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Hydrophobic Photolabeling Identifies BHA2 as the Subunit Mediating the Interaction of Bromelain-Solubilized Influenza Virus Hemagglutinin with Liposomes at Low pH†

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ABSTRACT: To investigate the molecular basis of the low-pH-mediated interaction of the bromelain-solubilized ectodomain of influenza virus hemagglutinin (BHA) with membranes, we have photolabeled BHA in the presence of liposomes with the two carbene-generating, membrane-directed reagents 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]TID) and a new analogue of a phospholipid, 1-palmitoyl-2-[11-[4-[3-(trifluoromethyl)diazirinyl]phenyl][2-^{3}H]undecanoyl]-*sn*-glycero-3-phosphocholine ([^{3}H]-PTPC/11). With the latter reagent, BHA was labeled in a strictly pH-dependent manner, i.e., at pH 5 only, whereas with [^{125}I]TID, labeling was seen also at pH 7. In all experiments, the label was selectively incorporated into the BHA2 polypeptide, demonstrating that the interaction of BHA with membranes is mediated through this subunit, possibly via its hydrophobic N-terminal segment. Similar experiments with a number of other water-soluble proteins (ovalbumin, carbonic anhydrase, α-lactalbumin, trypsin, and soybean trypsin inhibitor) indicate that the ability to interact with liposomes at low pH is not a property specific for BHA but is observed with other, perhaps most, proteins.

The fusion of membranes is an essential step in a broad range of biological processes such as intracellular transport, endocytosis, exocytosis, fertilization, and the entry of genome of enveloped animal viruses into the host cell. There have been extensive investigations concerning the mechanism and specificity of membrane fusion. Yet the molecular mechanisms are not, or only poorly, understood. In many cases, Ca^{2+} ions are required while in some others, the fusion process is triggered solely by proteins (Papahadjopoulos et al., 1977, 1979; White et al., 1983). Recently, Lucy proposed that fusion in biological systems is mediated by hydrophobic protein segments that result from endogenous proteolyses (Lucy, 1984). In the case of enveloped animal viruses, specific fusion proteins have been identified (White et al., 1983). On the other hand, there is evidence that membrane fusion can also be triggered effectively by several water-soluble proteins such as cytochrome *c* (Gad et al., 1982), clathrin (Hong et al., 1985), lysozyme (Arvinte et al., 1986), and α-lactalbumin (Kim & Kim, 1986), suggesting that fusion is a more general phenomenon than anticipated earlier.

Entry of influenza viruses into cells is mediated by a low-pH-induced membrane fusion event in endosomal vesicles (Skehel et al., 1982). The factor responsible for fusion is hemagglutinin (HA),[1] a viral spike protein whose structure is exceptionally well characterized. HA is a trimeric transmembrane protein in which each subunit consists of two disulfide-linked glycopeptides HA1 and HA2 with molecular weights of 58 000 and 26 000, respectively (Klenk et al., 1975; Wiley et al., 1977; Lazarowitz & Choppin, 1975). Exhaustive

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[1] Abbreviations: HA, influenza virus hemagglutinin; BHA, BHA1, and BHA2, bromelain-solubilized ectodomain of HA (BHA) and the two subunits derived therefrom; [^{125}I]TID, 3-(trifluoromethyl)-3-([^{125}I]-iodophenyl)diazirine; [^{3}H]PTPC/8, 1-palmitoyl-2-[11-[4-[(trifluoromethyl)diazirinyl]phenyl][10-^{3}H]-9-oxaundecanoyl]-*sn*-glycero-3-phosphocholine; [^{3}H]PTPC/11, 1-palmitoyl-2-[11-[4-[3-(trifluoromethyl)diazirinyl]phenyl][2-^{3}H]undecanoyl]-*sn*-glycero-3-phosphocholine; DPPC, dipalmitoyllecithin; TLC, thin-layer chromatography; DMF, dimethylformamide; THF, tetrahydrofuran; EDTA, ethylenediaminetetraacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; PBS, 150 mM NaCl and 5 mM sodium phosphate; SBTI, soybean trypsin inhibitor; PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; PS, 1,2-diacyl-*sn*-glycero-3-phosphoserine; PE, 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine.

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proteolysis of viruses with bromelain releases the water-soluble ectodomain of HA, called BHA, by cleaving off the hydrophobic membrane-anchoring segment that is located in the C-terminal region of HA2 (Brand & Skehel, 1972). For X:31 strain, the three-dimensional structure of BHA has been determined to a 3-Å resolution (Wilson et al., 1981).

The mechanism by which the hemagglutinin molecule promotes virus–membrane fusion is still obscure. It has been observed that, under mildly acidic conditions (pH <5.5), BHA undergoes a conformational change whereby it acquires amphipathic properties as exhibited by its ability to bind to liposomes and nonionic detergents (Skehel et al., 1982; Doms et al., 1985). This change is believed to result from exposure of a hydrophobic peptide, located at the N-terminal region of the BHA2 subunit. That this segment may have an important functional role is suggested by its remarkably high degree of conservation (Skehel & Waterfield, 1975; Ward, 1981; Skehel et al., 1982). Yet in spite of this, no actual demonstration of the penetration by this segment into the hydrophobic core of lipid bilayers has been made so far.

To tackle this problem, we have applied the technique of hydrophobic photolabeling (Brunner, 1981; Bayley, 1983; Robson et al., 1982; Bisson & Montecucco, 1985) using two carbene-generating probes, [^{125}I]TID (Brunner & Semenza, 1981) and [^{3}H]PTPC/11, a new analogue of a phospholipid. The main result of this study is that the BHA2 subunit is labeled specifically and, as predicted, in a pH-dependent manner, thus providing direct evidence that the low-pH-induced interaction of BHA with membranes is mediated through this subunit. It may be generally expected that specific labeling of BHA at low pH reflects a property that is unique for BHA and a few other proteins, among them some bacterial toxins (Montecucco et al., 1986; Escuyer et al., 1986). However, the present study strongly suggests that this is a more general phenomenon which is seen also with water-soluble proteins whose biological role is, almost certainly, unrelated to membrane fusion.

Experimental Procedures

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 Fluka was used. ^{1}H NMR spectra were recorded on a Bruker 300-MHz instrument. $CDCl_3$ was used as solvent and $(CH_3)_4Si$ (δ 0.00) as an internal standard. Chemical shifts are given in parts per million (ppm). In Figure 1 is shown a scheme of the reactions described below.

(I) (4-Bromophenyl)decanol (**2**). This compound was prepared by reduction of 10-(4-bromophenyl)decanoic acid (**1**) (Fieser et al., 1948) with diborane according to the procedure of Brown et al. (1970). 10-(4-Bromophenyl)decanoic acid (16.4 g, 50 mmol) was dissolved in 110 mL of THF (distilled over $LiAlH_4$) and added to a solution (95 mmol) of BH_3·THF (Aldrich) under an atmosphere of nitrogen at 0 °C. The reaction mixture was stirred for 1 h at 0 °C and then at room temperature overnight. The solution was hydrolyzed with aqueous HCl and extracted 3 times with ether. The organic layer was washed with water, dried over $MgSO_4$, and concentrated in vacuo. The product was crystallized from hexane. Yield: 14.2 g (97% of theory). TLC: R_f (CH_2Cl_2/methanol, 10:1 v/v) 0.76. ^{1}H NMR ($CDCl_3$) δ 1.28 (m, 12 H), 1.51–1.58 (m, 4 H), 2.54 (t, 2 H), 3.63 (t, 2 H), 7.05 and 7.39 (4 H, AA′/BB′).

(II) [[10-(4-Bromophenyl)decyl]oxy]-tert-butyldimethylsilane (**3**). The *tert*-butyldimethylsilyl protecting group was introduced as described by Shih and Bayley (1985). A mixture of 10-(4-bromophenyl)decanol (14.1 g, 45 mmol), imidazole (7.5 g, 111 mmol), and *tert*-butyldimethylsilyl chloride (7.2 g, 48 mmol) in 18 mL of DMF was stirred for 20 h at 40 °C. The reaction mixture was diluted with pentane and washed 3 times with water. The organic layer was dried with $MgSO_4$ and concentrated under reduced pressure. The crude product was chromatographed on silica gel 60 with hexane/ether (70:30 v/v) as the eluant. Yield: 19 g (99% of theory). TLC: R_f (hexane/ether, 7:3 v/v) 0.67. ^{1}H NMR ($CDCl_3$) δ 0.04 (s, 6 H), 0.89 (s, 9 H), 1.27 (m, 12 H), 1.48–1.60 (m, 4 H), 2.54 (t, 2 H), 3.59 (t, 2 H), 7.05 and 7.39 (4 H, AA′/BB′).

(III) [[10-[4-(2,2,2-Trifluoroacetyl)phenyl]decyl]oxy]-tert-butyldimethylsilane (**4**). This compound was synthesized by introducing the trifluoroacetyl group via the lithium compound as described by Nassal (1983). To a stirred solution of 18.0 g (42 mmol) of compound **3** in 210 mL of absolute ether was added dropwise 1.1 equiv (46 mmol) of *n*-butyllithium (1.6 M in hexane) within 2 h at −30 °C under nitrogen. The reaction mixture was allowed to warm up to 0 °C within

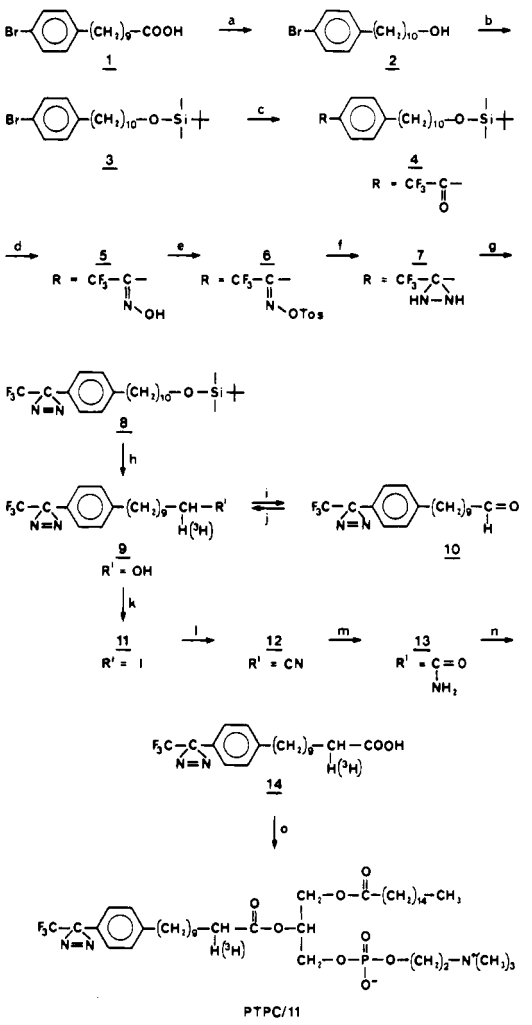

FIGURE 1: Steps in the synthesis of [^{3}H]PTPC/11: (a) BH_3·THF, room temperature, 15 h; (b) *tert*-butyldimethylsilyl chloride, imidazole, in DMF; (c) (1) *n*-butyllithium, (2) (trifluoroacetyl)piperidine, and (3) water/ammonium chloride; (d) hydroxylamine hydrochloride; (e) *p*-toluenesulfonyl chloride/pyridine; (f) liquid ammonia in ether; (g) iodine/triethylamine in methylene chloride; (h) methanolic HCl; (i) pyridinium dichromate in methylene chloride; (j) NaB^3H_4 in isopropyl alcohol/water; (k) methyltriphenoxyphosphonium iodide in DMF; (l) KCN/18-crown-6 in acetonitrile; (m) acetic acid saturated with HCl; (n) 70% nitrososulfuric acid/methylene chloride; (o) (1) PCl_5 in carbon tetrachloride and (2) lyso-PC/4-(dimethylamino)pyridine in chloroform/pyridine.

2 h and cooled down again to −50 °C. Subsequently, 1 equiv (42 mmol) of (trifluoroacetyl)piperidine in 42 mL of ether was added [(trifluoroacetyl)piperidine was prepared by reacting 1 equiv of piperidine with 1 equiv of trifluoroacetic acid methyl ester, washing twice with 0.1 N aqueous HCl, and distilling the organic layer in vacuo; bp 74 °C (14 mmHg)]. After 3 h of stirring at −50 °C the mixture was allowed to warm up to 0 °C and was then hydrolyzed with saturated aqueous NH_4Cl. The organic phase was washed 4 times with aqueous NH_4Cl and 4 times with water and dried over $MgSO_4$, and the solvent was evaporated in vacuo. The oily residue was used without further purification (for analytical purposes, a small amount was purified by column chromatography on silica gel 60 with hexane/CH_2Cl_2 as the eluant). Yield: 10.1 g (54%). TLC: R_f (hexane/CH_2Cl_2, 1:3 v/v) 0.79. ^1H NMR δ 0.04 (s, 6 H), 0.89 (s, 9 H), 1.27 (m, 12 H), 1.48–1.67 (m, 4 H), 2.70 (t, 2 H), 7.34 and 7.99 (4 H, AA′/BB′).

(*IV*) [[*10*-[*4*-[*1*-(*Hydroxyimino*)-*2,2,2-trifluoroethyl*]-*phenyl*]*decyl*]*oxy*]-*tert-butyldimethylsilane* (**5**). A mixture of compound **4** (10.0 g, 22 mmol) and 4.6 g (66 mmol) of hydroxylamine hydrochloride in 14 mL of pyridine was heated to 70–80 °C. After 4 h the solvent was evaporated under reduced pressure and the residue was dissolved in ether. The organic layer was washed 5 times with water, dried over $MgSO_4$, and concentrated in vacuo. The resulting product was a chromatographically pure yellow oil. Yield: 9.7 g (96%). TLC: R_f (hexane/CH_2Cl_2, 1:3 v/v) 0.28. ^1H NMR ($CDCl_3$) δ 0.05 (s, 6 H), 0.89 (s, 9 H), 1.27 (m, 12 H), 1.46–1.63 (m, 4 H), 2.60–2.66 (m, 2 H), 3.60 (t, 2 H), 7.20–7.44 (m, 4 H, AA′/BB′), 8.43 (s, 0.5 H), 8.64 (s, 0.5 H).

(*V*) [[*10*-[*4*-[*1*-[[(*p-Toluenesulfonyl*)*oxy*]*imino*]-*2,2,2-trifluoroethyl*]*phenyl*]*decyl*]*oxy*]-*tert-butyldimethylsilane* (**6**). Oxime **5** (9.7 g, 21 mmol) was dissolved in 44 mL of pyridine and refluxed with 6.0 g (31 mmol) of *p*-toluenesulfonyl chloride for 2 h. Subsequently, the solvent was evaporated in vacuo and the residue was diluted with CH_2Cl_2. The reaction mixture was washed 4 times with water, and then the organic phase was dried with $MgSO_4$ and concentrated in vacuo. The crude product was purified by column chromatography on silica gel with hexane/CH_2Cl_2 (1:3 v/v) as the solvent. Yield: 8.1 g (63%). TLC: R_f (hexane/CH_2Cl_2 1:3) 0.62. ^1H NMR δ 0.04 (s, 6 H), 0.89 (s, 9 H), 1.28 (m, 12 H), 1.46–1.65 (m, 4 H), 2.47 (m, 3 H), 2.64 (t, 2 H), 3.59 (t, 2 H), 7.24–7.39 (m, 6 H), 7.87 (d, 2 H).

(*VI*) [[*10*-[*4*-[(*Trifluoromethyl*)*diaziridinyl*]*phenyl*]-*decyl*]*oxy*]-*tert-butyldimethylsilane* (**7**). **6** (7.0 g, 11.4 mmol) in 20 mL of ether was cooled to −78 °C, and liquid ammonia (2–3 mL) was added into the thick-walled glass reaction vessel and sealed. The mixture was allowed to warm up to room temperature and to react overnight. Excess ammonia was then carefully evaporated, and the reaction mixture was diluted with ether and washed with water. The organic phase was dried over $MgSO_4$ and concentrated under reduced pressure. The yellowish residue was chromatographed on silica gel with hexane/CH_2Cl_2 (1:3 v/v). Yield: 4.2 g (81%). TLC: R_f (hexane/CH_2Cl_2, 1:3 v/v) 0.24. ^1H NMR ($CDCl_3$) δ 0.04 (s, 6 H), 0.89 (s, 9 H), 1.27 (m, 12 H), 1.48–1.60 (m, 4 H), 2.54 (t, 2 H), 3.59 (t, 2 H), 7.03 and 7.37 (4 H, AA′/BB′).

(*VII*) [[*10*-[*4*-[(*Trifluoromethyl*)*diazirinyl*]*phenyl*]-*decyl*]*oxy*]-*tert-butyldimethylsilane* (**8**). The diaziridine **7** was oxidized to the diazirine by using iodine in triethylamine as described by Church and Weiss (1970). To a stirred solution of 3.74 g (8.15 mmol) of diaziridine **7** in 5.2 mL of CH_2Cl_2 were added in small portions 1.4 mL of triethylamine and 1.5 g of iodine. Upon disappearance of the color of the iodine, additional portions of 0.5 mL of triethylamine and 0.4 g of iodine were added. The reaction mixture was diluted with ether (200 mL) and 10% citric acid (100 mL). $Na_2S_2O_5$ was added until disappearance of the iodine, and then the solution was extracted with several portions of ether. The organic phase was washed 3 times with water, dried over $MgSO_4$, and concentrated in vacuo. The oily residue was purified by column chromatography on silica gel with hexane/CH_2Cl_2 (1:3 v/v). Yield: 3.6 g (96%). TLC: R_f (hexane/CH_2Cl_2, 1:3 v/v) 0.67. ^1H NMR ($CDCl_3$) δ 0.04 (s, 6 H), 0.89 (s, 9 H), 1.27 (m, 12 H), 1.48–1.61 (m, 4 H), 2.60 (t, 2 H), 3.59 (t, 2 H), 7.09 and 7.19 (4 H, AA′/BB′).

(*VIII*) *10*-[*4*-[*3*-(*Trifluoromethyl*)*diazirinyl*]*phenyl*]*decanol* (**9**). The silyl protecting group was removed by treatment of compound **8** (3.19 g, 7 mmol) with a mixture of concentrated aqueous HCl and methanol (1:10 v/v) for 90 min at room temperature. The reaction mixture was diluted with 120 mL of ether, extracted with 5% sodium carbonate (until the pH was about 5), and subsequently washed with water. The organic phase was dried over $MgSO_4$ and evaporated. The crude product was chromatographed on silica gel with CH_2Cl_2/ether (19:1 v/v). Yield: 1.82 g (76%). mp 27–29 °C. TLC: R_f (hexane/ether, 1:1 v/v) 0.25. ^1H NMR δ 1.28 (m, 12 H), 1.51–1.60 (m, 4 H), 2.6 (t, 2 H), 3.63 (t, 2 H), 7.09 and 7.19 (4 H, AA′/BB′).

(*IX*) *10*-[*4*-[(*Trifluoromethyl*)*diazirinyl*]*phenyl*]*decyl Iodide* (**11**). The alcohol **9** (580 mg, 1.7 mmol) and methyltriphenoxyphosphonium iodide (1.54 g, 3.4 mmol) were dissolved in 8.5 mL of DMF. After the reaction mixture was stirred at room temperature for 2 h, it was diluted with 120 mL of ether and extracted twice with 1 M NaOH. The organic layer was washed with water until the pH was approximately 5, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was first purified by column chromatography on silica gel with hexane/ether (1:1 v/v) and subsequently by thick-layer chromatography on silica gel plates with hexane/CH_2Cl_2 as the solvent. Yield: 658 mg (86%). TLC: R_f (hexane/ether, 1:1 v/v) 0.72. ^1H NMR δ 1.27 (m, 12 H), 1.55–1.60 (m, 2 H), 1.78–1.85 (m, 2 H), 2.6 (t, 2 H), 3.17 (t, 2 H), 7.09 and 7.19 (4 H, AA′/BB′).

(*X*) *11*-[*4*-[*3*-(*Trifluoromethyl*)*diazirinyl*]*phenyl*]*undecanoic Acid* (**14**). This compound was synthesized from the iodide **11** via the intermediates nitrile **12** and amide **13**. To 226 mg (0.5 mmol) of the iodide **11**, dissolved in 5 mL of acetonitrile, were added 130 mg (2 mmol) of KCN and 132 mg (0.5 mmol) of 18-crown-6 (Fluka). The solution was stirred at room temperature for 2 h, diluted with 50 mL of ether, and extracted 3 times with water. The organic layer was dried with $MgSO_4$ and evaporated. The crude product was chromatographed on a silica gel column with hexane/ether (1:1 v/v) and hydrolyzed to the amide **13** by dissolving the nitrile (126 mg, 0.36 mmol) in 2.5 mL of acetic acid/water (10:1 v/v), saturating the solution with HCl gas at 0 °C, and reacting overnight at room temperature. The reaction product was then extracted twice with CH_2Cl_2. After the organic layer was dried over $MgSO_4$, it was concentrated in vacuo and further processed according to the procedure of Wade et al. (1982). The oily residue (amide **13**) was dissolved in 1 mL of CH_2Cl_2 and 1 mL of 70% nitrososulfuric acid (Du Pont NSA, 40% nitrososulfuric acid, 52% sulfuric acid, 7.8% water). After the reaction mixture was stirred for 80 min at 45 °C, it was hydrolyzed with ice and extracted with ether. The organic layer was dried over $MgSO_4$ and concentrated, and the residue was purified by a silica gel column chromatography

using hexane/ether/acetic acid (50:50:2 v/v). Yield: 103 mg (56%). TLC: R_f (hexane/ether/acetic acid, 50:50:2 v/v) 0.4. ^{1}H NMR δ 1.27 (m, 12 H), 1.53–1.67 (m, 4 H), 2.30–2.37 (m, 2 H), 2.57–2.62 (t, 2 H), 7.09 and 7.19 (4 H, AA'/BB').

(*XI*) [^3H]-*1-Palmitoyl-2-[11-[4-[3-(trifluoromethyl)diazirinyl]phenyl]undecanoyl]-sn-glycero-3-phosphocholine* ([^3H]*PTPC/11*). Tritium was introduced by reducing aldehyde **10** with NaB^3H_4. Aldehyde **10** was prepared from the alcohol **9** by using pyridine dichromate as an oxidant as described by Corey and Schmidt (1979). The tritium-labeled alcohol **9** was then converted via the iodide (**11**), nitrile (**12**), and amide (**13**) to the fatty acid as described above for the nonradioactive compounds.

The alcohol **9** (342.3 mg, 1 mmol) was dissolved in 1.4 mL of CH_2Cl_2, and 564.3 mg (1.5 mmol) of pyridinium dichromate was added. After the reaction mixture was stirred for 4 h at room temperature, it was centrifuged (2000*g*, 5 min), and the supernatant, containing the reaction product, was subjected to column chromatography using hexane/ether (1:1 v/v). Further purification was achieved by thick-layer chromatography (silica gel) using the same solvent. [Yield: 150 mg (44%). TLC: R_f (hexane/ether, 1:1 v/v) 0.59.] To 48 mg (0.141 mmol) of this aldehyde were added 720 μL of isopropanol/water (4:1 v/v) and 1 Ci of solid NaB^3H_4 (58.9 Ci/mmol). The reaction vessel was sealed with a septum, and the mixture was stirred at room temperature for 1 h. Subsequently, the reaction mixture was acidified with 3 M H_2SO_4 and diluted with ether. After several extractions with ether, the organic layers were combined and washed with water until the pH was approximately 5. The organic phase was dried over $MgSO_4$ and concentrated under reduced pressure. The crude product was chromatographed on a silica gel column with hexane/ether (1:1 v/v) as the solvent. The yield was approximately 630 mCi (63%) of chromatographically pure material (identical with the nonradioactive reference compound). The tritiated alcohol was processed to the acid **14** exactly as described above (yield: 266 mCi; 0.0181 mmol = 51%).

Acylation of 1-palmitoyl-*sn*-glycero-3-phosphocholine was carried out exactly as described previously (Meister et al., 1985) for the synthesis of [^{3}H]PTPC/8 by using the fatty acyl chloride of the unlabeled or tritiated fatty acid **14**.

Preparation of Sonicated Liposomes. Phospholipids were purchased from Lipid Products (South Nutfield, England). Solutions of [^{3}H]PTPC/11 in toluene/ethanol and egg lecithin in chloroform/methanol or of [^{3}H]PTPC/11 and a mixture of egg lecithin, phosphatidylserine, and phosphatidylethanolamine in the same solvent were dried at 10^{-2} mmHg for 1 h at room temperature. The phospholipid was then dispersed in PBS (pH 7.4) or MES–NaCl (pH 7.0) and the dispersion flushed with nitrogen. Sonication was performed in a sealed conical tube 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 to sediment multilamellar aggregates of the lipid.

Preparation of Bromelain-Solubilized Hemagglutinin (BHA). Influenza viruses PR8 [A/PR/8/34(H1N1)] were grown in the allantoic cavity of embryonated hen eggs and purified as described elsewhere (Gerhard, 1976). Bromelain-solubilized HA was produced by digestion of purified PR8 viruses with bromelain (Sigma) as described by Brand and Skehel (1972) with slight modifications. Virus particles were suspended in Tris buffer (0.1 M, pH 7.2), containing 1 mM EDTA, and incubated with bromelain (virus protein/enzyme, 1:1 w/w) in the presence of 50 mM β-mercaptoethanol. After incubation for 16 h at 37 °C, the viral cores were pelleted by centrifugation at 100000*g* for 1 h, and the BHA-containing supernatant was passed through a column of Sephacryl S-400 (30 × 2.5 cm). BHA was eluted with Tris–NaCl buffer (0.1 M Tris, 0.5 M NaCl, pH 7.8), and the peak fractions, containing pure BHA as judged by SDS–polyacrylamide gel electrophoresis, were pooled and stored at −20 °C.

Preparation of Human Erythrocyte Membranes. To remove leukocytes and platelets, heparinized blood (O, Rh+) was processed as described by Beutler et al. (1976). Erythrocyte membranes were prepared essentially according to Steck and Kant (1974). After hemolysis, the membranes were pelleted and washed 3 times in 5 mM sodium phosphate, pH 7.4, before resealing in PBS.

Determination of [^3H]*PTPC/11 Transfer from Liposomes to Erythrocyte Membranes.* Resealed ghosts (2 mg/mL) were incubated with sonicated liposomes prepared from radioactively labeled lipid. Aliquots of 10 μL were diluted with 140 μL of PBS and centrifuged in an airfuge at 90000*g* for 5 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 prior to centrifugation and, respectively, of the supernatant following sedimentation of the ghosts.

Photoactivation of the Reagents. This was carried out by means of the apparatus described elsewhere (Brunner & Semenza, 1981). The lipid dispersions, containing [^{3}H]-PTPC/11, were incubated and photolyzed in Eppendorf tubes which themselves were placed in a saturated solution of copper sulfate contained in a cylindrical thermostated quartz vessel. The samples were illuminated for a total of 1 min, a period shown previously to result in essentially complete photolysis of the diazirine.

Analytical Procedures. For labeling experiments with [^{3}H]PTPC/11, liposomes were prepared from a mixture of egg lecithin and [^{3}H]PTPC/11 in a molar ratio of 14:1 in MES–NaCl, pH 7.0, at a final concentration of 0.07–0.33 mg of lipid/mL, depending on the protein. The lipid dispersions were incubated with the proteins at a concentration of 0.06 mg of protein/mL, corresponding to a molar protein to lipid ratio of 1:100. After acidification with acetic acid the samples were incubated for 2 min at the indicated temperature, eventually neutralized, and illuminated for 1 min. After photolysis, 3 volumes of $CHCl_3/CH_3OH$ (1:2 v/v) was added to the samples to extract the lipid. The samples were kept for 60 min at room temperature, and the precipitated protein was sedimented by centrifugation (10 min at 10000*g*). The protein sediments were washed with $CHCl_3/CH_3OH$ (2:1 v/v). Subsequently, they were dried in a vacuum centrifuge and subjected to SDS–polyacrylamide gel electrophoresis under reducing conditions (Laemmli, 1970). After Coomassie Blue staining and densitometric recording of the bands, the gels were cut in 2-mm slices and each slice was incubated in 0.6 mL of NCS solubilizer (Amersham International plc, U.K.) for 2 h at 50 °C. Radioactivity was measured after addition of 3.5 mL of a toluene-based scintillation cocktail in a Beckman LS 1801 system. In the case of [^{125}I]TID-labeled BHA, most of the photolyzed (nonbound) reagent was first removed simply by repeated transfer of the protein/liposome mixture into Eppendorf tubes (they effectively adsorb the hydrophobic photolysis products).

RESULTS

Design and Synthesis of [^3H]*PTPC/11.* Hydrophobic photolabeling has emerged, in recent years, as a tool with great

potential for the elucidation of membrane phenomena such as fusion. However, most reagents currently used for this purpose, including all hydrophobic molecules and most analogues of phospholipids, undergo rapid diffusion (exchange) between membranes, a property that clearly limits the scope of their application. For example, in studies of vectorial processes such as membrane fusion, it would represent a particular advantage if reagents were available which, like natural phospholipids, do not undergo this rapid exchange and which could be inserted and confined to a subpopulation of the membranes and possibly to a single leaflet of a bilayer. [^{3}H]PTPC/8, a phospholipid analogue developed earlier, exhibits slow flip-flop, but transfer between membranes was found to be considerably more rapid than normally observed for phospholipids (Brunner et al., 1983). Since transfer appears to be due to individual lipid monomers and to be directly related to their solubility in water (Roseman & Thompson, 1980; Nichols & Pagano, 1981), we reasoned that this process could be markedly reduced by (i) eliminating nonessential polar groups within the photoactivatable fatty acyl chain and (ii) increasing their length. On the basis of these considerations, we have designed a new photosensitive fatty acid in which the ether bridge (connecting the fatty acyl chain with the photosensitive group) as present in [^{3}H]PTPC/8 (Brunner et al., 1983) is now replaced by a methylene group.

In Figure 1, the scheme for the synthesis of this new fatty acid analogue is depicted. Starting material is 10-(4-bromophenyl)decanoic acid (**1**), and a total of 14 steps are involved. An important facet of this synthesis is that tritium of very high specific radioactivity can be introduced much more easily than into the fatty acid used previously for the preparation of [^{3}H]PTPC/8. This is because all the tritiated intermediates (Figure 1, compounds **9–14**) are essentially nonvolatile and, therefore, can be purified and freed from residual solvent without losses or severe risks of contamination. We have prepared the fatty acid **14** and [^{3}H]PTPC/11 with a specific radioactivity of 15 Ci/mmol, corresponding to approximately 0.7 ^{3}H per molecule. The tritium atom is connected to the photoreactive group through stable bonds and therefore is not susceptible to exchange even if most harsh conditions, e.g., acid hydrolysis, are used in subsequent analytical procedures. For acylation of 1-palmitoyl-*sn*-glycero-3-phosphocholine, we followed the protocol used for the preparation of [^{3}H]PTPC/8 (Meister et al., 1985).

Properties of [^{3}H]PTPC/11. When stored as a solution in ethanol/toluene (1:1 v/v) at 4 °C and at less than 20 mCi/mL, [^{3}H]PTPC/11 shows no detectable degradation (<1%) within 3 months as judged by TLC and radioactivity scanning. In aqueous buffer, [^{3}H]PTPC/11 shows the typical behavior of a phospholipid (clear solution upon sonication).

To estimate and compare the rate at which [^{3}H]PTPC/11 is exchanged between membranes, we have measured the transfer of [^{3}H]PTPC/11 and that of [^{3}H]DPPC from sonicated liposomes containing the radiolabeled lipid to erythrocyte membranes. The main results are depicted in Figure 2. When liposomes prepared from [^{3}H]PTPC/11 alone were used, we consistently observed a characteristic initial phase of rapid transfer accounting for as much as about 20% of the label originally present in the liposomes. After about half an hour, the transfer slowed down markedly but was still more rapid than observed for [^{3}H]DPPC. We also carried out experiments in which [^{3}H]PTPC/11 was present as a tracer only in liposomes prepared from egg lecithin. Under these conditions, the initial rapid transfer was not observed and intermembrane exchange rates similar to those of [^{3}H]DPPC were obtained

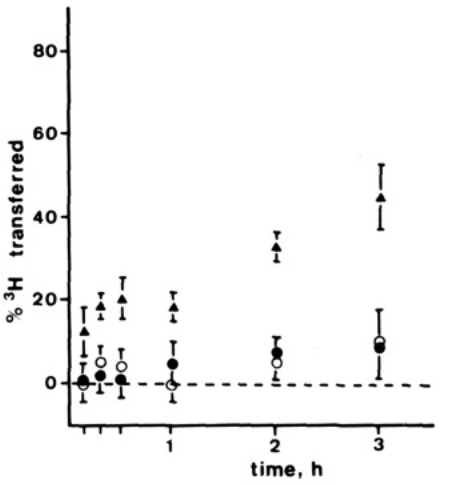

FIGURE 2: Transfer of [^{3}H]PTPC/11 from liposomes to erythrocyte ghosts. Small unilamellar liposomes prepared either from [^{3}H]-PTPC/11 alone (triangles) or from egg lecithin containing [^{3}H]-PTPC/11 as a radioactive tracer (filled circles) were incubated with resealed ghosts in PBS at 37 °C. The concentration of protein was 2 mg/mL and that of the liposome phospholipid 40 μM (10 μCi). After the time points indicated, aliquots of 10 μL were diluted with PBS and ghosts were sedimented differentially (5 min at 90000*g*). The fraction of radioactivity that cosedimented with ghost membranes corresponds to the phospholipid transferred [mean value of two transfer experiments with liposomes from [^{3}H]PTPC/11 and of four experiments with liposomes from egg lecithin traced with [^{3}H]PTPC/11; vertical bars represent standard deviations (±SD)]. In a control experiment (open circles), transfer of radioactivity from liposomes prepared from egg lecithin and a trace of [^{3}H]DPPC to erythrocyte ghosts was determined.

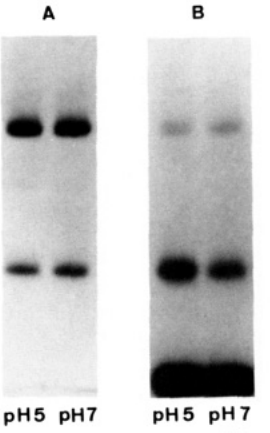

FIGURE 3: Patterns of labeling BHA with [^{125}I]TID at pH 5.0 and 7.0. (A) Coomassie Blue staining profiles of BHA (10 μg) run in a SDS–12% polyacrylamide gel after incubation with egg lecithin liposomes [containing 10 μCi of the photolabel (lipid/protein molar ratio 100:1) at pH 5.0 (lane labeled "pH 5") or 7.0 (lane labeled "pH 7")] and ultraviolet activation of the reagent. (B) Autoradiogram of the dried gel shown in (A) (Kodak X-Omat; exposure time 24 h at −80 °C).

(Figure 2). Evidently, transfer of [^{3}H]PTPC/11 is much slower than that measured for [^{3}H]PTPC/8 (Brunner et al., 1983).

Labeling of BHA with [^{125}I]TID. We first examined [^{125}I]TID labeling of BHA before and after incubation at pH 5. In all experiments, a lipid/protein ratio of 100:1 was used and the label was allowed to equilibrate for 3–5 min prior to photoactivation. Under all conditions used, the radioactivity incorporated into the HA1 was extremely small, amounting to less than 0.003% of the label that had been present originally. In contrast, BHA2 showed clear labeling, the precise extent of which was dependent on the pH and concentration

of the sample. At a protein and lipid concentration of 550 and 610 μg/mL, respectively, labeling of BHA2 at pH 5 was about twice (0.025%) that measured at pH 7 (0.013%) (Figure 3). However, when labeling was performed on a more diluted sample (60 μg of BHA/mL; 66 μg of lipid/mL), a clear increase in labeling of the pH 7 conformation of BHA was noticed, and practically identical patterns were obtained at pH 7 and 5.

Labeling of BHA with [^{3}H]PTPC/11. The main feature distinguishing [^{125}I]TID from [^{3}H]PTPC/11 in labeling BHA is that with the latter reagent the pH 5 form only was labeled. In order to evaluate which factors might affect label incorporation into the two subunits of BHA and, hence, might influence the label incorporation patterns among individual segments, the following parameters were varied.

(*i*) *Lipid/Protein Ratio.* Doms and collegues have shown recently (Doms et al., 1985) that a considerable excess of lipid is necessary (>5000 lipid molecules/BHA trimer) in order to obtain nearly complete attachment of the protein to the liposomes. Therefore, we also examined the effect of the lipid/protein ratio (from 50 to 5000 lipid molecules/BHA monomer) upon label incorporation into BHA. An approximately twofold increase in labeling was observed upon increasing the lipid/protein ratio from 50:1 to 5000:1 (in all experiments the ratio of [^{3}H]PTPC/11 to total lipid was kept constant). However, at high lipid/protein ratios (>500:1), it was difficult to achieve complete separation of the labeled protein from the bulk of nonbound label and, therefore, to derive accurate labeling yields. For the experiments described in the following, we have used a molar lipid/protein ratio of 100:1, conditions under which some of the BHA may not be bound to the liposomes.

(*ii*) *Time of Incubation.* We also varied incubation times at low pH prior to photolysis and found that maximal label incorporation is achieved within less than 2 min. Therefore, in all experiments, we used a 2-min incubation period.

(*iii*) *Effect of Temperature.* To evaluate a possible effect of the temperature, two samples each of BHA were incubated with the liposomes containing [^{3}H]PTPC/11 at either 0 or 37 °C (pH 5, 2 min). Following neutralization, one sample of each pair was photolyzed at 0 °C and the other at 37 °C, and the extent of label incorporation was measured. We found that labeling yields were, indeed, affected by the temperature, but solely by the temperature of the low-pH preincubation and not that of the photoactivation step. That is, incubation at 0 °C followed by reagent photolysis at either 37 or 0 °C resulted in about 5 times less labeling of BHA as compared with samples that had been incubated at 37 °C and then photolyzed at either 37 or 0 °C. Under the condition specified, low-pH incubation at 37 °C led to an extent of labeling corresponding to approximately 0.02% (±0.01; SD; n = 5). A control sample (incubation at pH 7.0, 37 °C) showed barely detectable labeling (approximately 0.002%). The complete set of results of an experiment is depicted in Figure 4. The small amount of label that appeared to be present in BHA1 may be due to some BHA2 dimers migrating to the same position in the gel. This possibility is supported by the observation that labeled BHA2 extracted from an SDS–polyacrylamide gel and reelectrophoresed consistently gave a weak band of radioactivity at the position of BHA1.

(*iv*) *Irreversibility of Conformational Change.* The acid-induced conformational change in BHA is irreversible as judged from labeling with [^{125}I]TID and [^{3}H]PTPC/11. Regardless of whether the BHA sample is both incubated and labeled at pH 5 or whether treatment at pH 5 is followed first

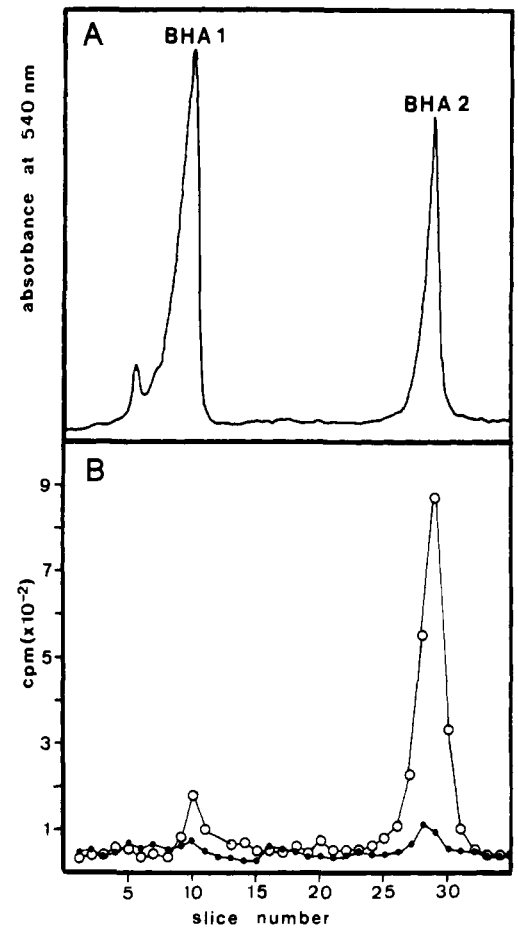

FIGURE 4: Profiles of labeling of BHA with [^{3}H]PTPC/11 in the presence of egg lecithin liposomes containing the photolabel at pH 5.0 and 7.0. Samples (10 μg) of BHA were incubated with the liposomes (protein/lipid molar ratio 1(100) in buffers of pH 7.0 (MES–NaCl) or 5.0 (MES–NaCl–acetic acid), at 37 °C for 2 min, and irradiated for 1 min. Following addition of 3 volumes of chloroform/methanol, the precipitated protein was subjected to SDS–polyacrylamide gel electrophoresis under reducing conditions (12% polyacrylamide). (A) Coomassie Blue staining densitogram. (B) [^{3}H]PTPC/11 radioactivity bound to BHA polypeptide chains. Filled circles refer to a sample incubated with the photolabel at pH 7.0, while open circles represent the result of an experiment in which the sample was first incubated for 2 min at 37 °C at pH 5.0 and then readjusted to pH 7.0 before ultraviolet irradiation. The two radioactivity profiles were normalized for identical quantities of BHA subunits as determined from the areas of the corresponding densitograms.

by a second incubation at pH 7 (for 2 min) and then by photolysis of the reagent, identical labeling patterns were obtained.

Labeling of Soluble Proteins with [^{3}H]PTPC/11. A difficulty in interpreting hydrophobic photolabeling data arises from the fact that even peripheral and soluble proteins can incorporate traces of radiolabel. Presumably, this "background" labeling results from reagent molecules either adsorbed to hydrophobic surface areas of these proteins or dissolved within their apolar fluid core. At present, it is not fully clear whether this type of labeling is seen only with relatively small, hydrophobic reagents or whether it might be significant also for analogues of phospholipids whose solubility in water is exceedingly low.

In order to address this problem, we have determined the extent to which some soluble proteins incorporate radioactivity following incubation with liposomes containing [^{3}H]PTPC/11. The proteins chosen were ovalbumin, carboanhydrase, α-lactalbumin, trypsin, and soybean trypsin inhibitor (SBTI). α-Lactalbumin appeared to be of particular interest because

Table I: Extent of Labeling of Some Water-Soluble Proteins in the Presence of Liposomes Containing [^{3}H]PTPC/11, Expressed as Percentage of the Total Radioactivity Introduced[a]

protein	p*I*	lipid composition of liposomes	treatment of the protein at pH[b] 7.0	5.0	3.5	5.0→7.0	3.5→7.0
ovalbumin	4.6	PC	0.015	0.06	nd[e]	nd	nd
		PC/PS/PE	0.005	0.05	nd	nd	nd
carbonic anhydrase	5.8	PC	0.002	0.004	nd	nd	nd
		PC/PS/PE	0.002	0.004	0.02	nd	nd
α-lactalbumin	5.1	PC	0.003	0.005	0.03	0.003	0.01
		PC/PS/PE	0.003	0.005	0.01	0.002	0.002
trypsin[c]	10.8	PC	<0.0005	<0.0005	0.0015	<0.0005	nd
SBTI	4.5	PC	0.001	0.001	0.003	0.001	0.002
BHA[d]	5.8	PC					
BHA1 subunit			<0.001	0.003	nd	0.003	nd
BHA2 subunit			0.002	0.02	nd	0.02	nd

[a] Incubations and photolyses were done in Eppendorf tubes, each containing 150 μL of a solution of 10 μg of protein and small unilamellar liposomes in a molar protein/lipid ratio of 1:100. Liposomes were prepared from egg lecithin (PC), or a mixture of PC, PS, and PE in a 2:1:1 ratio, and 15 μCi (1 nmol) of [^{3}H]PTPC/11. The protein was then precipitated and subjected to SDS–polyacrylamide gel electrophoresis. For quantitation, densitograms of Coomassie Blue stained gels were compared with densitograms of bands containing standard amounts (15 μg) of protein. Each gel track was then cut in 2-mm slices and subjected to scintillation counting. [b] Incubation buffers were 20 mM MES and 130 mM NaCl (pH 7.0); acid pHs (5.0, 3.5) were adjusted by the addition of 2 M acetic acid. Samples were incubated at the desired pH for 2 min at 37 °C and either photolyzed directly (pH 7.0, 5.0, and 3.5) or photolyzed after returning the pH to 7.0 by the addition of 2 M NaOH (5.0 → 7.0, 3.5 → 7.0). During photolysis, a temperature of 20 °C was maintained. [c] Labeling at pH 7.0 and 5.0 was within background levels. [d] Different temperatures (0 versus 37 °C) during photolysis had no measurable effects on labeling yields. [e] nd, not determined.

of its ability to induce membrane fusion at acid pH (Kim & Kim, 1986). The main results are summarized in Table I. It shows the extent of covalent incorporation into proteins of radiolabel derived from [^{3}H]PTPC/11 expressed as percentage of the radioactivity used for each experiment.

In spite of the limited accuracy of the data,[2] these experiments revealed several interesting features. First, we found that label incorporation at neutral pH is within a rather broad range. Thus, in the presence of liposomes consisting of phosphatidylcholine alone, ovalbumin incorporated at least 20 times more radioactivity than trypsin under otherwise identical conditions. Second, in all cases, lowering the pH of the incubation buffer resulted in an increase in labeling. Thus, ovalbumin was labeled about 10 times more strongly at pH 5 than at pH 7 (this increase is similar to that seen for BHA). With trypsin and SBTI, this pH dependence was less dramatic, and more acidic conditions were necessary to measure an effect. Third, Table I also shows that acid treatment of the proteins induced changes that were, at least partly, reversible within the 2-min period the protein was allowed, following acid treatment, to regain the pH 7 structure. Fourth, although a relationship might exist between the p*I* of the protein, composition of the lipid (presence of negatively charged phospholipids), and ionic strength of the buffer (not examined), on the one hand, and extent of labeling, on the other, such a correlation was not obvious from the data derived.

Discussion

The scope of hydrophobic photolabeling as a tool to investigate membrane phenomena is largely determined by the ability of the probe to distinguish in an unequivocal manner between domains or segments of proteins that are embedded in the lipid core of a membrane and those that are not. In agreement with the view put forward recently by Boulay et al. (1987), we conclude, in the present study, that [^{125}I]TID labeled regions in BHA that did not actually penetrate the liposomes. This conclusion is based on the finding that labeling was not strictly dependent upon low pH as would be predicted from corresponding binding studies (Skehel et al., 1982; Doms et al., 1985). Alternatively, the clear labeling of BHA2 at neutral pH may reflect the presence of a hydrophobic pocket or pocketlike structure in that subunit which may or may not be related to that region which upon acidification is responsible for binding BHA to the membranes. The ability of [^{125}I]TID to interact with and label distinct sites in soluble proteins has in fact been noticed previously. For example, calmodulin (Krebs et al., 1984) and its fragments (Buerkler & Krebs, 1985) are strongly labeled by [^{125}I]TID in a Ca^{2+}-dependent manner, which is believed to reflect exposure of a hydrophobic binding site. Likewise, bovine serum albumin strongly binds [^{125}I]TID and on photoactivation is heavily labeled regardless of whether membranes are present or not (unpublished data). Thus, the relatively small molecule [^{125}I]TID is not equally well suited in all cases to identify hydrophobic membrane binding sites. On the other hand, it is of considerable interest to define in more detail the presumed [^{125}I]TID binding site in the BHA molecule. In fact, the observed increase in labeling of BHA2 at pH 7 upon dilution of the sample may be indicative of the occurrence of some changes in the structure of BHA.

In contrast to what was found with [^{125}I]TID, labeling with the new phospholipid [^{3}H]PTPC/11 was strictly dependent upon the low-pH incubation. Most likely, the different behavior of these reagents results from the very much lower monomer solubility of [^{3}H]PTPC/11 in aqueous buffer and the very low rate at which this lipid can diffuse between membranes or between membranes and a soluble protein. In any case, it is evident that [^{3}H]PTPC/11 is much less prone than [^{125}I]TID to bind to hydrophobic domains of proteins located outside a lipid bilayer.

Our results obtained with [^{3}H]PTPC/11 are in agreement with the concept of HA structure and function and with the idea that fusion is triggered by the interaction of a hydrophobic peptide with the bilayer. It should now be possible to examine whether the interaction with the lipid bilayer is mediated solely through the hydrophobic NH_2 terminus of BHA2, as is generally assumed, or whether it involves other regions in the BHA subunit.

As hypothesized by Lucy (1984), membrane fusion may be mediated by hydrophobic segments of proteins, and the un-

[2] This is mainly due to residual free radioactivity which, following precipitation of the proteins, was still present and gave rise to somewhat variable levels of "background" radioactivity (within 50–100 cpm per 2-mm gel slice) along the gel track. Accordingly, the reproducibility was better for those proteins that were labeled to higher extents.

derlying mechanism may be fundamentally the same. Direct support of this view comes from a recent study by Lear and DeGrado (1987) in which it was shown that a synthetic peptide representing amino acid residues 1–20 of BHA2 binds to small unilamellar vesicles and promotes their fusion. However, the rate at which fusion is triggered by this peptide appears to be independent of pH between 5.0 and 7.0. The present finding that BHA was labeled only at low pH with the phospholipid reagent strongly suggests that this soluble protein may serve as a useful model to investigate some basic questions regarding the initial steps of hemagglutinin-induced membrane fusion.

It is clear that further labeling studies have to include also experiments on intact hemagglutinin (viruses). That the interaction of BHA with a target bilayer might not faithfully reflect what happens to HA during membrane fusion was indeed suggested recently by Boulay et al. (1986) on the basis of their [^{125}I]TID-labeling results. A different behavior of BHA and HA is also evident from the fact that, upon acidification, trimers of BHA at low concentration dissociate completely into monomers, whereas those of HA do not (Nestorowicz et al., 1985).

The observation that labeling of BHA was affected by the temperature of the low-pH incubation but not by that of the photolysis suggests that the labeling chemistry per se is largely independent of temperature between 0 and 37 °C. This result is consistent with data reported previously by Doms et al. (1985), indicating that binding of the acid form occurs more slowly at lower temperatures. On the other hand, prolonged incubation (up to 1 h) of BHA with liposomes at pH 5 and 0 °C did not appear to lead to a significant increase in labeling (data not shown), implying that the conversion of BHA to the acid form and/or binding to liposomes does not obey simple kinetic rules.

In the course of our work, we found it interesting to examine some other water-soluble proteins for their ability to interact with membranes and, hence, to become labeled by [^{3}H]-PTPC/11. The results obtained are presented in Table I. They seem to suggest that, under moderately acidic conditions, many, perhaps most, globular proteins can weakly interact with the lipid core of bilayers. If such interactions indeed occur, what could be their structural basis?

Before discussing this point, it seems appropriate to consider the possibility that the carbene generated from [^{3}H]PTPC/11 might have reacted with the protein in a hydrophilic environment at or near the surface of the bilayer rather than, as intended, within the apolar phase of the bilayer. In our opinion, this possibility can be excluded quite safely. Unlike the situation with other types of photolabeling reagents including aryl azides and 3-phenyl-3*H*-diazirines which in addition to the desired nitrenes and carbenes also generate intermediates of moderate or low reactivity, the photoreactive group used here is assumed to produce a single reactive species, the carbene. If generated in an aqueous phase, this species would be quenched instantaneously by water, presumably at diffusion-controlled rates, virtually excluding and possibility that a protein could be labeled to a measurable extent. This is presumably the reason why it has never been observed that addition of glutathione[3] to the aqueous buffer had any measurable effect on labeling yields or label distribution patterns. Any carbene exposed to water and hence accessible to glutathione would be scavenged with maximal efficiency by water alone. Therefore, [^{125}I]TID and [^{3}H]PTPC/11 labeling does occur only at sites from which water is largely excluded such as within the interior of a lipid bilayer and hydrophobic regions (pockets) of proteins.

In agreement with ideas put forward recently by Kim and Kim (1986), we also suggest that labeling reflects a general property of a protein that has been chracterized as "compact state with fluctuating tertiary structure" (Dolgikh et al., 1981, 1984). It seems quite reasonable that, in its molten-globule state, a protein may acquire properites that allow it more easily to interact with a lipid–water interface than in its native structure. This could mean that, under suitable conditions, and particularly at acid pH, most or many globular and water-soluble proteins could exhibit a certain potential to interact with the water–lipid interface of membranes and, perhaps, to trigger fusion. In addition to hydrophobic segments, amphipathic α-helices might be particularly prone to undergo such interactions with lipid bilayers. As implied by a recent study of Hurt and Schatz (1987), it is indeed very likely that such structures are present in a large number of soluble proteins but that in the compact, native structure their hydrophobic face is directed toward the interior of the proteins and, therefore, is unable to interact with membranes. That a moderate increase in acidity from pH 7 to pH 5 can trigger weak hydrophobic interactions of soluble proteins with membranes has not been expected or was thought to be restricted to some special cases of proteins such as bacterial toxins and viral fusion proteins that become "activated" in the low-pH environment of endosomal (lysosomal) vesicles. It remains a challenging task now to determine the specific properties and features that make hemagglutinin such a potent fusion agent and to find out in what respects its behavior is similar to or different from that of other proteinaceous fusogens such as α-lactalbumin. Possibly important in this context may be the "bivalency" of hemagglutinin which enables its simultaneous interaction with two membranes as well as a strict irreversibility of the low-pH-induced conformational change.

[3] Glutathione, a water-soluble powerful nucleophile, has occasionally been recommended to be included in the buffer in order to scavenge reactive species that escape the lipid bilayer.

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