Hydrophobic Labeling of a Single Leaflet of the Human Erythrocyte Membrane†

Josef Brunner,* Martin Spiess, Robert Aggeler, Peter Huber, and Giorgio Semenza

ABSTRACT: The photoactivatable phospholipid 1-palmitoyl-2-[10-[4-[(trifluoromethyl)diazirinyl]phenyl]-[9-^{3}H]-8-oxadecanoyl]-*sn*-glycero-3-phosphocholine ([^{3}H]PTPC) was synthesized with high specific radioactivity. When a sonicated dispersion of [^{3}H]PTPC was incubated with human erythrocyte membranes (ghosts), the radiolabel was inserted spontaneously into the erythrocyte membrane. Photo-cross-linking of [^{3}H]PTPC to membrane components and subsequent analysis of the distribution of radiolabel among polypeptide fragments of glycophorin allowed conclusions concerning the transbilayer distribution of [^{3}H]PTPC in the erythrocyte membrane. Thus, [^{3}H]PTPC was inserted exclusively into the outer leaflet of resealed ghosts, whereas with unsealed (leaky) ghosts, the photosensitive lipid was incorporated into both halves of the membrane simultaneously. These results are incompatible with fusion of [^{3}H]PTPC liposomes with the erythrocyte membrane being responsible for the lipid transfer observed and suggest instead that PTPC exchanges spontaneously between membranes. This property of PTPC could be related to the critical micelle concentration of this lipid [(6 ± 2) × 10^{-9} M at 23 °C].

The main goal of hydrophobic photolabeling of membranes is to provide information concerning the topological arrangements of intrinsic proteins. Labeling reagents used are either hydrophobic or amphiphilic in nature. While the former dissolve readily and react from within the entire lipid bilayer, amphiphilic reagents are intended to probe membranes at spatially defined subcompartments.

Using pure lipid systems, several groups have reported on the preferential labeling at defined depths (Breslow et al., 1978; Gupta et al., 1979; Vaver et al., 1979; Stoffel et al., 1982). The broad distributions of functionalization positions along fatty acyl chains may reflect the considerable degree of coiling and motion of the acyl chains. In extending this labeling approach to membranes containing proteins (reconstituted systems or biological membranes), it must be considered that the protein surface consists of different types of amino acid side chains which differ very much in their reactivity toward a photogenerated intermediate. Thus labeling of amino acid side chains at the protein–lipid interface must be a function of the probability of collisional encounters with the photoactivated group and of the intrinsic reactivities of the amino acid residues considered. So far very little information is available concerning the relative reactivities toward different amino acid side chains toward the carbenes and nitrenes used in photolabeling. While photolabeling of model membranes containing saturated fatty acyl chains occurs only by the most reactive intermediates generated photolytically from stable precursors, modification of proteins generally requires less reactive species. As carbenes and nitrenes are prone to intramolecular rearrangements, whereby often long-lived, reactive intermediates are generated, it is possible that these species are responsible for some of the labeling of proteins observed. Furthermore, such species may also account for the frequent differences in the labeling patterns of proteins obtained with different types of photoactivatable reagents.

Labeling data obtained with 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]TID)[1] (Brunner & Semenza, 1981; Spiess et al., 1982; Frielle et al., 1982) have encouraged us to investigate the possibility of labeling of a single leaflet of the human erythrocyte membrane. As suggested earlier (Brunner & Richards, 1980), analogues of phosphatidylcholine may be introduced into a target membrane by using phospholipid exchange protein. As in many membranes the rates of transversal diffusion (flip-flop) of phosphatidylcholines are slow as compared to intermembranous exchange in the presence of exchange protein, and it is likely that conditions can be found which allow the incorporation of a photosensitive lecithin into the outer leaflet of a closed membrane vesicle while maintaining the asymmetry during the insertion and subsequent photoactivation of the reagent.

Experimental Procedures

(*I*) *Chemical Syntheses.* All chemicals and solvents were commercial grades of highest purity. Thin-layer chromatography (TLC) was performed on silica gel 60 F_{254} plates from Merck. For column chromatography, silica gel (230–400 mesh ASTM) from the same supplier was used. ^{1}H NMR spectra were measured on a Varian HA-100 or were recorded in the Fourier transform mode on a Bruker CXP-300 instrument. Fourier transform spectra were taken by using 32K of data memory and a spectral width of 3.6 kHz. $CDCl_3$ or CD_3OD was used as solvent and $(CH_3)_4Si$ (δ 0.00) as an internal standard. Chemical shifts are given in parts per million (ppm). Infrared (IR) spectra were recorded on a Perkin-Elmer 257 spectrometer.

m-(Formylamino)trifluoroacetophenone (Methanesulfonyl)oxime. *m*-(Formylamino)trifluoroacetophenone oxime was prepared according to the published procedure (Brunner & Semenza, 1981): 580 mg (2.5 mmol) of the oxime was dissolved in 15 mL of dry acetone, and 430 mg (3.75 mmol) of methanesulfonyl chloride (Fluka) and 1.73 g of dry K_2CO_3 were added. The mixture was refluxed for 2 h and then transferred into a separation funnel. Thirty milliliters of ethyl acetate was added, and the dispersion was extracted 4 times with 20-mL portions of water. The organic solution

† From the Eidgenössische Technische Hochschule, Laboratorium für Biochemie, ETH-Zentrum, CH-8092 Zürich, Switzerland. *Received February 3, 1983.* This work was supported by SNSF, Berne.

[1] Abbreviations: PTPC, 1-palmitoyl-2-[10-[4-[(trifluoromethyl)diazirinyl]phenyl]-8-oxadecanoyl]-*sn*-glycero-3-phosphocholine; TID, 3-(trifluoromethyl)-3-(*m*-iodophenyl)diazirine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; $NaDodSO_4$, sodium dodecyl sulfate; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; IR, infrared; PLEP, phospholipid exchange protein; EDTA, ethylenediaminetetraacetic acid; cmc, critical micelle concentration; PBS, 150 mM NaCl in 5 mM sodium phosphate, pH 7.4; Tris, tris(hydroxymethyl)aminomethane; mp, melting point; bp, boiling point.

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was dried over $MgSO_4$ and evaporated: yield 738 mg (95%) of an oily residue. TLC (chloroform/methanol, 8:1 v/v) showed one major spot at an R_f value of 0.7. The product crystallized spontaneously by dissolving the oily residue in 5 mL of ether: yield 492 mg (63%) of TLC-pure mesylate; ^1H NMR (100 MHz; acetone-d_6) δ 3.36 (s, 3 H), 7.2–8.0 (m, 4 H), 8.40 (s, 1 H), and 9.50 (br s, 1 H).

2-[4-[(Trifluoromethyl)diazirinyl]phenyl]ethyl Trifluoromethanesulfonate (**8**). 2-[4-[(Trifluoromethyl)diazirinyl]-phenyl]ethanol (**5**) was prepared according to the procedure of Brunner et al. (1980). In a Reacti-Vial (3 mL) equipped with a Mininert-Valve (Pierce), 230 mg (1 mmol) of the alcohol (**5**) and 80.5 μL of absolute pyridine (Fluka, puriss) were dissolved in 0.25 mL of CH_2Cl_2 which had been distilled over P_2O_5. A solution of 330 mg of trifluoromethanesulfonic anhydride (Fluka, purum) dissolved in 1 mL of absolute CH_2Cl_2 was added gradually to the alcohol over a period of 30 min while the temperature was kept at 0 °C. Precipitated pyridinium salt of trifluoromethanesulfonic acid was removed by filtration of the reaction mixture through a filter of glass wool in the tip of a Pasteur pipet, and the filtrate was chromatographed on silica gel (15 g) with hexane/ether (5:1 v/v) as the solvent. On TLC plates, the triflate (**8**) had an R_f value of 0.68 (hexane/ether, 3:2 v/v) and was detectable by fluorescence quenching. Fractions containing TLC-pure triflate were pooled, and the solvent was evaporated in vacuo: residue yield 279 mg (77% of theory). This moisture-sensitive compound was not further characterized.

7-Acetoxyheptanoic Acid (**2**). 7-Hydroxyheptanoic acid (**1**) was synthesized following the procedure of Moonen et al. (1979). It was acetylated with acetic anhydride in pyridine. The procedure was the same as that described for 6-hydroxyheptanoic acid (Ellin et al., 1973). The ester was purified by distillation (bp 165 °C; 0.1 mm). The yield was 8.8 g (45% of theory). The IR spectrum showed two bands at 3300–2600 and 1730–1710 cm^{-1} due to the O—H (acid) and C=O of the ester and acid functions, respectively.

7-Acetoxyheptanoic Acid tert-Butyl Ester (**3**). A 1.12-g (5.9 mmol) sample of 7-acetoxyheptanoic acid and 2.85 mL (11.8 mmol) of *N,N*-dimethylformamide di-*tert*-butyl acetal (Fluka) (Thenot et al., 1972) were dissolved in 10 mL of benzene. Pyridine (0.5 mL) was added, and the mixture was refluxed for 20 h. While refluxing, an additional amount of 7.9 mL of the acetal dissolved in 1 mL of pyridine was added in small portions. The reaction mixture was concentrated on a rotavap evaporator and the residue chromatographed on a silica gel 60 column (50 g of silica gel) with hexane/ether (1:1 v/v) as the eluant: yield 1.02 g (70%) of the pure compound; ^1H NMR ($CDCl_3$) δ 1.42 (s, 9 H), 1.25–1.7 (m, 8 H), 2.04 (s, 3 H), 2.19 (t, 2 H), and 4.04 (t, 2 H).

7-Hydroxyheptanoic Acid tert-Butyl Ester (**4**). 7-Acetoxyheptanoic acid *tert*-butyl ester (956 mg; 3.9 mmol) and 235 mg (3.9 mmol) of ethylenediamine (Fluka) were placed into a hydrolysis tube (Pierce) and stirred at 100 °C for 50 h (Kricheldorf & Kaschig, 1976). The mixture was then diluted with ether, and the amide precipitated was separated by filtration. After concentration of the filtrate, the product was purified by column chromatography on silica gel 60 (30 g) with hexane/ether (1:1 v/v). A colorless oil (682 mg) was obtained; IR ($CHCl_3$) showed bands at 3600 and 1720 cm^{-1} which are due to the alcohol and ester carbonyl, respectively: ^1H NMR ($CDCl_3$) δ 1.42 (s, 9 H), 1.25–1.7 (m, 8 H), 2.19 (t, 2 H), and 3.6 (t, 2 H).

10-[4-[(Trifluoromethyl)diazirinyl]phenyl]-8-oxadecanoic Acid (**10**). Triflate (**8**) (279 mg; 0.77 mmol) and 7-hydroxyheptanoic acid *tert*-butyl ester (**4**) (155 mg, 0.77 mmol) were dissolved in 1.5 mL of CH_2Cl_2. Anhydrous potassium carbonate (530 mg; 3.8 mmol) was added, and the dispersion was stirred in a sealed vial for 80 h at 25 °C. The viscous dispersion was then diluted with ether and extracted with water, 1 M NaOH, and water again (3 times). The organic layer was dried over $MgSO_4$, and the solvent was evaporated to yield 260 mg of an oily residue which was dissolved in 2 mL of 40% (v/v) trifluoroacetic acid (Fluka) in methylene chloride. After 20 min, the solvent and most of the trifluoroacetic acid were removed by rotavap evaporation (40 °C), and the residue was chromatographed on silica gel (75 g) with hexane/ether/glacial acetic acid (40:60:1 v/v/v) as the solvent. Fractions which on TLC plates (same solvent as above) showed spots at R_f 0.55 were pooled and extracted 5 times with water. After drying over $MgSO_4$, the organic solvent was evaporated and the residue dried at room temperature (10^{-2} mmHg) for several hours (yield 59% of theory): ^1H NMR ($CDCl_3$) δ 1.66 (m, 8 H), 2.65 (t, 2 H), 3.1 (t, 2 H), 3.8 (t, 2 H), 3.9 (t, 2 H), and 7.2–7.5 (4 H); UV (ethanol) λ_{max} 365 nm (ϵ_{365} ~300).

2-[4-[(Trifluoromethyl)diazirinyl]phenyl]ethanal (**6**). Dipyridine–chromium (VI) oxide complex was prepared according to the procedure of Collins et al. (1968). The complex was dissolved (92 mg·mL^{-1}) in absolute CH_2Cl_2 (distilled over P_2O_5); 26.9 mL of this solution was added to a mixture of 2-[4-[(trifluoromethyl)diazirinyl]phenyl]ethanol (**5**) (368 mg; 1.6 mmol) which was dissolved in 70 mL of absolute CH_2Cl_2. The reaction mixture was stirred vigorously for 2 h at 25 °C while a dark precipitate was formed. The supernatant was decanted and concentrated by evaporation. This solution was diluted in ether, and after removal of insoluble material, the filtrate was concentrated by rotavap evaporation to a volume of approximately 2–3 mL. Column chromatography (30 g of silica gel) with ether/hexane (2:3 v/v) yielded approximately 100 mg of a yellow product which on TLC plates (same solvent) had an R_f value of 0.60. This compound was rechromatographed on 10 g of silica gel with hexane/methylene chloride (2:1 v/v) as the solvent: yield 45 mg of TLC-pure material. Upon reduction with $NaBH_4$ in aqueous ethanol, a sample of the product was quantitatively converted to the starting material (compound **5**) as determined by TLC.

10-[4-[(Trifluoromethyl)diazirinyl]phenyl]-[9-^3H]-8-oxadecanoic Acid (**10**). A total of 30–35 mg of 2-[4-[(trifluoromethyl)diazirinyl]phenyl]ethanal (**6**) was dissolved in 0.1 mL of 2-propanol (Fluka, puriss). This solution was mixed with a freshly prepared solution of NaB^3H_4 (250 mCi; 14.9 Ci·$mmol^{-1}$; Amersham) in 100 μL of 2-propanol/water (4:1 v/v) and reacted at room temperature for 1 h. Subsequently, 5 mL of ether was added, and the organic phase was washed with 3 mL of water containing 0.05 mL of 3 M H_2SO_4 and then repeatedly with water alone until the pH was approximately 5. The ether layer was dried over $MgSO_4$ and concentrated by careful distillation by using a microdistillation apparatus equipped with a 10-cm Vigreux column and a cold trap containing liquid nitrogen in order to condense any volatile radioactive components. The slightly yellow residue (containing alcohol, excess of the aldehyde, and some residual ether) was diluted with 100 μL of CH_2Cl_2 containing 5.4 μL of dry pyridine (this amount of pyridine corresponds to that of the alcohol present assuming that 1 equiv of $NaBH_4$ quantitatively reduced 4 equiv of the aldehyde and that no losses occurred during workup of the reaction mixture). By use of a Hamilton syringe, this alcohol/pyridine solution was transferred in small portions into a 1-mL Reacti-Vial (Pierce)

equipped with a Mininert-Valve which contained a solution of 20 μL of trifluoromethanesulfonic anhydride in 0.3 mL of absolute CH_2Cl_2. The temperature was maintained at 0 °C for 60 min. The precipitate formed was removed by filtrating the reaction mixture through glass wool which had been placed into the tip of a Pasteur pipet. It was rinsed twice with 100 μL of CH_2Cl_2, and the combined filtrates were supplemented with 150 μL of 7-hydroxyheptanoic acid *tert*-butyl ester and 250 mg of anhydrous and finely ground potassium carbonate and stirred for 2 h in a sealed vial. The dispersion was diluted with ether and filtered through a small column of silica gel (5 g) in order to remove inorganic salt (solvent ether). The filtrate was concentrated by rotavap evaporation and the residue treated with 2 mL of 40% (v/v) trifluoroacetic acid in CH_2Cl_2 in order to remove the protective group. The product (compound **10**) was purified by column chromatography as described above for the nonradioactive material. The yield of pure acid was 14.9 mg which corresponds to 55% of the theory as calculated on the basis of the NaB^3H_4 introduced. The specific radioactivity was calculated to be 3.6 Ci·mmol^{-1}.

Phospholipid Syntheses. 1,2-Diacyl-*sn*-glycero-3-phosphocholines were synthesized by using the general procedure of Gupta et al. (1977). 1-Palmitoyl-*sn*-glycero-3-phosphocholine (lysolecithin) was purchased from R. Berchtold, Biochemisches Labor, Bern. Acylation of lysolecithin using anhydrides of the tritiated or nonlabeled fatty acid **10** was followed by TLC (chloroform/methanol/H_2O, 65:25:4 v/v/v). Phosphodiesters were visualized by the molybdenum blue spray (Goswami & Frey, 1971). Isolation and purification of a component showing the same R_f value (0.26) as a reference sample of DPPC were accomplished by column chromatograpy using the same solvent system as above. This product was identified as 1,2-diacyl-*sn*-glycero-3-phosphocholine by high-resolution ^{1}H NMR spectroscopy.

(*II*) *Preparation of Sonicated Liposomes.* Solutions of [^{3}H]PTPC in toluene/ethanol or of a mixture of egg lecithin and [^{3}H]DPPC in chloroform/methanol were evaporated, and the phospholipid films were dried at 10^{-2} mmHg for 2 h at 25 °C. The phospholipid was then dispersed in PBS and the dispersion flushed with nitrogen. Sonication was performed in a sealed ampule under nitrogen by using a bath sonifier (Laboratory Supplies Co., Hicksville, NY; Model T-80-80-1RS). Sonicated dispersions were centrifuged at 100000*g* for 30 min in an airfuge (Beckman) to sediment multilamellar aggregates of the lipid.

(*III*) *Measurement of Critical Micelle Concentration* (*cmc*). The cmc of [^{3}H]PTPC was determined by dialysis using a "Dianorm" dialysis apparatus (Diachema, AG, Switzerland). The two cylindrical half-cells (1 mL each) manufactured of Teflon were separated by a high permeability dialysis membrane (Diachema, AG, Ref. No. 10.16; molecular weight cutoff 10 000) and allowed to rotate slowly at 22 °C. One half-cell contained liposomes prepared from [^{3}H]PTPC at a concentration of 4.4×10^{-6} M, and the other half-cell was filled with dialysis buffer, 0.02 M Tris-HCl (pH 8.3)/0.1 M NaCl/5 mM NaN_3/1 mM EDTA [ionic strength 0.133 (Reynolds et al., 1977)]. The liposomes used were prepared by sonication from [^{3}H]PTPC which had been purified by chromatography on a silica gel column and assayed for purity by thin-layer chromatography. The radiochemical purity was more than 99%. Dialysis buffer was replaced several times (in intervals of approximately 30 min) by fresh buffer before measurements were started. This procedure was included because even purified lipid contained traces of radioactive contaminants which can be removed by dialysis. For cmc measurments, aliquots were taken from both half-cells after time intervals of 10–20 min. The cmc was determined from the rate of dialysis by using the intersection of tangents corresponding to the initial and final (after approximately 2 h) rates (Haberland & Reynolds, 1973).

(*IV*) *Preparation of Human Erythrocyte Membranes.* (*A*) *Unsealed Ghosts.* Human red cells from citrate/phosphate/dextrose-treated, freshly collected blood (O, Rh+) were washed 3 times by centrifugation in 150 mM NaCl/5 mM sodium phosphate, pH 7.4 (PBS). Hemolysis and washing of the membranes were performed according to the procedure of Steck & Kant (1974).

(*B*) *Resealed Ghosts.* Human red cells were washed as described above. Hemolysis and resealing were done according to the procedure 2 of Steck & Kant (1974) with the modification that after hemolysis the membranes were pelleted and washed once by sedimentation in 5 mM sodium phosphate, pH 7.4, before resealing in PBS was initiated. Ghosts were purified by centrifugation on a dextran cushion (Dextran T-70, Pharmacia) of a density of 1.03 g·mL^{-1} for 2 h at 90000*g*. Membranes floating on the barrier were removed and washed twice by sedimentation in PBS. Preparations of resealed ghosts were at least 95% pure as judged from the accessibility of NADH–cytochrome *c* oxidoreductase (Steck & Kant, 1974).

(*V*) *Determination of* [^{3}*H*]*PTPC Transfer from Liposomes to Erythrocyte Membranes.* Resealed ghosts (2 mg·mL^{-1}) were incubated with sonicated liposomes prepared from radioactively labeled lipid. Aliquots of 10 μL were diluted with 100 μL of PBS and centrifuged in an airfuge at 100000*g* for 1 min. Under these conditions, erythrocyte membranes sedimented while liposomes did not. The fraction of phospholipid transferred was calculated from the radioactivity of aliquots of the diluted membrane suspension before centrifugation and of the supernatant after centrifugation.

(*VI*) *Photolysis Experiments.* Membranes containing the photosensitive reagent were transferred into a cylindrical quartz cuvette thermostated at 2–4 °C. Light from a 350-W high-pressure mercury lamp (Illumination Industries, Inc., type 350-1008) was focused onto the center of the cuvette. To screen out IR and shortwave UV light, the beam was directed through filters of circulating cold water (30 mm) and a saturated solution of copper sulfate (20 mm). Samples were photolyzed for 60 s.

(*VII*) *Isolation of Glycophorin A.* Photolabeled erythrocyte membranes were washed once in 5 mM Tris-HCl, pH 7.5, and were lyophilized. Glycophorin was isolated according to the procedure of Marchesi & Andrews (1971). For delipidation, glycophorin was extracted with chloroform/methanol/HCl (150:150:1 v/v/v) followed by a second phenol extraction (Van Zoelen et al., 1977).

(*VIII*) *Cyanogen Bromide Cleavage of Glycophorin.* Samples of glycophorin (corresponding to approximately 50–100 μg of protein) dialyzed against water were lyophilized. Cyanogen bromide (CNBr) cleavage was performed essentially as described by Segrest et al. (1973) in 50 μL of 70% (v/v) formic acid containing 10 mg·mL^{-1} CNBr. Incubation mixtures were kept at 25 °C for 48 h, then diluted 10-fold with water, and lyophilized. A second portion of water was added to the dry material and the lyophilization repeated to remove all traces of reagent. Protein fragments were dissolved in sample buffer and subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis.

(*IX*) *Analytical Procedures.* Protein was determined according to a modified Lowry procedure (Peterson, 1977) with bovine serum albumin as a standard. For $NaDodSO_4$/poly-

acrylamide gel electrophoresis, protein samples were denatured in 2% $NaDodSO_4$ and 5% mercaptoethanol at 100 °C for 2 min. Proteins were separated on a discontinuous electrophoresis system as described previously (Brunner et al., 1979) using polyacrylamide slab gels (12 × 2.7)[2] (thickness 2 mm, length 9 cm). Lanes were cut into 2-mm slices and incubated in scintillation vials with 750 μL of NCS-Solubilizer (Amersham) for 2 h at 50 °C before 6 mL of scintillation solution was added and counted.

Results

Design and Synthesis of a Photoactivatable Phospholipid of High Specific Radioactivity. An inconvenience of hydrophobic photolabeling of membranes is the frequent requirement of substantial quantities of radioactivity for providing a basis of subsequent analysis of labeled membrane proteins. As shown for [^{125}I]TID with erythrocyte membranes, less than 3% of the radioactivity originally introduced became bound to proteins, (Brunner & Semenza, 1981), and in the case of small intestinal sucrase–isomaltase, approximately 0.012% of the radioactivity was found covalently associated with the membranous segment (Spiess et al., 1982). The low efficiency of carbenes to label proteins recommended the synthesis of a phospholipid of high specific radioactivity.

Since analysis of labeled proteins may include such harsh conditions as acid hydrolysis, it was further advantageous to introduce the radiolabel at a position close to and tightly bound to the photoreactive unit. Thus radiolabeling of the polar head group did not appear to be a suitable solution because of the susceptibility of the ester bonds to hydrolysis. On the basis of these considerations, we have chosen the phospholipid and the synthetic route depicted in Figure 1. In essence, the synthesis consists of the preparation of a modified fatty acid which in ω-position is linked via an ether bond to the radioactively labeled photoactivatable unit. In the course of this synthesis, tritium was introduced by reducing aldehyde **6** with NaB^3H_4 of high molar radioactivity (14.9 Ci·mmol^{-1}). On the basis of the amount of radioactivity introduced, the overall yield in the synthesis of fatty acid **10** was as much as 55%. Since the molar radioactivity of the product is approximately one-quarter that of the tritiated borohydride used, the specific radioactivity of the final compound was calculated to be 3.6 Ci·mmol^{-1}. By use of this radiolabeled photosensitive fatty acid, 1-palmitoyl-*sn*-glycero-3-phosphocholine was acylated following the general procedure of Gupta et al. (1977).

By use of the detailed reference data (Lammers et al., 1978), the photosensitive phospholipid isolated was identified as 1,2-diacyl-*sn*-glycero-3-phosphocholine by high-resolution ^{1}H NMR. All resonances could be clearly assigned and chemical shifts and coupling constants were in excellent agreement with the literature data. The *sn*-2-methine proton H_h [the assignment is that of Lammers et al. (1978)] showed a broad signal at δ 5.2. The protons H_f showed two doublets at δ 4.16 and 4.42 with coupling constants of 12 and 7 Hz, respectively.

Treatment of a sample of the radioactive phospholipid, [^{3}H]PTPC, with phospholipiase A_2 followed by TLC separation of the reaction products showed a rapid degradation of [^{3}H]PTPC with concomitant formation of a radioactive fatty acid. The radioactive phospholipid, [^{3}H]PTPC, is stable in the dark for an extended period of time when stored at 4 °C as a solution in ethanol/toluene (1:1 v/v) at a concentration

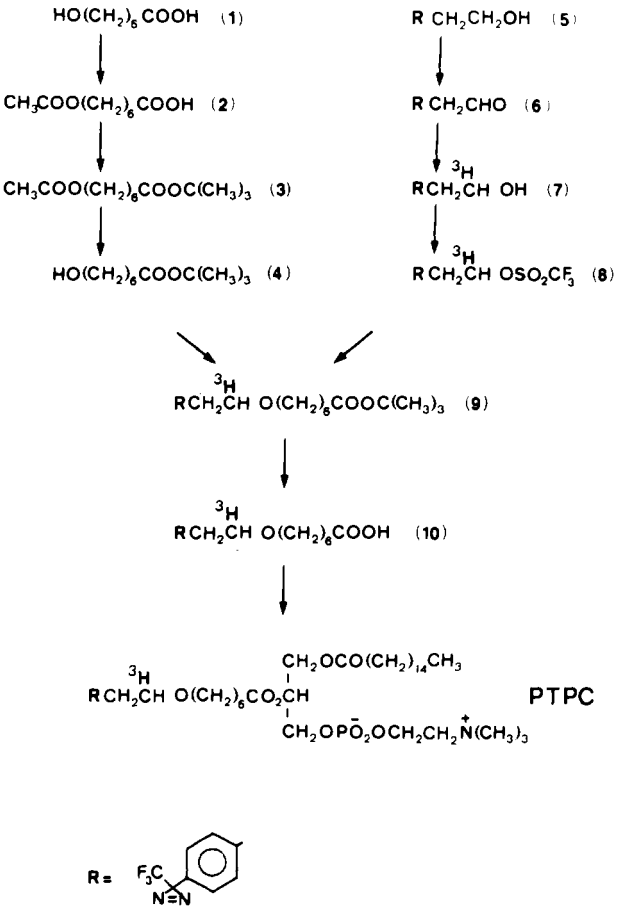

FIGURE 1: 1-Palmitoyl-2-[10-[4-[(trifluoromethyl)diazirinyl]-phenyl]-[9-^{3}H]-8-oxadecanoyl]-*sn*-glycero-3-phosphocholine ([^{3}H]-PTPC). Reaction scheme for the synthesis of [^{3}H]PTPC.

of not more than 1 mCi·mL^{-1}.

Properties of the Photosensitive Phospholipid. Sonication of an aqueous dispersion (1 mM) of PTPC gave a solution which scattered light only slightly. The lipid particles formed had an internal cavity of 0.33 mL·mmol^{-1} of phospholipid as determined from the fraction of [^{35}S]taurine which could be entrapped and separated from the bulk of the taurine originally added to the buffer. The figure determined for the internal space of the phospholipid particles is very near that (0.31 mL·mmol^{-1}) which had been determined previously for small unilamellar liposomes prepared from egg lecithin (Brunner et al., 1976).

By use of dialysis rates (Haberland & Reynolds, 1973), the critical micelle concentration (cmc) of PTPC was determined. At a concentration of the phospholipid of 4.4 × 10^{-6} M, the cmc is (6 ± 2) × 10^{-9} M at 23 °C in 0.02 M Tris-HCl (pH 8.3)/0.1 M NaCl/5 mM NaN_3/1 mM EDTA (ionic strength 0.133).

Incubation of a sonicated dispersion of [^{3}H]PTPC with erythrocyte membranes resulted in a spontaneous transfer of radioactivity to the erythrocyte membrane. In contrast, when liposomes made of [^{3}H]DPPC/egg lecithin mixtures were used instead of the photosensitive lipid, there was no spontaneous intermembrane transfer of the radiolabel. The results in Figure 2 demonstrate that under the conditions used, transfer of [^{3}H]PTPC was rather efficient and incorporation of 50% of the total label added was accomplished within approximately 5 min.

Photolabeling of the Erythrocyte Membrane. In order to investigate the transverse distribution of [^{3}H]PTPC in the human erythrocyte membrane, a procedure has been devised

[2] According to Hjertén (1962) the first figure in the parentheses indicates the total concentration of monomers [percent (w/v)] and the second that of *N,N'*-methylenebis(acrylamide), as percentage of the total monomer concentration.

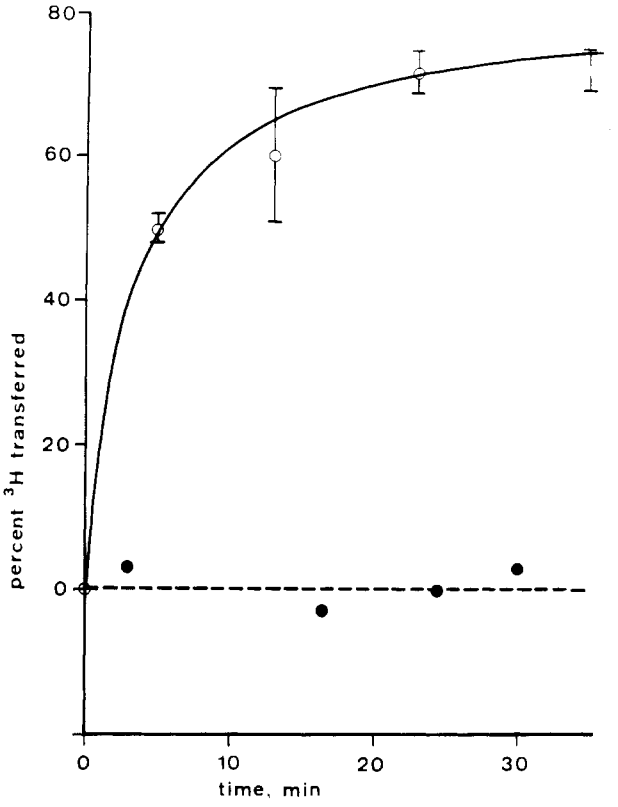

FIGURE 2: Intermembrane phospholipid exchange. Resealed ghosts were incubated with sonicated liposomes prepared from radioactively labeled phospholipid at 37 °C. The concentration of protein was 2 mg·mL^{-1} and that of phospholipid approximately 40 μM, and the incubation buffer was PBS. After the time points indicated, ghosts were sedimented differentially (1 min at 100000g). The fraction of radioactivity that cosedimented with ghost membranes corresponds to the phospholipid transferred. Each time point (open circles) represents the mean value of four transfer experiments (±SD) performed with [^{3}H]PTPC (3.6 Ci·mmol^{-1}). In a control experiment, resealed ghosts were incubated with liposomes (same concentrations as above) which had been prepared from a mixture (molar ratio 1:1) of [^{3}H]DPPC and egg yolk lecithin. The transfer of [^{3}H]DPPC is represented by the closed circles.

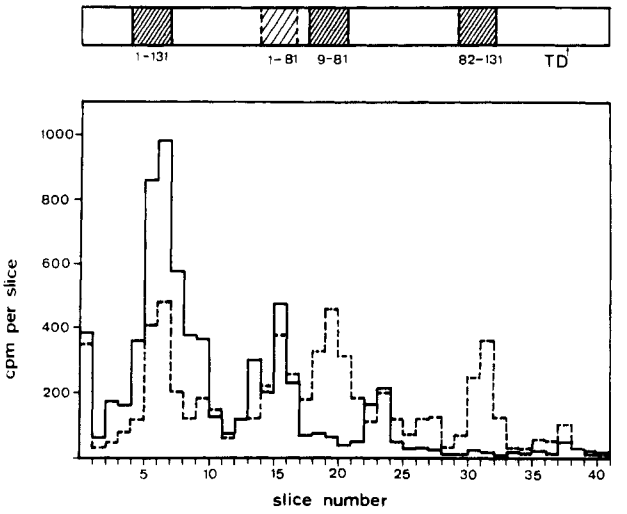

FIGURE 3: Distribution of radioactivity among cyanogen bromide fragments derived from glycophorin labeled with [^{125}I]TID. Glycophorin isolated from [^{125}I]TID-labeled ghosts was purified by $NaDodSO_4$ gel electrophoresis. Protein corresponding to PAS I was extracted from the gel by using 5 mM $NaHCO_3$ and 0.05% $NaDodSO_4$. Excess of $NaDodSO_4$ was removed by gel filtration on Sephadex G-25 (Amons & Schrier, 1981). Samples of purified, [^{125}I]TID-labeled glycophorin were treated for 35 h at 20 °C with formic acid alone (control) or with a solution of cyanogen bromide (10 mg·mL^{-1}) in formic acid. Products were analyzed by $NaDodSO_4$ gel electrophoresis followed by radioactivity counting of 2-mm slices of the gel. The radioactivity profiles shown correspond to products obtained from treatment of glycophorin with formic acid alone (continuous line) and from treatment with cyanogen bromide/formic acid (broken line). Positions of the products are indicated by the gel pattern at the top of the figure.

which includes photo-cross-linking of the photosensitive lipid to membrane components followed by an analysis of the distribution of radiolabel among suitable fragments of glycophorin A. Glycophorin A, the major sialoglycoprotein of this membrane, spans the lipid bilayer once in a "C-in, N-out" direction (Segrest et al., 1973). This transmembrane domain has been studied by using a variety of techniques including photolabeling approaches (Ross et al., 1982). Recent data (Ross et al., 1982) suggest a transmembrane segment comprising residues 63–94 which has predominantly α-helical structure. The two methionine residues (residues 8 and 81) in glycophorin A permit fragmentation of the polypeptide chain by cyanogen bromide. According to the model of the transmembrane domain, methionine-81 must be located near the middle of the membrane-spanning segment. Cyanogen bromide treatment of glycophorin, therefore, yields fragments which contain that part of the membranous segment which traverses either the outer half of the lipid bilayer (fragment 9–81) or the inner half of the membrane (fragment 82–131). The analytical approach used in this study relied upon the assumption that if the reactive lipid is present in the outer half of the membrane, labeling of glycophorin should be confined to a region contained in fragment 9–81. Alternatively, if labeling is restricted to the inner half, the sites of predominant modification of glycophorin A would be found within fragment 82–131. This suggests that from measurements of the ratios of radioactivity among the cyanogen bromide fragments, relevant information concerning the transverse distribution of the label can be derived.

(A) With [^{125}I]TID. Photolabeling of the erythrocyte membranes with the lipid-soluble [^{125}I]TID has shown that glycophorin incorporates approximately 0.3% of the radioactivity originally present (Brunner & Semenza, 1981). In addition, it was demonstrated that the radiolabel covalently bound to glycophorin is confined to a segment designated as T-6 (Furthmayr et al., 1978) that contains the hydrophobic transmembrane domain.

In order to evaluate the intrinsic reactivities of the two halves of the membrane-spanning helix toward the carbene, the labeling pattern was analyzed in more detail. First, [^{125}I]TID-labeled glycophorin was isolated and purified by $NaDodSO_4$/polyacrylamide gel electrophoresis. This step was included in order to delipidate protein rigorously and to remove traces of noncovalently bound radioactive contaminants. Second, glycophorin thus purified was then treated with cyanogen bromide in formic acid or with formic acid alone (control). Third, products obtained were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis followed by the determination of radioactivity in 2-mm gel slices. The radioactivity profiles thereby obtained are shown in Figure 3. Glycophorin treated with formic acid alone (continuous line) gave three to four radioactive bands which correspond to the distinct molecular forms of glycophorin as obtained by boiling in $NaDodSO_4$ (Furthmayr & Marchesi, 1976). Glycophorin treated with cyanogen bromide gave two bands (in addition to those from uncleaved glycophorin) that correspond to cyanogen bromide fragments 9–81 and 82–131, respectively. The assignment was based on the relative electrophoretic mobilities of these bands and staining with Coomassie blue (both bands were stained) and periodic acid/Schiff's reagent

(only that fragment with the lower electrophoretic mobility was stained) (Segrest et al., 1973). A third cleavage product, fragment 1–81, appears to have an electrophoretic mobility that is identical with or very similar to that of the PAS II species of uncleaved glycophorin.

The main results of this analysis can be summarized as follows: (i) [^{125}I]TID labeled the transmembrane domain of glycophorin A at at least two sites, one being located within fragment 9(1)–81 and the other within fragment 82–131. Therefore, both halves of the transmembrane domain contain amino acid residues that are reactive toward the carbene. (ii) Treatment of [^{125}I]TID-labeled glycophorin with formic acid alone did not give rise to low molecular weight components which traveled with or near the tracking dye front of the gel. (iii) In the gel system used, the mobilities of fragments 9–81 and 82–131 are such that they can be distinguished clearly from any of the forms of uncleaved glycophorin.

(*B*) *With* [^{3}H]PTPC. In essence, two experiments were performed in which erythrocyte membranes [leaky or resealed ghosts; 2 (mg of protein)·mL^{-1}] were incubated with sonicated liposomes prepared from [^{3}H]PTPC at 37 °C for 5 min and photolyzed subsequently for 1 min. The concentrations of [^{3}H]PTPC were 0.017 (leaky ghosts) and 0.034 mg·mL^{-1} (resealed ghosts).

Upon photolysis, glycophorin was extracted by using the method of Marchesi & Andrews (1971). As glycophorin isolated by this procedure still contained rather significant amounts of bound lipids, the glycoprotein was subjected to extractions with chloroform/methanol/HCl and phenol. Both extraction procedures were shown to essentially completely remove bound lipids (Van Zoelen et al., 1977). NaDodSO$_4$/polyacrylamide gel electrophoresis of samples of glycophorin showed that despite the delipidation steps included, glycoprotein still contained radioactive contaminants that could be released and separated from glycophorin by boiling in NaDosSO$_4$ followed by gel electrophoresis. In both preparations of glycophorin (from labeled leaky and resealed ghosts), the amount of noncovalently bound radioactive components corresponded to approximately 25% of the total radioactivity.

In order to demonstrate that short-time incubation (5 min) of erythrocyte membranes with [^{3}H]PTPC liposomes resulted in incorporation of [^{3}H]PTPC molecules in the erythrocyte membrane bilayer and in subsequent labeling within the hydrophobic core, glycophorin [isolated from labeled (leaky) ghosts] was treated with trypsin and T-6 peptide recovered by acid precipitation (Furthmayr et al., 1978). As the radioactivity in the sample was quantitatively (>95%) sedimented with the insoluble T-6 peptide, labeling must have occurred within this peptide and hence within the lipid bilayer of the erythrocyte membrane. This conclusion was consistent with results of further experiments in which it was shown that none of the extrinsic proteins contained substantial quantities of radioactivity and that extensive treatment of labeled and washed membranes with papain did not result in a release of more than 5% of the radioactivity. On the basis of the specific radioactivity of [^{3}H]PTPC-labeled glycophorin, it was calculated that approximately 0.2–0.4% of the radioactivity transferred became covalently bound to the glycoprotein. This demonstrates that the labeling efficiency of PTPC is the same or nearly the same as that of [^{125}I]TID which dissolves and reacts exclusively from within the hydrophobic phase.

Aliquots of glycophorin were subjected to cyanogen bromide treatment. Products were analyzed by NaDodSO$_4$ gel electrophoresis followed by scintillation counting of 2-mm slices. The main results were shown in Figure 4. At the top it shows

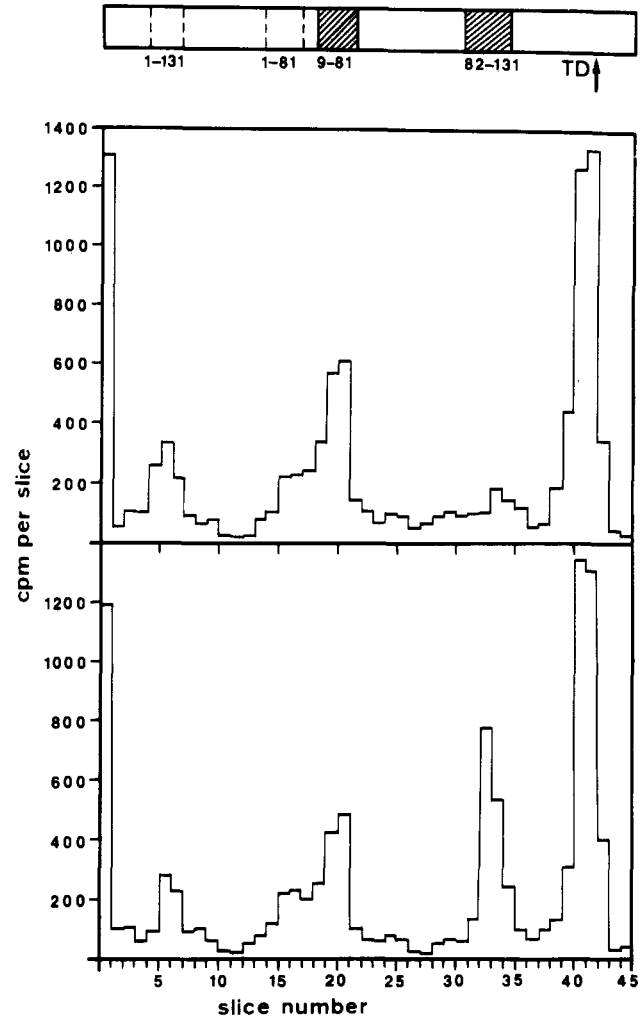

FIGURE 4: Distribution of radioactivity among cyanogen bromide fragments derived from glycophorin labeled with [^{3}H]PTPC in resealed ghosts (upper profile) and in unsealed ghosts (lower profile). [^{3}H]PTPC was transferred into erythrocyte membranes and photocross-linked to membrane components. Glycophorin was isolated from [^{3}H]PTPC-labeled membranes and subjected to cyanogen bromide cleavage. Cleavage products were separated by NaDodSO$_4$/polyacrylamide gel electrophoresis. Gels were cut into 2-mm slices, and radioactivity was determined by scintillation counting. The radioactivity profiles shown correspond to identical numbers of total cpm.

a schematic drawing of the NaDodSO$_4$/polyacrylamide gel pattern of the cleavage products of glycophorin as established by Coomassie blue staining and periodic acid/Schiff's reagent (original data not shown). The upper radioactivity profile was that derived from glycophorin cross-linked to [^{3}H]PTPC in resealed ghosts, and the lower one corresponds to fragments of glycophorin labeled in leaky ghosts.

Both profiles are virtually identical except for a radioactive band (lower profile) corresponding to cyanogen bromide fragment 82–131. There was little if any radioactivity at the same position in the upper profile, demonstrating that segment 82–131 was not labeled by [^{3}H]PTPC upon transfer of the reagent into resealed ghosts. In both profiles, radioactivity was measured at positions that correspond to (uncleaved) glycophorin and fragments 1–81 and 9–81. Therefore, the distributions of radiolabel among the fragments of glycophorin were consistent with those predicted by assuming that phospholipid transfer occurred as monomers through the aqueous phase.

Not unexpectedly, both samples contained radioactive products that traveled with the tracking dye front. The origin

of this material is not known, but it is likely due to cross-linked lipids. In fact, [^{125}I]TID-labeling experiments (see above) support this view since neither formic acid alone nor formic acid/cyanogen bromide was able to release substantial amounts of radioactive products from rigorously delipidated glycophorin. Regardless of how samples were treated with $NaDodSO_4$ prior to electrophoresis (room temperature or 100 °C for 2 min), there was always a significant fraction of radioactivity that did not enter the separation gel. This material is likely to originate from fragments of glycophorin that formed aggregates. Since the composition of these presumed aggregates is not known, the actual distribution of radiolabel among the cyanogen bromide fragments of glycophorin cannot be determined precisely. However, despite this difficulty, the main result of this study, namely, the finding that segment 82–131 was not labeled by [^{3}H]PTPC in resealed ghosts, is secure since all additional electrophoresis experiments have given consistent results.

Discussion

The present paper describes the selective labeling of the outer leaflet of the human erythrocyte membrane with a photosensitive phospholipid. This reagent was synthesized with high specific radioactivity and was found to be inserted spontaneously into the erythrocyte membrane. This observation demonstrates that the application of photosensitive phospholipids is restricted to model membranes or cultured cells (Hu & Wisnieski, 1979) or that their insertion into biomembranes requires phospholipid exchange protein (Brunner & Richards, 1980).

Spontaneous Lipid Transfer. Spontaneous transfer of phospholipids between membranes is well documented (Martin & MacDonald, 1976; Duckwitz-Peterlein et al., 1977; Kremer et al., 1977; Papahadjopoulos et al., 1976), and kinetic studies support a mechanism by which lipids are transferred as monomers through the aqueous phase (Roseman & Thompson, 1980; Correa-Freire et al., 1982; Massey et al., 1982). The rate of transfer of fluorescent phosphatidylcholines was dramatically affected by the fatty acyl chain composition and increases in the transfer rate correlated with changes in the aqueous solubility of the monomers (Massey et al., 1982). Interestingly, the cmc of PTPC [$(6 \pm 2) \times 10^{-9}$ M] is only about 1 order of magnitude higher than that of DPPC (4.6×10^{-10} M; Smith & Tanford, 1972), a lecithin which did not show detectable transfer under similar experimental conditions. This indicates that transfer rates may not strictly correlate with the aqueous solubility of the lipid. In correlating cmc's with transfer rates, it should be noted also that the cmc's considered had not been determined under identical conditions. Clearly further work is necessary to determine the factors that could have been responsible for the high transfer rates observed with PTPC. As the rate-limiting step is the dissociation from the lipid surface [discussed by Massey et al. (1982)], this suggests that similar transfer rates would be obtained with other "acceptor" membranes.

Transbilayer Diffusion (Flip-Flop) of Phosphatidylcholines. Labeling of the outer leaflet of a closed membrane vesicle rests decisively upon formation of a nonequilibrium distribution of the photosensitive lipid in the target membrane. Therefore, membranes exhibiting very high flip-flop rates for phosphatidylcholines may not be amenable to this type of labeling. Flip-flop rates have been determined for various membranes. Thus, Bloj & Zilversmit (1976) have determined the half-time for equilibration in rat erythrocyte resealed ghosts to be approximately 2.3 h at 37 °C. In the intact erythrocyte, the corresponding value is approximately 7 h. While diffusion rates are even smaller in human erythrocyte membranes or in liposomes (Johnson et al., 1975), values in the order of minutes were measured for excitable membranes (McNamee & McConnell, 1973), and very rapid translocation rates seem to exist also for microsomal membranes (Zilversmit & Hughes, 1977). If the time required for spontaneous intermembrane transfer of phospholipid is long compared to the half-time of phospholipid flip-flop, phospholipid exchange protein might represent a convenient tool in that it accelerates intermembranous exchange while probably not affecting flip-flop rates. Since photoactivation of the reagent may be achieved by a single powerful flash, high flip-flop rates need not represent a major obstacle in the selective labeling of the outer leaflet of a membrane vesicle.

Interpretation of Photolabeling Patterns. The critical role of the photoactivatable unit for photolabeling is now generally recognized [for reviews, see Bayley & Knowles (1977) and Chowdhry & Westheimer (1979)]. Thus, it appears that despite the numerous successful applications of aryl azides, these reagents give satisfactory results only when certain types of amino acid side chains are available to the reactive intermediate(s). As a result of the low efficiency of arylnitrenes in their reaction with paraffinic side chains (Bayley & Knowles, 1978a; Gupta et al., 1979; Brunner & Richards, 1980), much of the more recent work is focused on the development of suitable precursors of carbenes (Smith & Knowles, 1975; Chowdhry et al., 1976; Brunner et al., 1980). Among these, diazirines appear to meet most of the requirements although, as discussed below, some problems remain.

The transmembrane domain of glycophorin has been subjected to various photolabeling studies in which three types of diazirines were used. These are (i) adamantanyldiazirine, (ii) 3-aryl-3*H*-diazirines [Ross et al. (1982) and previous work cited therein], and (iii) 3-(trifluoromethyl)-3-aryldiazirines (TID and PTPC). A common feature of these diazirines is that upon photolysis 35–60% of the diazirine is photoisomerized to the corresponding diazo isomer. While diazoadamantane and aryldiazomethane are powerful alkylating reagents, aryltrifluorodiazoethane is unreactive under the usual labeling conditions (Brunner et al., 1980). The occurrence of long-lived, reactive intermediates may be responsible for some of the differences frequently observed in labeling with different reagents.

TID and PTPC labeled the two halves of the membrane-spanning segment of glycophorin to nearly the same extent. This is in contrast to a recent study (Ross et al., 1982) in which labeling by other carbene-generating lipids [PL I and PL III according to Ross et al. (1982)] was confined to segment 9–81. Edman degradation in that study showed that cross-linking occurred primarily at glutamic acid-70. This surprising selectivity was interpreted to be the result of two main factors, (i) the increasing chain mobility toward the methyl terminus (to explain that phospholipids differing in the presumed penetration of the reactive group reacted with the same residue) and (ii) an increased reactivity (accessibility) of residues such as glutamic acid or tryptophan when compared with that of other amino acid side chains. However, since PTPC (which is structurally closely related to PL I) did label segment 82–131 (this study), it may be appropriate to consider an alternative interpretation. In our opinion it is possible that PL I and PL III (Ross et al., 1982) *did* label both halves of the membrane-spanning segment. However, as labeling by the carbene may have been much less efficient than esterification of glutamic acid-70 by the diazo isomers of PL I and PL III, the former contribution may have escaped detection in the ana-

lytical procedure used. That the diazo isomers did substantially contribute to the overall labeling was recognized by Ross et al. (1982). Of course, this view assumes that the carbenes generated from PL I and PL III are as reactive as those obtained from TID or PTPC. Support for this comes from earlier studies which indicate that PL I gives higher yields in cross-linking to saturated acyl chains in liposomes than does a phospholipid containing the 3-(trifluoromethyl)-3-aryldiazirine group (Gupta et al., 1978; Brunner & Richards, 1980). In analogy, (trifluoromethyl)carbene is slightly less reactive than the unsubstituted counterpart, methylene (Atherton & Fields, 1968).

Adamantanyldiazirine, which like PL I and PL III generates a reactive diazo isomer (Bayley & Knowles, 1978b), labeled both halves of the membranous domain of glycophorin to nearly the same extent (Goldman et al., 1979). Thus, this result seems to be inconsistent with our concern about long-lived reactive intermediates. However, although the distribution of radiolabel among fragments 9–81 and 82–131 were essentially the same as that obtained with [^{125}I]TID or [^{3}H]PTPC (in leaky ghosts), there is substantial evidence that labeling by adamantanyldiazirine did not occur at the same sites. In fact, as shown by a more recent study, the presence of water-soluble thiols reproducibly reduced the extent of labeling of glycophorin (Bayley & Knowles, 1981), indicating that groups lying just outside the hydrophobic core have reacted with label. Such an effect was not observed with [^{125}I]TID (Brunner & Semenza, 1981). There are also considerable differences in the relative extents to which components of the erythrocyte membrane were labeled with adamantanyldiazirine and TID (Bayley & Knowles, 1981; Brunner & Semenza, 1981). The same is also the case for Na,K-ATPase (unpublished results).

Acknowledgments

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Appendix

Under Experimental Procedures, a simple synthesis of *m*-(formylamino)trifluoroacetophenone (methanesulfonyl)-oxime with 65% yield is described. This compound can be converted quantitatively to the corresponding diaziridine by treatment with ammonia (Brunner & Semenza, 1981). We consider this modification in the synthesis of TID precursor as a substantial improvement which should allow large-scale preparations of the photosensitive reagent.

Registry No. **1**, 3710-42-7; **2**, 86013-76-5; **3**, 86013-77-6; **4**, 86013-78-7; **5**, 73899-21-5; **6**, 86013-79-8; **8**, 86013-80-1; **10**, 86013-81-2; [^{3}H]**10**, 86013-82-3; PTPC, 86013-83-4; [^{3}H]PTPC, 86013-84-5; *m*-(formylamino)trifluoroacetophenone (methanesulfonyl)oxime, 86013-85-6; *m*-(formylamino)trifluoroacetophenone oxime, 79684-38-1; 1-palmitoyl-*sn*-glycero-3-phosphocholine, 17364-16-8.

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Structure and Kinetics of the Photoproduct of Carboxymyoglobin at Low Temperatures: An X-ray Absorption Study†

B. Chance,* R. Fischetti, and L. Powers

ABSTRACT: Photolysis and recombination of carboxymyoglobin at low temperatures have been studied by a variety of methods. This paper combines optical and structural studies of carboxymyoglobin photolysis and recombination in the temperature range 4–120 K. The absorbance changes indicate ablation of the characteristic optical transitions of carboxymyoglobin and formation of a photoproduct (Mb*CO) differing from deoxymyoglobin. When the X-ray absorption changes in the 7150–7200-eV region of the X-ray absorption spectrum are used as an indicator of structural change, the photoproduct at 4 K as measured with respect to the unphotolyzed sample is 60% of that observed for the chemically produced deoxy form. Saturation of the change is obtained with repetitive flashes totaling several thousand joules of energy from a xenon flash lamp by using a thin sample (1 mm) at 4 mM concentration as measured by both optical transmission and X-ray absorption criteria. The kinetics of the reaction show the change to occur at 10 K within the resolving time currently available (2 s) in the X-ray absorption measurements. The amplitude of the light-induced change decreases to half its maximal value at 40 K and to zero at 90 K. Steady illumination suggests at least two recombination processes. Analysis of the extended X-ray absorption fine structure (EXAFS) data on Mb*CO indicates small distance changes in the first shell of Fe–N and Fe–C that can be attributed to lengthening of the pyrrole nitrogen bonds and proximal histidine motion, together with a small displacement of the CO molecule on photolysis—a form here designated Mb*CO. This structure of the geminate state, Mb*CO, may elucidate the nature of elementary steps in chemical reactions and in tunneling processes.

In 1928, the wavelength-specific photolysis of iron carbonyls was used by Otto Warburg to identify "Atmungsferment" (Warburg, 1948; Bücher, 1947), later called cytochrome oxidase (Keilin, 1966). This ingeneous indirect method involved photolysis of the heme–CO compound in the presence of oxygen and was later perfected for a variety of oxidases including cytochrome *o* (Chance et al., 1953). Spectral observation of this photolysis was obtained at near freezing temperatures (Chance, 1953) and at lower temperatures (Chance et al., 1965). Curiously enough, the temperature dependence of recombination of CO and myoglobin could be observed at much lower temperatures than cytochrome oxidase as shown by Yonetani et al. (1973), who recorded the kinetics of the recombination reaction to 4.2 K (Iizuka et al., 1974). These kinetics were studied in detail by Frauenfelder and his colleagues (Austin et al., 1973, 1975; Alberding et al., 1976a,b, 1978; Alben et al., 1980), who emphasized the nonexponential nature of the reaction kinetics. While the optical method gave no clue as to the nature of the structural change that accompanies the flash-induced photolysis of carboxymyoglobin (MbCO), their results suggested that it would be best demonstrated at 4.2 K where the recombination rate is slow and the motion of the CO molecule away from the heme is limited.

The existence of an optical property of the photoproduct (Mb*CO) different from deoxymyoblobin (Mb) was supported by a shift of the absorption band of Mb*CO with respect to that of Mb by 14 nm, from 758 to 772 nm at 4.2 K (Iizuka et al., 1974). As the temperature increases toward 30 K, the

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