| Arg          | 5.7 (6)                 | 3.6 (3.0)| 5.8 (6)   | 1.9 (2)   | 2.0 (2)   |
| Trp          | 0.0 (0)                 | 0.0 (0)   | 0.0 (0)   | 0.0 (0)   | 0.0 (0)   |

* a, obscured by amino sugars.

b Determined in a separate hydrolysis with 3 M mercaptoethanesulfonic acid.
Edman sequencing of glycoporphin A cross-linked to phospholipids I or III gave similar results. Glutamic acid-70 was, therefore, accessible to the reactive intermediates of all the three phospholipids (I-III) and the location of the $^{14}$C either in the C-1 or C-2 fatty acyl chain did not significantly affect these results.

**Characterization of the Major Radioactive Compound Released during the Sequencing of Cross-linked Peptide B**

The product released in cycle 7 of the Edman degradation shown in Fig. 7A. The thiazoline derivative of glutamic acid is linked to radioactive phospholipid I residue through a benzylic ester linkage. The cross-link arises from the carbene insertion and reaction of the diazo intermediate following photolysis. VII and VIII are the radioactive products expected on base treatment of VI. These were found to be identical with authentic samples as synthesized in the test.

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**Release of the Radioactive Benzylic Alcohol (VIII) by Base Treatment of Cross-linked Glycoporphin A**—The experiments described above showed that much of the cross-linked phospholipid is attached to glutamic acid-70 in glycoporphin A by an ester linkage. If so, compound VIII (Fig. 8) should be released by direct base treatment of cross-linked glycoporphin A.

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**Fig. 8.** Structure (VI) of the expected radioactive compound released at cycle 7 of Edman degradation shown in Fig. 7A. The thiazoline derivative of glutamic acid is linked to radioactive phospholipid I residue through a benzylic ester linkage. The cross-link arises from the carbene insertion and reaction of the diazo intermediate following photolysis. VII and VIII are the radioactive products expected on base treatment of VI. These were found to be identical with authentic samples as synthesized in the test.

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**Fig. 9.** Autoradiogram of the compounds generated by base-catalyzed hydrolysis of free phospholipid, peptide A, and peptide B (Fig. 8). The thin layer chromatogram (silica gel) was developed with Solvent 4. Lane 1: as a control, the material in the free phospholipid peak (Fig. 3) was treated with base. Lane 2: peptide A cross-linked with phospholipid II was treated with base and the hydrolysis products were separated on a Sephadex LH-20 column. The lower molecular weight radioactive material was applied on TLC. Lane 3: same as lane 2 except that peptide B was used. The thin layer chromatogram (silica gel) was developed with Solvent 4. SF, solvent front.
Vesicles containing glycophorin A and phospholipid II were photolyzed and the cross-linked glycophorin A was separated from the free lipid on a Sephadex LH-60 column. The free lipid, cross-linked glycophorin A, and the cross-linked peptides A and B prepared as in Fig. 6 were treated with base as described under "Experimental Procedures." The release of radioactivity from glycophorin A, peptide A, and peptide B was up to 75-85%. Radioactive compounds released by base treatment of the [14C]DCC-glycophorin A adduct showed that the hydrophobic fragment T6 (residues 62-96) was the only segment cross-linked. Edman sequence analysis showed that greater than 95% of the radioactivity was associated with the segment containing residues 62-81 (T6 and CB-1).

Cross-linking Experiments Using Phospholipids IV and V (Fig. 1)—Sequencing experiments as described above were carried out with peptide B generated from glycophorin A which had been cross-linked with either phospholipid IV or V. Unsatisfactory results were obtained. Essentially all of the radioactivity was released prior to the addition of phenylisothiocyanate, indicating that the cross-links formed by these phospholipids were not stable to sequencing conditions. Therefore, the chemical nature of cross-links produced in these experiments is not clear.

Cross-linking of the phospholipids with glycophorin A reconstituted into DMPC vesicles—Glycophorin A (0.21 nmol) reconstituted into DMPC vesicles was incubated with 2-78 nmol of [14C]DCC added as an ethanol solution (the final concentration of ethanol in the mixture did not exceed 1%). Under these conditions, about 0.6 nmol of [14C]DCC/nmol of glycophorin A (Fig. 10) was incorporated as judged by the radioactivity chromatographing with the protein in the excluded volume. The point of linkage of [14C]DCC was analyzed as described for phospholipid III in red blood cell ghosts. A small percentage (0.05-0.10%) of the [14C]DCC reacted with glycophorin A corresponding to the thiazolinone VI (Fig. 8). Their identity was confirmed by sequence analysis, [14C]DCC (11.5 nmol/nmol of glycophorin A) was incubated with the protein vesicles and peptide B was prepared as described above by papain cleavage of the vesicles. The results of stepwise degradation, which are given in Fig. 7B, showed that all of the radioactivity was released at cycle 7 and thus, glutamic acid-70 was the sole site of reaction of [14C]DCC with glycophorin A.

Fig. 10. Reaction of [14C]DCC with glycophorin A in DMPC vesicles. The [14C]DCC dissolved in ethanol (overall concentration < 1%) was added to glycophorin (0.21 nmol) vesicles and the mixture was incubated overnight at room temperature. The free carbodiimide was removed by chromatography on a Sephadex LH-60 column (0.7 x 18 cm) equilibrated with 88% formic acid/ethanol/water (20:30:30, v/v/v). For further details, see "Methods."
sequence information and the lability to alkali showed that the carboxyl group of glutamic acid-70 was the predominant site of cross-linking.

The present work also showed that the cross-linking involved not only the insertion of the carbene intermediate formed from the diazirine into the γ-carboxyl group of the glutamic acid residue but also proceeded via a long-lived diazo intermediate. The photoisomerization of aryl diazirines to diazo compounds (40) and the reaction of the latter with aspartyl and glutamyl residues of proteins (41) are known. The cross-linking reactions of glycoporphin A using adamantyl diazirine in a recent study (42) may also have proceeded via a similar diazo intermediate.

The cross-linking reactions using radioactive phospholipids IV and V, although they have not been investigated as thoroughly, further delineated the surface boundaries of the bilayer. Thus, following the cyanogen bromide degradation, the fragments 9-81 and 82-131 (Fig. 5B) were found labeled by the cross-linking reactions. On trypptic cleavage of the cross-linked protein, most of the radioactivity was in the fragment T8 (residues 62-96). Thus, the residues 62 and 96 must be at the bilayer surface.

Surprisingly, Glu-70 in glycoporphin A was the predominant site of cross-linking both with phospholipids I and III which carry photosensitive fatty acids of differing chain lengths. This may be a result of two factors. First of all, there is a greater fatty acyl chain mobility toward the methyl terminus. The neutron diffraction data of Zaccari et al. (43) have provided evidence that the orientational disorder and the angular fluctuations of the segments increased at the end of the chains. The photolabel thus resides in this fluid region of the fatty acyl chain. Secondly, even though aryl diazirines are capable of labeling all amino acids, suitably positioned reactive residues such as glutamic acid or tryptophan (44) receive the bulk of the cross-linking. Segments of proteins which lack these residues might be poorly labeled even though they are in the vicinity of the photogenerated carbene.

To confirm the intramembranous location of the glutamic acid, the membrane-permeant reagent DCC was used as an independent probe. DCC is known to react specifically with glutamic or an aspartic acid residues within the membrane-embedded subunits of the ATPase complexes (13, 45-48). [14C]DCC did indeed react with reconstituted glycoporphin and the reaction was specific for Glu-70. Thus, the pattern of fatty acyl chain mobility toward the methyl terminus. The presence of polar or charged group, hydrogen bonds, and salt bridges is being increasingly demonstrated in membranes. For example, in a number of ATPases, amphipathic and Segrest and Jackson (55) suggested that the polar faces interact to form lateral aggregates in the membrane. Glutamic acid-72 is in the middle of the polar face and, hence, would be buried between two glycoporphin A molecules through Glu-72 is a possibility.

Arginine, the most strongly charged amino acid, occupies positions 61, 96, and 97 and must be at the exterior of the bilayer. As stated earlier, the number of amino acids by 0.5-1.0 (54). Adopting an α-helical structure, Corey-Pauling-Koltun models show that Glu-70 (in the protonated form) can simultaneously hydrogen bond with Ser-69 and His-66. Also Glu-63 and His-67 can form hydrogen bonds with each other. Such hydrogen bonds would be very strong within the hydrophobic bilayer. The intramembranous α-helix of glycoporphin A is amphipathic and Segrest and Jackson (55) suggested that the polar faces interact to form lateral aggregates in the membrane. Glutamic acid-72 is in the middle of the polar face and, hence, would be buried between two glycoporphin A molecules.

The intramembranous region includes residues 63-94, an α-helical segment which includes a 20° kink at Pro-71. The positions of key residues are shown by residue number and some charged residues are further highlighted by a + or -. Proposed hydrogen bonds are drawn as dashed lines. N and C refer to NH2 and COOH termini.

The presence of polar or charged group, hydrogen bonds, and salt bridges is being increasingly demonstrated in membrane proteins. For example, in a number of ATPases, amphipathic subunits contain aspartic or glutamic acid residues. A model recently proposed for another integral membrane pro-
tein, bacteriorhodopsin, places a number of charged residues within the membrane (56). This model, as well as the present proposal for glycophorin A, emphasizes intra- and interhelical electrostatic interactions which mask the polar groups from the hydrophobic membrane.

REFERENCES

1. Furthmayr, H. (1977) J. Supramol. Struct. 1, 121-134
2. Tomita, M., Furthmayr, H., and Marchesi, V.T. (1978) Biochemistry 17, 475-477
3. Marchesi, V.T., Tillack, T.W., Jackson, R.L., Segrest, J.P., and Scott, R.E. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 1445-1449
4. Cottomore, S.F., Furthmayr, H., and Marchesi, V.T. (1977) J. Mol. Biol. 113, 539-553
5. Radhakrishnan, R., Gupta, C.M., Ermi, B., Curetolo, W., Robson, R.J., Majundar, A., Ross, A.H., Takagaki, Y., and Krohana, H.G. (1980) Ann. N.Y. Acad. Sci. 346, 165-198
6. Gupta, C.M., Radhakrishnan, R., and Krohana, H.G. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 4315-4319
7. Radhakrishnan, R., Robson, R.J., Takagaki, Y., and Krohana, H.G. (1981) Methods Enzymol. 12D, 408-433
8. Gupta, C.M., Radhakrishnan, R., Gerber, G.E., Olsen, W.L., Quay, S.C., and Krohana, H.G. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 2595-2599
9. Gupta, C.M., Costello, C.E., and Krohana, H.G. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 3139-3143
10. Bligh, E.G., and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911-917
11. Applebury, M.L., Zuckermann, D.M., Lamola, A.A., and Jovin, T.M. (1974) Biochemistry 13, 3448-3458
12. Stoffel, W. (1975) Methods Enzymol. 35B, 533-541
13. Fillungane, R.H. (1975) J. Bacteriol. 124, 870-883
14. Marchesi, V.T., and Andrews, E.P. (1971) Science 174, 1247-1248
15. Furthmayr, H., Tomita, M., and Marchesi, V.T. (1975) Biochem. Biophys. Res. Commun. 65, 113-121
16. Kagawa, Y., and Racker, E. (1971) J. Biol. Chem. 246, 5477-5487
17. Warren, L. (1959) J. Biol. Chem. 234, 1971-1975
18. Furthmayr, H., Galardy, R.E., Tomita, M., and Marchesi, V.T. (1978) Arch. Biochem. Biophys. 185, 21-29
19. Klapper, D.G., Wilde, C.E., and Capra, J.D. (1978) Anal. Biochem. 58, 126-131
20. Bridgen, P.J., Cross, G.A.M., and Bridgen, J. (1976) Nature 263, 613-614
21. Steck, T.L., Weinstein, R.S., Strauss, J.H., and Wallach, D.F.H. (1970) Science 175, 255-257
22. Kahane, I., Furthmayr, H., and Marchesi, V.T. (1976) Biochim. Biophys. Acta 429, 464-476
23. O'Farrell, P.H. (1975) J. Biol. Chem. 250, 4007-4021
24. Swank, R.T., and Munke, K.D. (1971) Anal. Biochem. 39, 462-477
25. Fairbanks, G., Steck, T.L., and Wallach, D.F.H. (1971) Biochemistry 10, 2606-2617
26. Inouye, H., and Beckwith, J. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 1440-1444
27. Hirs, C.H.W. (1967) Methods Enzymol. 11, 325-329
28. Udenfriend, S., Stein, S., Bohlen, P., Daisman, W., Leimgruber, W., and Weigle, M. (1972) Science 178, 871-872
29. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275
30. Ames, B.N., and Dubin, D.T. (1960) J. Biol. Chem. 235, 769-775
31. Grant, C.W., and McConnell, H.M. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 4653-4657
32. Van Zeele, E.J., Verkleij, A.J., Zwaal, R.F.A., and van Deenen, L.L.M. (1978) Eur. J. Biochem. 86, 539-546
33. Steck, T.L. (1972) in Membrane Research (Fox, C.F., ed) pp. 71-93, Academic Press, New York
34. Lifter, J., Hew, C.L., Yoshioka, M., Richards, F.F., and Kottisberg, W.H. (1974) Biochemistry 13, 3567-3571
35. Bayley, H., Huang, K.S., Radhakrishnan, R., Ross, A.H., Takagaki, Y., and Krohana, H.G. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 2225-2229
36. Rouesselet, A., Guthmann, C., Matrican, J., Bienvenue, A., and Devaux, P.F. (1976) Biochim. Biophys. Acta 426, 357-371
37. Tanaka, K., and Ohnishi, S. (1976) Biochim. Biophys. Acta 426, 218-231
38. Bouna, S.R., Drislane, F.W., and Huestis, W.H. (1977) J. Biol. Chem. 252, 6759-6763
39. Ong, R.L., Marchesi, V.T., and Prestegard, J.H. (1981) Biochemistry 20, 4283-4292
40. Smith, R.A.G., and Knowles, J.R. (1975) J. Chem. Soc. Perkin Trans. II, 686-694
41. Offord, R.E. (1969) Nature (Lond.) 221, 37-40
42. Bayley, H., and Knowles, J.R. (1980) Biochemistry 19, 3883-3892
43. Zaccari, G., Bulati, G., Seelig, A., and Seelig, J. (1979) J. Mol. Biol. 134, 691-706
44. Brunner, J., and Richards, F.M. (1980) J. Biol. Chem. 255, 3319-3329
45. Beechey, R.B., Holloway, C.T., Knight, I.G., and Roberton, A.M. (1966) Biochem. Biophys. Res. Commun. 23, 75-80
46. Cattell, K.J., Knight, I.G., Lindop, C.R., and Beechey, R.B. (1970) Biochem. J. 117, 1011-1013
47. Kielh, R., and Hatfield, Y. (1980) Biochemistry 19, 541-548
48. Sebold, W., Machleidt, W., and Wachter, E. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 785-789
49. Marchesi, V.T. (1979) in Advances in Inflammation Research (Weissman, G., Samuelson, B., and Paoletti, R., eds) pp. 19-31, Raven Press, New York
50. Rand, R.P., and Luzzati, V. (1968) Biophys. J. 8, 125-137
51. Schultz, T.H., and Marchesi, V.T. (1979) Biochemistry 18, 275-280
52. Cramer, J.A., Marchesi, V.T., and Armitage, I.M. (1980) Biochim. Biophys. Acta 595, 235-243
53. Egmond, M.R., Williams, R.J.P., Weleb, E.J., and Rees, D.A. (1979) Eur. J. Biochem. 97, 73-85
54. Schultz, G.E., and Schirmer, R.H. (1979) Principles of Protein Structure, p. 70, Springer-Verlag, New York
55. Segrest, J.P., and Jackson, R.L. (1977) in Membrane Proteins and Their Interactions with Lipids (Capaldi, R.A., ed) pp. 21-46, Marcel Dekker, New Yoir
56. Engelmann, D.M., Henderson, R., McLachlan, A.D., and Wallace, B.A. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 2023-2027

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A H Ross, R Radhakrishnan, R J Robson and H G Khorana

J. Biol. Chem. 1982, 257:4152-4161.

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