Regioselective Photolabeling of Glycophorin A in Membranes

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Abstract: We have developed a chemical method for directly identifying the amino acid residues of the transmembrane domain of a protein that are located right in the center of the membrane. Glycophorin A (GPA), the major sialoglycoprotein of human erythrocytes, was the first membrane protein whose primary sequence was elucidated, but its three-dimensional structure is still not known. GPA has been reconstituted into liposomes formed from dimyristoylphosphatidylcholine, dimyristoylphosphatidylserine, cholesterol, and a bola-amphiphilic phospholipidic photoactivatable probe (radioactive probe **1**) by a detergent-mediated method. Electron microscopy confirmed the formation of spherical vesicular structures, and sucrose-density gradients revealed that the proteoliposomes comprised only one membrane fraction. Proteinase-K digestion of GPA in the proteoliposomes suggested that the orientation of GPA in reconstituted proteoliposomes was virtually identical to that observed in natural erythrocyte membranes. After photo-irradiation of the reconstituted proteoliposomes and in situ tryptic digestion, the photolabeled amino acid residues were analyzed by Edman degradation and their radioactivity was measured. Val80 and Met81, which had been assumed to be located near the center of the transmembrane domain of GPA, were indeed highly selectively photolabeled by probe **1**. The new method might be applied to analyze the three-dimensional arrangement of the transmembrane domain of protein complexes that are made up from several subunits.

Keywords: cross-linking · isotopic photolabeling · liposomes · membrane proteins · membrane topography · vesicles

Introduction

Studies of the topographical arrangement of membrane proteins are important for understanding the structural and functional properties of biological membranes. However, this internal topography is difficult to study. Although the crystalline structure determination of membrane-bound proteins at atomic resolution has been improved very recently,[1] it remains crucial to obtain direct evidence on the topographical arrangement of membrane proteins, and thus to establish a new experimental strategy. We present here a chemical approach to this problem.

We have conceived and synthesized a novel *transmembrane*-type photochemical probe **1a** (Scheme 1). The tandem use of **1a** and cholesterol for photolabeling experiments in DMPC (1, 2-dimyristoyl-*sn*-glycero-3-phosphocholine) vesicles (liposomes) led to a remarkable improvement in the regioselectivity of cross-linking between **1a** and DMPC, and between **1a** and cholesterol: the myristoyl chains, functionalized at C-11, C-12, and C-13, made up to 95% of the total photolabeled myristate, while cholesterol was principally (85%) functionalized at the C-25 position on the side-chain. These probes are now available for studies of some membrane proteins.[2]

Glycophorin A (GPA), the major sialoglycoprotein of the human erythrocytes, is one of the best characterized mem-

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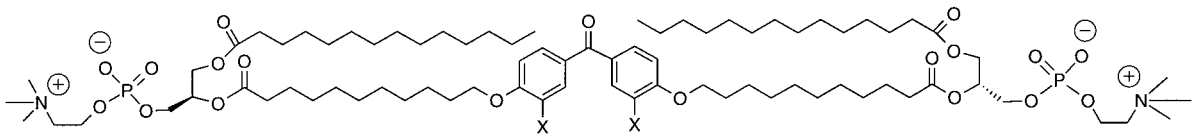

1a (X=H), **1b** (X=D), **1c** (X=T), **1d** (X=I)

Scheme 1. Structure of photoactivatable probe **1**.

brane proteins and is the first membrane protein for which the sequence was elucidated. Human-erythrocyte GPA consists of a single polypeptide chain of 131 amino acids and 16 oligosaccharide chains that form 60% of its molecular weight.[3] The protein possesses a tripartite structure: an N-terminal carbohydrate-rich domain pointing to the outside of the erythrocyte membrane, a hydrophobic transmembrane domain of about 20 amino acid residues, and a C-terminal hydrophilic domain located in the cytoplasm (Figure 1). However, the three-dimensional structure of GPA within membranes is not yet clear.

We thus chose GPA as our first target to test the applicability of our methodology.

ERVQLAHHFSEPEITLIIFGVMAGVIGTILLISYGIRRLIKKSPS
60 70 80 90 100

Figure 1. Primary sequence of glycophorin A for the residues of 60–104. The proposed transmembrane region (residues Thr74–Ile91)[10] is underlined.

Abstract in French: *Nous avons développé une méthode chimique pour identifier, dans le cas d'une protéine membranaire, les résidus localisés au centre des membranes. La glycophorine A (GPA), la sialoglycoprotéine majeure des érythrocytes humains, est la première protéine membranaire dont la séquence ait été élucidée, mais sa structure tridimensionelle n'ait pas encore été clairement résolue. La GPA a été reconstituée par utilisation de détergent dans des protéoliposomes composés de dimyristoylphosphatidylcholine, de dimyristoylphosphatidylsérine, de cholestérol et d'une sonde bola-amphiphilique phospholipidique photoactivable (sonde radioactive **1**). La microscopie électronique confirme la formation de structures vésiculaires sphériques et le gradient de densité en sucrose a révélé que les protéoliposomes sont composés d'une seule fraction membranaire. La digestion de la GPA par la protéinase K dans des protéoliposomes montre que l'orientation de la GPA dans des vésicules reconstituées est presque identique à celle observée dans les membranes d'érythrocytes. Après photo-irradiation des protéoliposomes reconstitués et digestion trypsique in situ, les résidus photomarqués ont été déterminés en parallèle par dégradation d'Edman et mesure de la radioactivité. Les résidus Val80 and Met81, qui doivent être localisés au centre du domaine transmembranaire de la GPA, ont été très sélectivement photomarqués par la sonde **1**. La méthode présentée ici pourrait être appliquée pour l'analyse de l'arrangement tridimensionnel de complexes protéiques transmembranaires composés de plusieurs sous-unités.*

Results and Discussion

Reconstitution and characterization of proteoliposomes incorporating glycophorin A:

Comparative studies on the reconstitution of proteoliposomes: The first step was to establish a reproducible reconstitution method for proteoliposomes composed of four components (phospholipids, cholesterol, probe, and protein), in which the molar ratio of the components was approximately the same as in the initial mixture of the preparation. For this purpose, we reconstituted proteoliposomes by three methods: 1) mild sonication followed by extrusion, 2) sonication, 3) detergent solubilization and dilution. After preparation of the proteoliposomes (for details, see the Experimental Section), the molar ratios of their components (phospholipids, cholesterol, and glycophorin A) were determined by sucrose-density-gradient centrifugation followed by titration of phosphate, cholesterol, and protein. The results are shown in Table 1.

In the absence of GPA, sonication resulted in liposomes in which cholesterol is well incorporated. But this method was not adapted to the preparation of proteoliposomes, as most of the GPA was not incorporated into the lipid membrane. On the contrary, proteoliposomes prepared by mild sonication followed by extrusion were collected as a single peak after sucrose-density-gradient centrifugation (Figure not shown); however, the cholesterol concentration of the membrane fraction was lower than in the initial mixture (20 mol%) (Table 1). The best results were obtained by detergent solubilization and dilution (Figure 2). A single peak was found in fractions 7–10 of the sucrose-density gradient, which corresponded to proteoliposomes containing phospholipids (65.1 mol%), cholesterol (34.7 mol%), and GPA (0.2 mol%). These values are approximately the same as those of the initial mixture of the preparation. The high content of cholesterol in proteoliposomes may be justified, as natural erythrocyte membranes contain 23 wt.% (about 33 mol%) of cholesterol in the total lipid.[4] Besides, in the case of the detergent solubilization and dilution, the addition of 5 mol% of a negatively charged phospholipid, 1,2-dimyristoyl-*sn*-glycero-3-phosphoserine (DMPS), to 61 mol% of DMPC allowed reproducible preparation of stable proteoliposomes. This might be due to the favorable electrostatic interactions between the negative (DMPS) and positive charges (His66, His67 on the outer surface and Arg96, Arg97 on the inner surface of proteoliposomes). Since human erythrocyte membranes include approximately 5 mol% of phosphatidylserine,[4] the presence of PS seemed to be an important factor in maintaining the membrane structure that contained glycophorin A. Among the detergents tested for reconstitution

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Table 1. Composition [mol %] of phospholipid, cholesterol, and glycophorin A in liposome or proteoliposome fractions purified by sucrose-density gradient centrifugation.[a]

Methods	Phospholipid [mol %]	Cholesterol [mol %]	Glycophorin A [mol %]
sonicated liposome without glycophorin A	68.4 (67)	31.6 (33)	–
proteoliposome prepared by sonication and extrusion	79.6 (67.1)	20.1 (32.6)	0.3 (0.3)
proteoliposome prepared by sonication	70.1 (67.1)	29.9 (32.7)	< 0.05 (0.2)
proteoliposome prepared by detergent	65.1 (66.3)	34.7 (33.5)	0.2 (0.2)

[a] The value in parentheses represents the mol % of each component in the initial mixture. In the first three methods DMPC was employed as phospholipid, whereas in the forth method, a mixture of DMPC (60 mol %) and DMPS (5 mol %) was used.

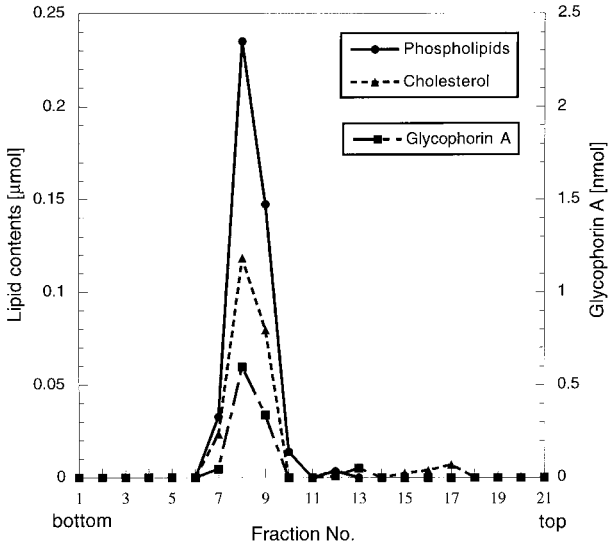

Figure 2. Sucrose-density gradient centrifugation profile of proteoliposomes prepared by a detergent solubilization and dilution method.

[one zwitterionic detergent: 3-(3-cholamidopropyl)dimethylammonio-1-propane sulfonate (CHAPS) and three nonionic ones: *n*-dodecyl-D-maltoside (dodecylmaltoside), *tert*-octylphenoxypolyethoxyethanol (Triton-X-100), *n*-octyl-β-D-glucopyranoside (octylglucoside)] octylglucoside gave the best results.

Proteoliposome characterization by electron microscopy: Proteoliposomes were observed by electron microscopy with freeze–fracture techniques to confirm the formation of vesicles that had been prepared by detergent solubilization and dilution. Proteoliposomes were mainly formed as spherical unilamellar vesicles, with an average diameter about 150 nm (see Figure S1 in the Supporting Information).

Orientation of glycophorin A in membranes: To analyze the membrane topography of a protein, it is essential to reconstitute proteoliposomes in such a manner that the protein is only inserted into the membranes with an orientation identical to that in the natural membrane. In order to determine the orientation of the glycophorin A incorporated in proteoliposomes that were prepared by sonication then extrusion or by detergent solubilization and dilution, the GPA was digested in situ by proteinase K according to the method of Challou et al.[5a] Small peptides, derived from the digestion of the N-terminal part, were separated in the supernatant solution by centrifugation. The peptide fragments in precipitated proteoliposomes were applied to SDS-PAGE gel. Figure 3 shows the electrophoresis profile of peptide fragments stained by Coomassie Brilliant Blue (CBB): lane 1 is for standard molecular size markers for proteins, and lane 2 is for a control experiment sample without glycophorin A. As the amount of proteinase K employed was small (0.4 mg) relative to the detection limit ($\approx$1 mg) on the gel by CBB staining, no peptide band corresponding to proteinase K (M_W = 18.5 kDa) was observed in lane 2. Lane 3 is for a

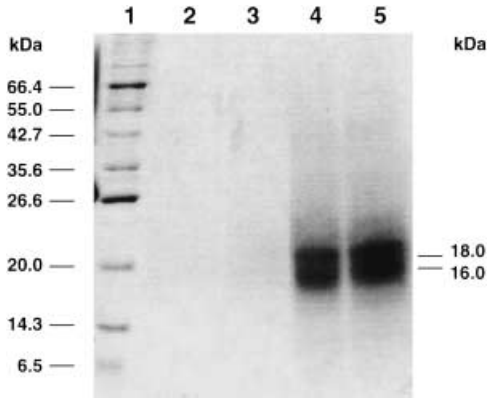

Figure 3. SDS-PAGE gel of peptide fragments obtained from in situ digestion of proteoliposome by proteinase K. Lanes: 1) protein molecular weight markers, 2) digestion of proteoliposomes without GPA (control experiment), 3) digestion of GPA in water (control), 4) digestion of proteoliposomes formed by sonication followed by extrusion, 5) digestion of proteoliposomes formed by detergent solubilization and dilution. Peptide bands were stained by Coomassie Brilliant Blue.

peptide sample obtained from digestion of glycophorin A by proteinase K in a buffer solution. No bands were observed, probably because GPA molecules in the aqueous solution were digested in small fragments (less than 5 kDa). Peptides obtained by proteinase-K digestion of proteoliposomes (lanes 4 and 5), prepared respectively by mild sonication followed by extrusion or by detergent solubilization and dilution, exhibited in both cases molecular masses of 16 kDa and 18 kDa, corresponding to parallel "head-to-head" dimers of the TC fragment (transmembrane part + following C-terminal part of GPA) of GPA (Figure 4). The N-terminal sequences of both bands were identified as a mixture of two peptides: one starting at His66 and another starting at Ser69 in the GPA sequence. From the molecular size and N-terminal sequence of these peptide bands, we concluded that glycophorin A was inserted in such a manner that its N-terminal is exposed to the outside of the proteoliposomes and that

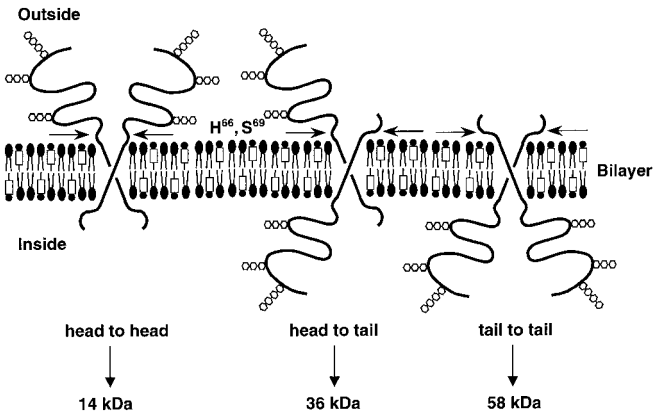

Figure 4. Possible conformations that could be adopted by glycophorin A dimers within membranes. Proteinase K in situ digestion allows discrimination between the three conformations ("head-to-head", "head-to-tail", and "tail-to-tail") that generate peptide fragments of 14 kDa, 36 kDa, and 58 kDa (theoretical values), respectively.[12] The present work supported the head-to-head conformation (N-terminal outside) of GPA incorporated in proteoliposomes, which is the same conformation adopted as in natural erythrocyte membranes. Arrows represent the cleavage positions of GPA by proteinase K, which are located near the lipid/water interface. If GPA is inserted in lipid bilayers with its N-terminal on the outside of the membrane, these cleavage positions are His66 and Ser69.

proteinase K digested this part of the protein molecule. The theoretical molecular masses of the two peptidic dimers His66 – Gln131 (C-terminal) and Ser69 – Gln131 (C-terminal) are calculated as 14.4 kDa and 13.6 kDa, respectively. These apparently larger sizes on SDS-gel than their calculated values could be due to the high number of acidic residues (Asp, Glu) within the C-terminal domain and/or to a high affinity of the transmembrane domain with SDS; this may slow down the migration of these fragments during SDS-gel electrophoresis. On the other hand, this C-terminal domain possesses six proline residues, which have a significant effect on the conformation of the polypeptide backbone; this might explain the presence of two different bands (16 kDa and 18 kDa).

Starting from proteoliposomes prepared by hydrating a film of DMPC (without cholesterol or negatively charged phospholipid) with an aqueous solution of GPA, and following the analytical procedures described above, Ruysschaert's group[5a] obtained three bands of peptides (16 kDa, 18 kDa, and 38 kDa) on SDS-PAGE. Their results indicate the presence of two conformations for GPA-dimers in proteoliposomes (a head-to-head dimer and an antiparallel head-to-tail dimer, Figure 4). In our case, only two bands (16 kDa, 18 kDa) were observed, and neither the 38 kDa band nor the 58 kDa band, which would correspond to a tail-to-tail dimer, were detected. These results suggest that GPA is incorporated in proteoliposomes in only one orientation, that is, in the same one (N-terminal outside) as in natural erythrocyte membranes.

On the other hand, Khorana's group[6] reconstituted proteoliposomes made of DMPC, a radioactive photosensitive probe, and GPA (without cholesterol or phosphatidylserine) by detergent (sodium cholate) solubilization and dialysis (with Tris buffer and Bio-Beads). They determined the orientation of GPA by the addition of neuraminidase and papain: 75% of GPA molecules were oriented with the N-terminal exposed to the outside of the proteoliposomes.

Estimation of the content of octylglucoside in proteoliposome membrane: In this study, we used octylglucoside[7] to solubilize phospholipids, cholesterol, and glycophorin A. Reconstitution of proteoliposomes was then followed by dilution.[8] The values of partition coefficient of octylglucoside (OG) between water and vesicle membranes were reported by Rigaud's group[7c] as: 1012 for $0 < [OG](H_2O) < 15.6$ mM and 1168 for $16.5 < [OG](H_2O) < 21$ mM. Therefore, the final molar fraction of octylglucoside in the membrane can be theoretically calculated from these values[9] (see calculation in the Supporting Information). By this calculation, the molar fraction of octylglucoside in proteoliposome membranes was 0.0055. The presence of this concentration of octylglucoside in lipid membranes might not have any serious effects on the lipid bilayers for enzymatic reactions or for photolabeling experiments.

In conclusion, detergent (octylglucoside) solubilization and dilution provided proteoliposomes (DMPC/DMPS/cholesterol/photosensitive probe/GPA) that were formed as spherical vesicles and in which glycophorin A was inserted in the same orientation as that found in natural erythrocyte membranes. We employed these proteoliposomes for the photolabeling experiments.

Determination of photolabeled amino acids of glycophorin A located in the center of a lipid bilayer:

Analytical procedures for determining photolabeled amino acids in the transmembrane domain of glycophorin A: The peptide TC fragment was isolated from proteoliposomes that had been prepared by detergent solubilization and dilution. Scheme S1 (in the Supporting Information) presents the successive analytical steps leading to the identification of the photolabeled amino acids in the TC fragment: 1) proteoliposome reconstitution by detergent solubilization and dilution as described above, 2) photo-irradiation, 3) tryptic digestion, 4) delipidation, 5) purification of peptide fraction, and 6) localization of labeled amino acids in TC. We present here the results.

Analysis of photolabeled amino acids in the TC fragment: After photo-irradiation of the proteoliposomes, the photolabeled TC fragment was prepared by tryptic digestion, followed by centrifugation and delipidation (Scheme S1). A major peptide fragment was found at about 20 kDa (see Figure S2 in the Supporting Information), which may correspond to a dimer of a TC fragment. The calculated molecular mass of the peptide fragment [Val62 – Gln131 (C-terminal)] is 15.2 kDa. The apparent larger size (20 kDa) on the SDS-gel than calculated might be due to the attachment of the probe **1c** (about 1.5 kDa) and/or to the same reason as discussed earlier for proteinase K digestion. This peptide fragment was then extracted from the gel and analyzed by Edman degradation, which showed that the fragment was composed of only a single N-terminal sequence, starting at Val62 (35 amino acids). The radioactivity distribution of the photo-

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labeled TC fragment was also measured, and the radioactivity in each PTH (phenylthiohydantoin) amino acid was plotted against the amino acid sequence (from Val62 to Arg96) of GPA. A significant radioactivity was detected at Val80 and to a lesser extent at Met81 (Figure 5). This indicated that the

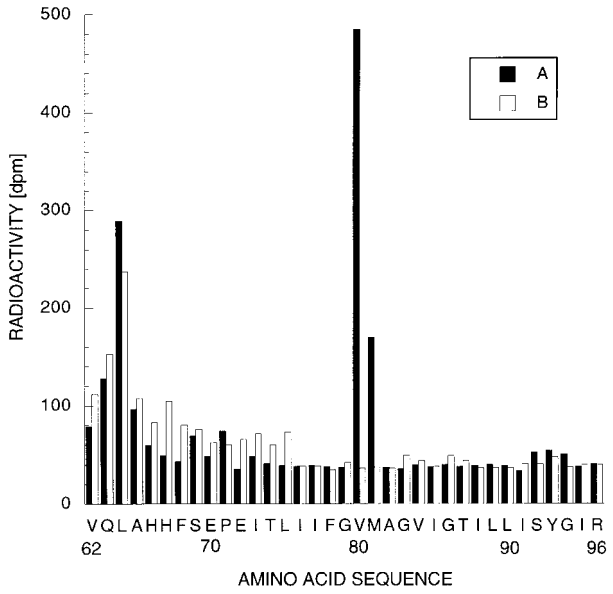

Figure 5. Edman degradation of TC fragments and the distribution of radioactivity in the TC fragment of GPA (35 amino acids starting from Val62). The black bars represent the radioactivity of the fraction from the experiment in the presence of GPA. The white bars represent the radioactivity of the fraction in a control experiment without GPA. Residues Val80 and Met81 were selectively photolabeled, whereas the radioactivities in other amino acid fractions corresponded to background level except from Val62 to Ala65; this is surely due to contamination with labeled phospholipids.

center of the transmembrane domain of glycophorin A had been selectively photolabeled *within* the membrane. No radioactivity was found in the sequence located in the interface region between lipid and water phases. Radioactivity was also detected in association with four residues (from Val62 to Ala65) located in the N-terminus of TC fragment. According to Ruysschaert,[5a] these residues are located on the outside of the membrane. In order to understand the significance of this detection, a control experiment was performed in the absence of glycophorin A by exactly the same procedures as described in Scheme S1. In this control experiment, we only observed significant incorporation of radioactivity in the N-terminal moiety (residues 62–66), corresponding to the first Edman degradation cycles, but not in the middle positions of the transmembrane domain (Val80, Met81). Therefore, the radioactivity observed in the N-terminus of the TC fragment seemed to be derived from probe **1c** phospholipid adducts, which were not eliminated after ethanol extraction (see Scheme S1). The separation procedure of protein from lipid fractions should be improved.

From the data of Engelman's three-dimensional model in micelles for the dimeric transmembrane domain of GPA,[10] Ala82 could also be a candidate for cross-linking by the probe. There might be two possible explanations for the lack of radioactivity associated with Ala82: 1) Phe78 and/or Ile85 could sterically hinder the neighboring residues on the preceding or subsequent α-helical turns. Or, 2) a photochemical reaction may have occurred at Ala82, but a retro-aldol-type degradation of the α-coupling product formed might have followed.[11, 12]

Photolabeling yield of glycophorin A in proteoliposomes: In order to estimate the photolabeling yield of GPA in proteoliposomes, its TM fragment was separated according to Scheme S2 (see Supporting Information). The efficiency of photolabeling of the TM fragment was estimated by its radioactivity (the radioactivity of "proteoliposomes" is taken to be 100%). The TM fragment was associated with 7% of the total radioactivity. This value is quite good in comparison with the 1–2% obtained by Khorana's group[6] or to the 0.1% by Montecucco and Schiavo.[13]

Comparison with previous studies: As described above, the analysis of photolabeled amino acids in the TC fragment showed that, in addition to Met81, Val80 had been highly regioselectively functionalized. Our results are in good agreement with the structural features of the transmembrane domain of GPA, as proposed earlier on the basis of ATR-FTIR and NMR spectroscopy.[5, 10b] This represents a marked improvement over the results reported earlier. For example, in order to define the membrane-embedded region of GPA, Khorana's group had reconstituted proteoliposomes made of DMPC, GPA, and phospholipidic probes carrying a carbene precursor that could be photoactivated. But, after photoirradiation of the proteoliposomes, most of the cross-linked positions were found to be at Glu70, which should be situated at the lipid–water interface according to the results of the FTIR studies of Ruysschaert's group.[5a] Khorana's results might be due to the extensive disorder of the phospholipid matrix and of the probe itself above the phase-transition temperature. In our case, the tandem use of the transmembrane probe and of cholesterol led to a well-ordered bilayer structure and, hence, to an excellent selectivity for the photolabeling of the transmembrane domain of GPA in proteoliposomes.

An independent synthesis of another transmembrane phospholipidic probe (bearing a trifluoromethylphenyldiazirine as the photosensitive group) and its application to the membrane topography of a protein were reported by Delfino et al.[14] However, when this probe was incorporated in vesicles, only about 50% of the molecules were found to be in the transmembrane conformation, the other half being in a "U" form. In that study, the effect of cholesterol was not examined. In our study,[15] with solid state ^{2}H NMR, we showed that in vesicles containing DMPC, deuterated probe **1b**, and a "physiological" amount of cholesterol, the extended transmembrane conformation of the probe was very much predominant.

On the other hand, the desirable characteristics of benzophenones as photophores have recently been "rediscovered", but their application has been limited to *photoaffinity* labeling, that is, to determine receptor–ligand binding sites.[16]

Conclusion

We have developed a methodology for the topographical analysis of glycophorin A in proteoliposomes. Our data show for the first time that the center of the transmembrane domain of a protein can be selectively functionalized. Although glycophorin A is a well-known protein, and our experiments were carried out in "artificial" well-ordered bilayer systems, our findings validate this new chemical method, which could be useful for analyzing the three-dimensional arrangement of the transmembrane domain of protein complexes made up of several subunits.

Experimental Section

Synthesis of the tritiated photochemical probe 1c: The diiodo precursor **1d** (10 mg, 5.7 μmol)[15c] was dissolved in MeOH/THF (1:1 *v*/*v*, 3 mL). To this was added DMPC (44 mg, 278 μmol), sodium acetate (5 mg, 60 μmol), and 10% Pd/C (30 mg). The mixture was tritiated with 2.3 Ci of tritium gas at 55 °C and 1.2 atm for 1 h with rapid stirring. Unreacted tritium gas was then adsorbed onto a small alloy bed ($LaNi_4Mn$). Labile tritium was removed by several methanol evaporations, then following catalyst filtration, the crude product was stored in methanol (30 mL; total radioactivity = 128 mCi). The crude product was purified by semipreparative HPLC (Zorbax NH_2 column, 10 mm × 250 mm, eluted at 1 mL min^{-1} with acetonitrile/methanol/10 mM pH 4.8 phosphate buffer (50:40:10, *v*/*v*/*v*)). This gave 35 mCi of tritiated product **1c** with a radiochemical purity of 98.6% (by analytical HPLC under the same conditions as above on a 4.6 mm × 250 mm Zorbax NH_2 column). The radiochemical purity (100%) was also checked by thin layer chromatography on silica gel with the solvent system CH_3Cl_3/MeOH/H_2O (65:25:4, *v*/*v*/*v*). The specific activity was determined to be 40 Ci mmol^{-1} by UV spectroscopy. UV (MeOH): λ_{max} (ε) = 200 nm (30000), 223 nm (14500), 295.5 nm (22500). A proton-decoupled tritium NMR spectrum (320 MHz, $CDCl_3$/CD_3OD (9:1, *v*/*v*)) showed 100% of the label in the ring (δ = 6.90). Probe **1c** was stocked in a methanolic solution at −20 °C.

Glycophorin A: GPA was isolated from human blood type MM according to methods described earlier.[17] In order to get a purer GPA, affinity column chromatography with a column packed with low-ratio wheat-germ agglutinin-conjugated Sepharose 4B was then performed according to the procedure described earlier.[18] The purity of isolated GPA was checked on a SDS-PAGE gel by CBB staining, silver staining, periodic acid Schiff staining or by immunoblotting with an anti-GPA antibody. GPA purified by this method consists mainly of a GPA dimer with an estimated molecular weight of about 72 kDa.

The following chemicals were purchased from the companies indicated in parenthesis: CHAPS, cholesterol standard solution, dodecylmaltoside, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), octylglucoside, protein molecular-weight markers, proteinase K, Sepharose 4B, TPCK-treated trypsin, Triton-X-100 (Sigma, St. Louis, MO); cholesterol (Aldrich, Steinheim, Germany); DMPC (Stegena, Cambridge, MA); DMPG (1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol), DMPS (Avanti, Alabaster, AL); Bio-Beads SM-2, detergent compatible protein assay dye reagent (Bio-Rad, Hercules, CA); scintillation solution (Ultima gold M, Packard Bioscience Company, Meriden, CT).

Preparation of proteoliposomes:

Method 1: *Preparation by weak sonication followed by extrusion*: DMPC (5.00 mg, 7.38 μmol), cholesterol (1.40 mg, 3.62 μmol), **1c** (102 μL, 1.28 nmol, 51 μCi; from a methanol stock solution: 18.6 μg mL^{-1}, 0.50 mCi mL^{-1}), and GPA (1.0 mg, 32 nmol) were dissolved by vortexing in chloroform/methanol (1.0 mL, 1:2, *v*/*v*) in a 20 mL test tube. The organic solvent was evaporated under a N_2 gas stream, and the residual lipid film was dried under vacuum for 12 h. The molar ratio of the dried lipid film was PC/cholesterol/**1c**/GPA 67.09:32.61:0.01:0.29. HEPES buffer (2.0 mL, 50 mM HEPES, pH 7.5) was added to the lipid film. After vortexing, the aqueous lipid solution was sonicated in a bath-type sonicator (Sonorex RX100H Bandelin) for 30 min at 60 °C. The liposomes were extruded ten times through polycarbonate membranes (Costar, pore size 200 nm) in a Matesson Model 3030-580 instrument, followed by centrifugation at 120000 g for 2 h at 4 °C. The precipitate was resuspended in HEPES buffer (1.0 mL, 50 mM HEPES, pH 7.5) to form proteoliposomes.

Method 2: *Preparation by sonication*: DMPC (10.8 mg, 15.9 μmol), cholesterol (3.0 mg, 7.8 μmol), **1c** (220 μL, 2.76 nmol, 110 μCi; from a methanol stock solution: 18.6 μg mL^{-1}, 0.50 mCi mL^{-1}), and GPA (1.5 mg, 48.3 nmol) were dissolved by vortexing in chloroform/methanol (1.0 mL, 1:2, *v*/*v*) in a 20 mL test tube. The organic solvent was evaporated under a stream of N_2 gas, and the residual lipid film was dried under vacuum for 12 h. The molar ratio of the dried lipid film was PC/cholesterol/**1c**/GPA 67.09:32.70:0.01: 0.20. HEPES buffer (2.0 mL, 50 mM HEPES, pH 7.5) was added to the lipid film. After vortexing, the aqueous lipid suspension was sonicated by a probe type sonicator (Sonifer B-30, Branson Ultrasons, duty cycle 50 power 6) for 10 min at 60 °C to form proteoliposomes.

Method 3: *Preparation by detergent solubilization and dilution*: DMPC (24.80 mg 36.6 μmol), DMPS (2.10 mg, 3.0 μmol), cholesterol (7.7 mg, 19.8 μmol), and probe **1c** (480 μL, 6.00 nmol, 240 μCi; from a methanol stock solution: 0.50 mCi mL^{-1}) dissolved in chloroform/methanol (1.0 mL, 1:2 (*v*/*v*)) were dispersed by vortexing in a 20 mL test tube, dried under a gentle stream of N_2 gas, and pumped in vacuo for 12 h. The lipid film was then dissolved in HEPES buffer (2.0 mL, 50 mM, pH 7.5), vortexed, and sonicated by a probe-type sonicator (Sonifer B-30, Branson Ultrasons, duty cycle 50 power 6) for 5 min at 60 °C. A solution of octylglucoside (0.5 mL, 0.3 M) and GPA (3.72 mg, 120 nmol) in HEPES buffer (0.5 mL, 50 mM, pH 7.5) was added to the sonicated vesicles (1.0 mL). The molar ratio of the components in the final suspension was DMPC/DMPS/cholesterol/**1c**/GPA 61.00:5.00:33.33:0.01:0.2, and the final concentration of octylglucoside was 100 mM. The suspension was incubated for 15 min at room temperature and HEPES buffer (30 mL, 50 mM × 20 dilution) was added. The diluted solution was incubated for 15 min on ice, followed by centrifugation for 10 h at 50000 g and 4 °C. The precipitate was collected and resuspended in HEPES buffer (2.0 mL, 50 mM) to form proteoliposomes.

Proteoliposome characterization by sucrose-density gradient centrifugation: Proteoliposomes were characterized by discontinuous sucrose-gradient centrifugation. The sucrose-density gradient ranged from 2 to 30% (*w*/*w*) aqueous solution in centrifugation tubes. Centrifugation was performed at 4 °C and 125000 g for 17 h with a Beckman No. TL100, TST49 Swing Bucket Rotor. After the centrifugation, the sucrose-gradient solutions were fractionated in 1.0 mL fractions in a tube. The fractions were titrated to quantify phospholipid, cholesterol, and glycophorin A. The phosphate was titrated according to the method of Chen et al.[19] The amount of cholesterol was measured by the cholesterol oxidase calorimetric method with the Sigma cholesterol assay kit. Glycophorin A assay: 200 μL aliquots of a sample solution or the standard solution were placed in a new cuvette (1.0 mL) for UV spectroscopy. To the solution were added 0.6 mL of Millipore "pure water" and 0.2 mL of detergent compatible protein assay dye reagent (Bio-Rad). The cuvette was incubated at room temperature for 5 min and its optical density at 595 nm was measured, from which the amount of glycophorin A in the sample was calculated.

To select a detergent for the reconstitution of proteoliposomes (see above), we had previously compared four detergents: CHAPS, dodecylmaltoside, Triton-X-100 and octylglucoside, concerning the sucrose-density gradient profile and the efficiency of GPA incorporation.

Comparison of detergents for the reconstitution of proteoliposomes: Vesicles were prepared from DMPC (64.3 mol%), cholesterol (33 mol%), and DMPG (2.5 mol%). After solubilization of GPA (150 μg, 0.2 mol%) with one of the four different detergents [CHAPS (10 mM), dodecylmaltoside (15.7 mM), Triton X-100 (15 mM), or octylglucoside (40 mM) in NH_4OAc (250 μL; 50 mM)], this solution was added to the vesicles. After incubation for 15 min, the detergent was slowly removed by repeated addition of SM-2 Bio-Beads (Bio Rad) and the formed vesicles (100 μL) were applied to a sucrose-density gradient. The recovered fractions were extracted with $CHCl_3$ and assayed for phosphorus. GPA was monitored by a Bio-Rad detergent compatible protein assay.

Proteoliposome characterization by electron microscopy: The reconstituted proteoliposomes prepared by detergent solubilization and dilution as described above were frozen onto gold holders in liquid nitrogen. The specimens were fractured in a JOEL JFD-9010 freeze – fracture apparatus

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at −160 °C and were shadowed with platinum and carbon, with etching. The replicas were observed in a JOEL JEM-1010 electron microscope. The observed areas were imaged randomly.

Preparation of the TC fragment of photolabeled glycophorin A in proteoliposome: The proteoliposome solution prepared by detergent solubilization and dilution as described above and containing glycophorin A (1.0 mg) in an Eppendorf tube was irradiated by UV (365 nm, 180 watts) for 10 min at room temperature. To the irradiated sample was then added a solution of trypsin in Tris-HCl (10 μL, 50 mM, pH 8.3, 1.0 mg mL^{-1}, GPA/Trypsin 50:1, *w*/*w*), and the solution was incubated at 37 °C for 6 h, and separated in a centrifuge at 20000 g and 4 °C for 2 h. The precipitate was collected and washed twice with cold ethanol to remove the lipid fraction. The residual peptides were applied to a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (15% *w*/*v* acrylamide SDS-PAGE).[20] After CBB staining of the gel, a band on the SDS-PAGE gel of about 20 kDa was cut out, and the gel piece was extracted by incubation with an aqueous SDS solution (0.1%, *w*/*v*) at 37 °C for 1 h to afford the peptide sample. This fragment was found to be a TC (transmembrane domain + C-terminal domain of GPA) fragment after microsequencing.

Analysis of photolabeled amino acids by Edman degradation and measurement of radioactivity: The N-terminal amino acid sequence of the TC peptide fragment was determined by Edman degradation sequencing (sequencer, Applied Biosystems 473A). Each PTH-amino acid was analyzed on a HPLC column (PTH C18, 220 × 2.1 mm, 5 μm bead size) at 55 °C with detection at 269 nm. In addition, the PTH amino acid fractions in each Edman cycle were collected in a scintillation tube, scintillation solution (3 mL; ULTIMA GOLD MV, Packard Bioscience Company) was added, and the fraction was kept at room temperature for 10 h. The tritium radioactivity in each tube was counted by a scintillation counter.

Digestion of GPA in proteoliposomes by proteinase K: To analyze the orientation of GPA in the proteoliposome membrane, proteinase K digestion was performed on proteoliposomes prepared by a sonication followed by extrusion method or a detergent solubilization and dilution method. Proteinase K solution (10 μL, 40 μg mL^{-1}) was added to a proteoliposome solution (0.5 mL containing 20 μg of GPA), prepared by methods 1 and 3 cited above, GPA/proteinase K 50:1 (*w*/*w*), and the mixture was incubated at 37 °C for 4 h. The solution was centrifuged at 20000 g and 4 °C for 2 h. Cold ethanol (0.5 mL) was added to the precipitate, and the mixture was vortexed. The ethanol solution was centrifuged at 20000 g and 4 °C for 1 h. This ethanol extraction was repeated, and the precipitate was dried under vacuum for 2 h. A sample buffer [10 μL; SDS (4%, *w*/*v*), β-mercaptoethanol (10%, *v*/*v*), Tris (12 mM), glycerol (20%, *v*/*v*), bromo-phenol blue (0.002%, *w*/*v*), pH 6.8] was added to the dried precipitate and the mixture was incubated for 5 min at 60 °C. The peptide fragment in the sample solution was separated on a polyacrylamide gel (6% stacking gel and 15% resolving gel).[20] Electrophoresis was carried out at room temperature for 4 h. After electrophoresis, the gel was stained by CBB.

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[1] a) E. M. Landau, J. P. Rosenbusch, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 14532–14535; b) S. H. White, W. C. Wimley, *Annu. Rev. Biophys. Biomol. Struct.* **1999**, *28*, 319–365; c) P. F. Lenne, B. Berge, A. Renault, C. Zakri, C. Venien-Bryan, S. Courty, F. Balavoine, W. Bergsma-Schutter, A. Brisson, G. Grubel, N. Boudet, O. Konovalov, J. F. Legrand, *Biophys. J.* **2000**, *79*, 496–500; d) K. Palczewski, T. Kumasaka, T. Hori, C. A. Behnke, H. Motoshima, B. A. Fox, I. L. Trong, D. C. Teller, T. Okada, R. E. Stenkamp, M. Yamamoto, *Science* **2000**, *289*, 739–745.

[2] Y. Ogawa, W. Hahn, P. Garnier, N. Higashi, D. Massotte, H. H. Metz-Boutigue, B. Rousseau, J. Sunamoto, G. Ourisson, Y. Nakatani, *Angew. Chem.* **2001**, *113*, 970–972; *Angew. Chem. Int. Ed.* **2001**, *40*, 944–946.

[3] M. Tomita, H. Furthmayr, V. T. Marchesi, *Biochemistry* **1978**, *17*, 4756–4770.

[4] D. E. Vance, J. E. Vance in *New Comprehensive Biochemistry*, *Vol. 20* (Eds.: A. Neuberger, L. L. M. van Deenen), Elsevier, Amsterdam, **1991**, pp. 5–7.

[5] a) N. Challou, E. Goormaghtigh, V. Cabiaux, K. Conrath, J. M. Ruysschaert, *Biochemistry* **1994**, *33*, 6902–6910; b) S. O. Smith, J. Hamilton, A. Salmon, B. J. Bormann, *Biochemistry* **1994**, *33*, 6327–6333.

[6] A. H. Ross, R. Radhakrishnan, R. J. Robson, H. G. Khorana, *J. Biol. Chem.* **1982**, *257*, 4152–4161.

[7] a) L. T. Mimms, G. Zampighi, Y. Nozaki, C. Tanford, J. A. Reynolds, *Biochemistry* **1981**, *20*, 833–840; b) M. T. Paternostre, M. Roux, J. L. Rigaud, *Biochemistry* **1988**, *27*, 2668–2677; c) J. L. Rigaud, M. T. Paternostre, A. Bluzat, *Biochemistry* **1998**, *37*, 2677–2688; d) M. Keller, A. Kerth, A. Blume, *Biochem. Biophys. Acta* **1997**, *1326*, 178–192.

[8] C. Breyton, C. Tribet, J. Olive, J. P. Dubacq, J. L. Popot, *J. Biol. Chem.* **1997**, *272*, 21892–21900.

[9] a) M. L. Jackson, C. F. Schmidt, D. Lichtenberg, B. J. Litman, A. D. Albert, *Biochemistry* **1982**, *21*, 4576–4582; b) M. Paternostre, O. Meyer, C. Grabielle-Madelmont, S. Lesieur, M. Ghanam, M. Ollivon, *Biophys. J.* **1995**, *69*, 2476–2488.

[10] a) H. E. Treutlein, M. A. Lemmon, D. M. Engelman, A. T. Brünger, *Biochemistry* **1992**, *31*, 12726–12733; b) K. R. MacKenzie, J. H. Prestegard, D. M. Engleman, *Science* **1997**, *276*, 131–133.

[11] E. Deseke, Y. Nakatani, G. Ourisson, *Eur. J. Org. Chem.* **1998**, 243–251.

[12] C. Montecucco, G. Schiavo, *Biochem. J.* **1986**, *237*, 309–312.

[13] U. Schöllkopf, U. Groth, W. Hartwig, *Liebigs Ann. Chem.* **1981**, 2407–2418.

[14] J. M. Delfino, S. L. Schreiber, F. M. Richards, *J. Am. Chem. Soc.* **1993**, *115*, 3458–3479.

[15] a) Y. L. Diyizou, A. Genevois, T. Lazrak, G. Wolff, Y. Nakatani, G. Ourisson, *Tetrahedron Lett.* **1987**, *28*, 5743–5746; b) M. Yamamoto, W. A. Warnock, A. Milon, Y. Nakatani, G. Ourisson, *Angew. Chem.* **1993**, *105*, 302–304; *Angew. Chem. Int. Ed. Engl.* **1993**, *32*, 259–261; c) M. Yamamoto, V. Dollé, W. Warnock, Y. Diyizou, M. Yamada, Y. Nakatani, G. Ourisson, *Bull. Soc. Chim. Fr.* **1994**, *131*, 317–329; d) Y. Nakatani, M. Yamamoto, Y. Diyizou, W. Warnock, V. Dollé, W. Hahn, A. Milon, G. Ourisson, *Chem. Eur. J.* **1996**, *2*, 129–138.

[16] a) G. Dormàn, G. D. Prestwich, *Biochemistry* **1994**, *33*, 5661–5673; b) G. D. Prestwich, G. Dormàn, J. T. Elliott, D. Marecak, A. Chaudhary, *Photochem. Photobiol.* **1997**, *65*, 222–234.

[17] a) J. T. Dodge, C. Mitchell, C. Hanahan, *Arch. Biochem. Biophys.* **1963**, *100*, 119–130; b) V. T. Marchesi, E. P. Andwers, *Science* **1971**, *174*, 1247–1248.

[18] K. Furukawa, J. E. Minor, J. D. Hegarty, V. P. Bhavanandan, *J. Biol. Chem.* **1986**, *261*, 7755–7761.

[19] P. S. Chen, T. Y. Toribara, H. Warner, *Anal. Chem.* **1956**, *28*, 1756–1758.

[20] U. K. Laemmli, *Nature* **1970**, *227*, 680–685.

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