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# Characterization of the Membrane Binding Domain of γ-Glutamyltranspeptidase by Specific Labeling Techniques†

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ABSTRACT: The amphipathic form of γ-glutamyltranspeptidase was labeled either by reductive methylation of primary amino groups or by galactose oxidase/$NaB^3H_4$ labeling of galactose residues. The labeled enzyme was asymmetrically reconstituted into unilamellar phosphatidylcholine vesicles and subjected to partial papain proteolysis, and the resulting products were resolved by Sepharose 4B chromatography. Chromatography of the vesicle-associated material on Sephadex LH-60 yields an 8700 molecular weight peptide which is labeled by both techniques. This peptide, therefore, contains lysine residues and/or the $NH_2$-terminus of the large subunit and a galactose-containing oligosaccharide side chain. This peptide appears to be identical with peptide I which is labeled by 3-(trifluoromethyl)-3-(*m*-[$^{125}$I]iodophenyl)diazirine [Frielle, T., Brunner, J., & Curthoys, N. P. (1982) *J. Biol. Chem. 257*, 14979–14982]. A second hydrophobic peptide (peptide II) which is also labeled by the membrane-permeable, photoactivatable probe is not significantly labeled either by reductive methylation or by galactose oxidase/$NaB^3H_4$ labeling. Sephadex G-25 chromatography of the $^3$H-labeled hydrophilic peptides released from the vesicles by papain proteolysis yields a [$^3$H]galactose-labeled peptide of 2600 molecular weight (peptide III) and a 1300 molecular weight peptide labeled by reductive methylation (peptide IV). Peptide I, but not peptide IV, partitions into a series of primary aliphatic alcohols and can be reconstituted into vesicles. The hydrophilic peptides are probably derived either from a peripheral sequence of the membrane binding domain or from the region which connects the hydrophilic domain of the large subunit with the membrane binding domain.

Rat renal γ-glutamyltranspeptidase is an amphipathic integral membrane glycoprotein that is asymmetrically associated with the brush border membrane of the proximal tubule (Tsao & Curthoys, 1980). The enzyme possesses a limited hydrophobic domain that is responsible for its membrane association (Curthoys & Hughey, 1979). The catalytic activity is contained within a separate hydrophilic domain that is highly glycosylated and positioned away from the membrane surface by a sequence of amino acids that is sensitive to papain. As a result, the γ-glutamyltranspeptidase purified following solubilization by limited papain proteolysis is soluble in aqueous buffers and has a molecular weight for 69 000 (Hughey & Curthoys, 1976). In contrast, purification following solubilization with Triton X-100 yields an amphipathic enzyme that is soluble only at concentrations of detergent above the critical micelle concentration. After correction for the mass of bound detergent, the molecular weight of the Triton X-100 purified enzyme was estimated to be 87 000 (Hughey & Curthoys, 1976). Only the amphipathic form of the enzyme can be asymmetrically reconstituted into unilamellar phosphatidylcholine vesicles (Hughey et al., 1979).

γ-Glutamyltranspeptidase is composed of two nonidentical subunits. The molecular weight of the smaller subunit, which contains the γ-glutamyl binding site (Tate & Meister, 1977), is unaltered by papain treatment. However, the large subunit of the Triton-purified enzyme is 21 000 daltons greater than that of the papain-purified enzyme (Horiuchi et al., 1978). The isolated small subunits of the two forms of enzyme both contain $NH_2$-terminal threonine residues, whereas the $NH_2$-terminal residues of the large subunits are nonidentical (Tsuji et al., 1980). Furthermore, the reaction of the reconstituted enzyme with the membrane-soluble, photactivatable reagent 3-(trifluoromethyl)-3-(*m*-[$^{125}$I]iodophenyl)diazirine ([$^{125}$I]TID)[1] results in the labeling of only a limited segment of the large subunit (Frielle et al., 1982). Thus, the $NH_2$-terminal segment of the large subunit apparently contains the hydrophobic membrane binding domain of γ-glutamyltranspeptidase.

Amino acid sequence analyses indicate that the membrane binding domains of NADPH–cytochrome P-450 reductase (Black & Coon, 1982) and the intestinal brush border membrane enzymes aminopeptidase N (Feracci et al., 1982) and the sucrase-isomaltase complex (Spiess et al., 1982) possess $NH_2$-terminal hydrophilic sequences which contain one to three lysine residues. The $NH_2$-terminal segment of the sucrase–isomaltase complex also contains an oligosaccharide side chain which is linked to a threonine residue at position 11 (Frank et al., 1978). In each case, the hydrophilic sequence is contiguous with a hydrophobic sequence which is integrated into the lipid bilayer. It has been proposed that the charged res-

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[1] Abbreviations: [$^{125}$I]TID, 3-(trifluoromethyl)-3-(*m*-[$^{125}$I]iodophenyl)diazirine; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PγGT, γ-glutamyltranspeptidase purified following papain proteolysis; $V_R$, retention volume; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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idues may anchor the nascent polypeptides in the membrane during biosynthesis (Roelofsen & Zwaal, 1976)

Papain proteolysis of the reconstituted, [$^{125}$I]TID-labeled γ-glutamyltranspeptidase yields two vesicle-associated [$^{125}$I]TID-labeled peptides which constitute portions of the membrane binding domain (Frielle et al., 1982). Amino acid analysis of the isolated, [$^{125}$I]TID-labeled peptides indicates that they contain several lysine residues. The similarities between the sucrase–isomaltase complex and γ-glutamyltranspeptidase suggest that the membrane binding domain of the transpeptidase may also possess an oligosaccharide side chain. In the present study, γ-glutamyltranspeptidase labeled either by reductive methylation of primary amino groups (Means, 1977) or by galactose oxidase/$NaB^3H_4$ labeling of galactose residues (Morell & Ashwell, 1972) was reconstituted into unilamellar phosphatidylcholine vesicles and subjected to limited papain proteolysis. Only the larger of the two resulting hydrophobic peptides was significantly labeled by either of these procedures. These studies also establish that papain proteolysis releases at least two distinct hydrophilic peptides from the transpeptidase. A portion of this study has been presented previously in preliminary form (Frielle & Curthoys, 1982).

## Materials and Methods

The amphipathic form of γ-glutamyltranspeptidase was purified following Triton X-100 solubilization (Hughey & Curthoys, 1976) and was assayed at room temperature with γ-glutamyl-*p*-nitroanilide as the substrate (Tate & Meister, 1974). $NaB^3H_4$ (7-10 Ci/mmol), obtained from New England Nuclear, was dissolved in 0.1 N NaOH at a concentration of 12 mg/mL and stored at −20 °C. A 40 mM solution of formaldehyde was prepared by heating solid paraformaldehyde until solubilized. Galactose oxidase of *Dactylium dendroides* (Worthington) was immobilized by the method of Axen et al. (1967) using CNBr-activated Sepharose (Sigma). Type III-E phosphatidylcholine (10 mg/mL in hexane) was purchased from Sigma and stored under $N_2$ at −20 °C. The sodium salt of cholic acid was purchased from Merck. Papain (Sigma) was activated prior to use by preincubation of a 1 mg/mL solution in 50 mM imidazole, pH 7.2, containing 10 mM DTT and 2.5 mM EDTA for 15 min at 37 °C.

Galactose residues were labeled essentially according to the method of Morell et al. (1972) but without prior treatment with neuraminidase. Galactose residues were oxidized by incubation of 200 μg of enzyme in 200 μL of 50 mM imidazole and 20% ethanol, pH 7.2, with 200 μL of a suspension of galactose oxidase/Sepharose (4 units/mL) for 3 h at 37 °C. The protein was transferred to 20 mM sodium borate and 20% ethanol, pH 9.0, by centrifuge desalting on a column of Sephadex G-50 (Penefsky, 1971) and incubated at 4 °C. A 2-μL aliquot of the $NaB^3H_4$ solution was added, and the sample was immediately neutralized. The labeled enzyme was recovered by centrifuge desalting.

[$^3$H]Galactose was identified by hydrolysis of labeled protein and peptides in 2 N trifluoroacetic acid for 2 h at 100 °C. The hydrolysates were rotoevaporated to dryness, dissolved in water, and applied to 0.5 × 1 cm Dowex AG50W-X8 columns equilibrated in water. The neutral eluates were chromatographed on Whatman 1 paper by using an ethyl acetate/pyridine/water (120:50:40) solvent system. Identification of galactose was achieved by comparison with chromatograms of [$^{14}$C]galactose and of unlabeled standard monosaccharides.

The amphipathic form of γ-glutamyltranspeptidase was labeled by reductive methylation using a modification of the method of Rice & Means (1971). A 200-μL sample containing 200 μg of enzyme and 20 mM sodium borate/20% ethanol, pH 9.0, was incubated at 4 °C. To the solution was added 20 μL of 40 mM formaldehyde followed by the addition of 4 μL of the $NaB^3H_4$ solution. The solution was immediately neutralized and the labeled enzyme recovered by centrifuge desalting.

γ-Glutamyltranspeptidase labeled by either procedure was reconstituted into unilamellar phosphatidylcholine vesicles by using a previously described modification (Hughey et al., 1979) of the cholate dialysis method of Brunner et al. (1976). The reconstituted enzyme was incubated in a solution containing 100 mM NaCl, 10 mM Tris-HCl, pH 7.2, 2 mM DTT, and 2.5 mM EDTA for 18 h at 37 °C with a 40:1 ratio of transpeptidase:papain (w/w). The vesicles were resolved from the proteolyzed enzyme and retention-volume peptides by chromatography on a 1 × 50 cm column of Sepharose 4B.

Fractions from the Sepharose 4B profile were pooled for further analysis. The vesicle fractions were dialyzed vs. water, lyophilized, and dissolved in ethanol/formic acid (70:30). The residual transpeptidase and the hydrophobic peptides were resolved by chromatography of the vesicle material on a 2 × 50 cm column of Sephadex LH-60 (Gerber et al., 1979). The material from the retention-volume fractions of the Sepharose 4B column was lyophilized and analyzed by chromatography on a Sephadex G-25 (fine) column (1 × 100 cm) equilibrated with 8 M acetic acid.

The peptides isolated from γ-glutamyltranspeptidase prelabeled by reductive methylation were allowed to partition between 0.3 mL of 50 mM imidazole, pH 7.2, and 0.3 mL of various aliphatic alcohols. The phases were mixed thoroughly and centrifuged to facilitate separation. Aliquots were taken from each phase for determination of radioactivity. The radioactivity of the interphase was calculated by subtracting the radioactivity contained in each phase from the total in the sample. Reconstitution of the isolated peptides was carried out by using the same method as employed for the reconstitution of the transpeptidase. The $^3$H-labeled peptides were subjected to electrophoresis using the 6 M urea, 0.1% SDS, and 20% polyacrylamide gel system of Ito et al. (1980). Peptides were visualized by sodium salicylate fluorography of the dried gels using Kodak X-Omat AR-5 film (Chamberlain, 1979).

## Results

The amphipathic form of γ-glutamyltranspeptidase can be labeled by treatment with galactose oxidase and $NaB^3H_4$ to a specific radioactivity of $2 \times 10^6$ cpm/mg. Specific labeling of galactose residues was confirmed by descending paper chromatography of a 2 N trifluoroacetic acid hydrolysate of the labeled enzyme (Figure 1). Approximately 95% of the $^3$H radioactivity chromatographs as a single peak with a mobility identical with that of the [$^{14}$C]galactose standard. Similarly, chromatography of hydrolysates prepared from individual peptide fractions generated in later experiments also indicated that only galactose residues are labeled (data not shown).

The [$^3$H]galactose-labeled enzyme retains both full activity and the ability to be reconstituted into phosphatidylcholine vesicles. As shown in Figure 2A, papain proteolysis of the [$^3$H]galactose-labeled, reconstituted enzyme solubilizes 90% of the enzyme activity. However, the released enzyme which elutes at a fraction characteristic of the papain-purified γ-glutamyltranspeptidase (PγGT) contains only 40% of the initial $^3$H radioactivity. $^3$H-labeled peptide material is found in the retention volume ($V_R$) of the Sepharose 4B column. This material is probably derived from a hydrophilic portion of the

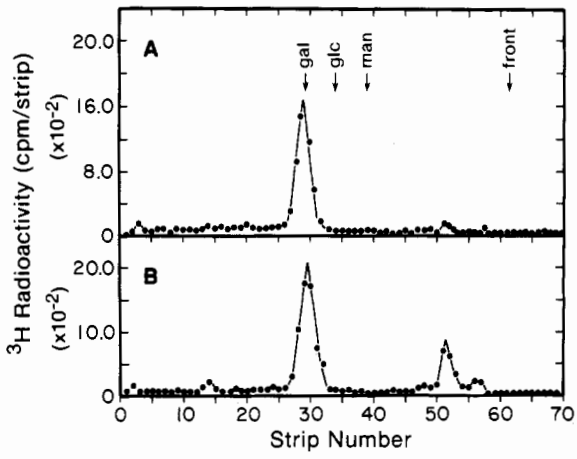


FIGURE 1: Descending paper chromatography of a 2 N trifluoroacetic acid hydrolysate of γ-glutamyltranspeptidase labeled by galactose oxidase/$NaB^3H_4$. The hydrolysate of the labeled enzyme (A) and a sample of [$^{14}$C]galactose which had been treated in the same manner (B) were chromatographed separately. Unlabeled galactose, glucose, and mannose were also treated with 2 N trifluoroacetic acid and chromatographed as described under Materials and Methods.

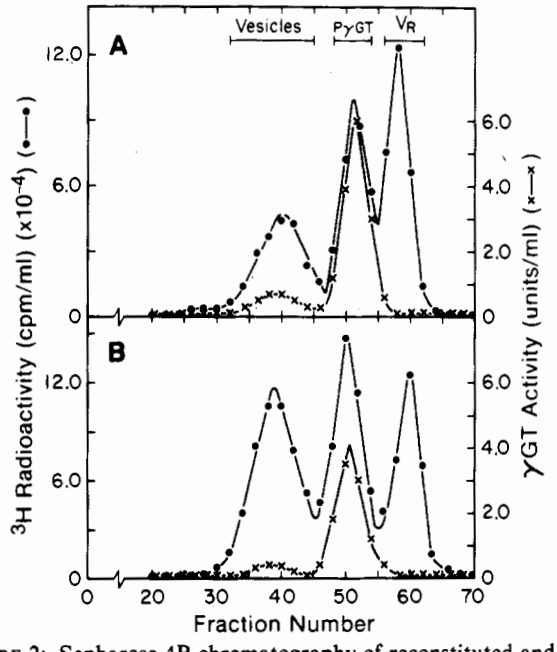


FIGURE 2: Sepharose 4B chromatography of reconstituted and papain-treated γ-glutamyltranspeptidase. Enzyme labeled either by galactose oxidase/$NaB^3H_4$ treatment (A) or by reductive methylation (B) was reconstituted into phosphatidylcholine vesicles and treated with papain. The proteolyzed vesicles were then chromatographed on a 2 × 50 cm Sepharose 4B column.

transpeptidase that is exposed on the external suface of the vesicles and not from the hydrophobic portion of the transpeptidase that is integrated into the lipid bilayer. This conclusion is supported by the observation that papain proteolysis of reconstituted [$^{125}$I]TID-labeled enzyme does not yield labeled material which elutes in the retention volume of the Sepharose 4B column (Frielle et al., 1982). The data presented in Figure 2A also provide evidence that papain proteolysis generates $^3$H-labeled material that remains associated with the vesicles. Approximately 40% of the $^3$H radioactivity initially incorporated into the transpeptidase elutes with an elution volume characteristic of phosphatidylcholine vesicles (Frielle & Curthoys, 1982). However, only 10% of the total enzyme activity coelutes with the vesicles. Thus, the vesicles must contain $^3$H-labeled peptides in addition to the residual [$^3$H]galactose-labeled enzyme.

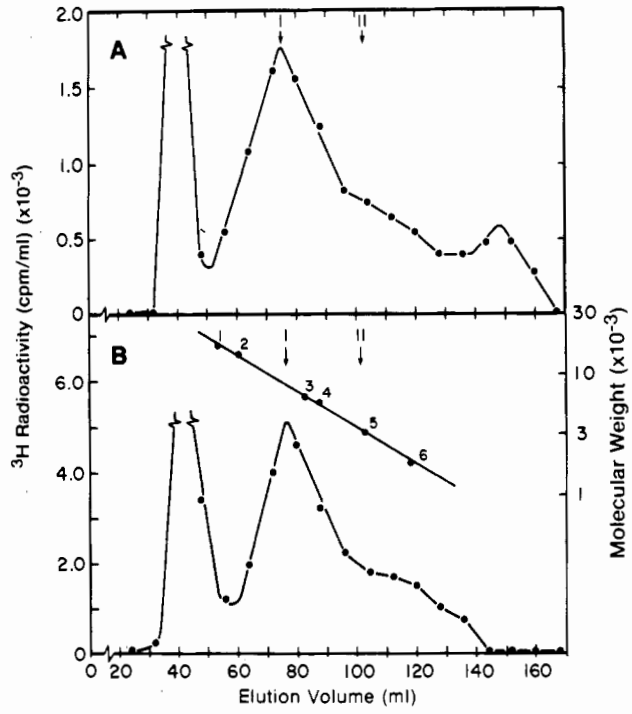


FIGURE 3: Sephadex LH-60 chromatography of the vesicle-associated material derived from enzyme labeled either by galactose oxidase/$NaB^3H_4$ treatment (A) or by reductive methylation (B). Papain-proteolyzed vesicles recovered from the Sepharose 4B column (Figure 2) were lyophilized, solubilized in ethanol/formate (70:30), and chromatographed on a 2 × 50 cm Sephadex LH-60 column. Molecular weight standards are (1) myoglobin ($M_r$ 17000), (2) lysozyme ($M_r$ 14300), (3) aprotinin ($M_r$ 6500), (4) insulin ($M_r$ 5700), (5) glucagon ($M_r$ 3490), and (6) gramicidin D ($M_r$ 1850).

Reductive methylation of the amphipathic form of γ-glutamyltranspeptidase results in a specific radioactivity of 25 μCi/mg without loss of enzymatic activity. This represents approximately 4 mol of $NH_2$-terminal or lysine ε-amino groups modified per mol of enzyme. The ability of γ-glutamyltranspeptidase to be reconstituted into unilamellar phosphatidylcholine vesicles is not affected by prior reductive methylation. Chromatography of the reconstituted enzyme following papain treatment results in a column profile (Figure 2B) which is qualitatively similar to the profile generated by using enzyme labeled by galactose oxidase/$NaB^3H_4$. Approximately 85% of the enzyme activity is solubilized by papain proteolysis. Significant amounts of $^3$H radioactivity elute with the vesicles and in the retention volume of the column, suggesting that the papain-generated peptides which elute in these fractions contain primary amino groups.

To resolve the vesicle-associated peptides from residual [$^3$H]galatose-labeled transpeptidase not released by papain treatment, vesicle-containing fractions from the Sepharose 4B column (Figure 2A) were dialyzed, lyophilized, and chromatographed on Sephadex LH-60 in 70% ethanol:30% formic acid (Figure 3A). The peak of $^3$H radioactivity corresponding to an elution volume of 40 mL has been identified by polyacrylamide gel electrophoresis as residual transpeptidase (Frielle & Curthoys, 1982). The $^3$H-labeled material which elutes at 75 mL is a vesicle-associated peptide that contains galactose residues. This peptide corresponds to peptide I, which is labeled by [$^{125}$I]TID and derived from the $NH_2$-terminal segment of the large subunit (Frielle et al., 1982). On the basis of its elution volume, this peptide has a molecular weight of 8700.

The proteolyzed vesicles derived from reconstituted enzyme labeled by reductive methylation (Figure 2B) were also

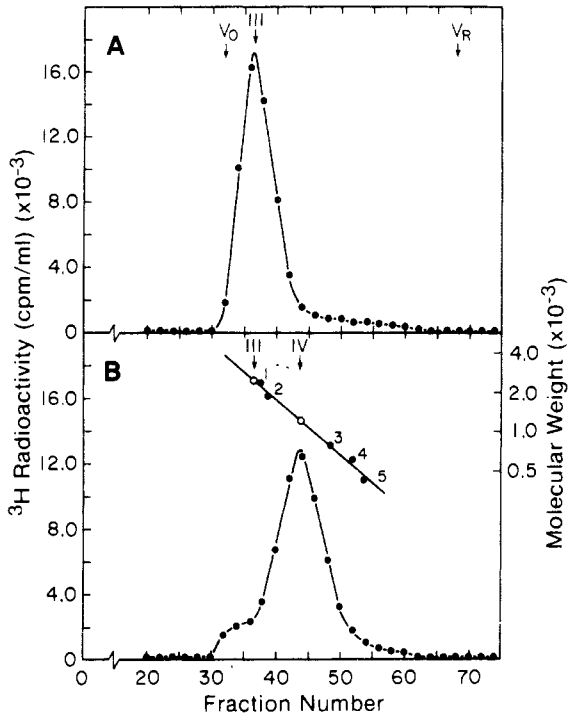


FIGURE 4: Sephadex G-25 chromatography of the hydrophilic peptides released from enzyme labeled either by galactose oxidase/$NaB^3H_4$ treatment (A) or by reductive methylation (B). The $^3$H-labeled material which elutes in the retention volume of the Sepharose 4B column (Figure 2) was lyophilized, solubilized in 8 M acetic acid, and chromatographed on a 1 × 100 cm Sephadex G-25 column. Molecular weight standards are (1) insulin A chain ($M_r$ 2340), (2) gramicidin D ($M_r$ 1850), (3) Phe-Phe-Phe-Phe-Phe ($M_r$ 750), (4) Phe-Phe-Phe-Phe ($M_r$ 610), and (5) Phe-Gly-Gly-Phe ($M_r$ 430).

chromatographed by using Sephadex LH-60 (Figure 3B). The peak of $^3$H radioactivity that elutes at 40 mL was again identified as γ-glutamyltranspeptidase, which is not released from the vesicles by papain proteolysis. The second peak of $^3$H radioactivity possesses the same elution volume as that of peptide I. When subjected to electrophoresis, this material exhibits the same mobility as peptide I labeled either with [$^{125}$I]TID or with galactose oxidase/$NaB^3H_4$. Consequently, peptide I must consist of a hydrophilic sequence, containing an oligosaccharide side chain and primary amino groups, which is contiguous with a hydrophobic sequence that intercalates into the lipid bilayer. A second [$^{125}$I]TID-labeled peptide (peptide II) that elutes at 102 mL is not significantly labeled by either galactose oxidase/$NaB^3H_4$ or reductive methylation. However, fluorescamine analyses of the column profiles indicate that unlabeled peptide material is present in the fractions where peptide II elutes (data not shown). Thus, peptide II contains no galactose residues or primary amino groups which are readily available for labeling.

As shown in Figure 2A, papain proteolysis results in the release of [$^3$H]galactose-labeled peptide material from the vesicles. The retention-volume fractions from the Sepharose 4B column were pooled and analyzed further by Sephadex G-25 chromatography in 8 M acetic acid (Figure 4A). Chromatography of the [$^3$H]galactose-labeled material results in the elution of a single peak of $^3$H radioactivity corresponding to a glycopeptide of approximately 2600 molecular weight (peptide III). The retention-volume material derived from enzyme labeled by reductive methylation was also subjected to chromatography on Sephadex G-25 (Figure 4B). The material elutes as a single peak, with an elution volume corresponding to a molecular weight of 1300 (peptide IV). The two peptides apparently do not share any common primary structure since incubation of peptide III with papain does not generate peptide IV or any other lower molecular weight peptides.

Table I: Partitioning of Papain-Generated Peptide Fractions between Water and Various Primary Aliphatic Alcohols

| fraction | % of total $^3$H radioactivity | | | |
|---|---|---|---|---|
| | 1-butanol | 1-hexanol | 1-octanol | 1-decanol |
| peptide I | | | | |
| organic phase | 78 | 76 | 74 | 66 |
| aqueous phase | 9 | 13 | 17 | 25 |
| interphase | 13 | 11 | 9 | 9 |
| peptide IV | | | | |
| organic phase | 2 | 1 | 1 | 1 |
| aqueous phase | 98 | 99 | 99 | 99 |
| interphase | 0 | 0 | 0 | 0 |

The relative hydrophobicity of $^3$H-labeled peptide I obtained from the Sephadex LH-60 column and peptide III recovered from the retention volume of the Sepharose 4B column was estimated by determining the ability of the isolated peptides to partition between an aqueous phase and an organic phase consisting of various primary aliphatic alcohols (Table I). The retention-volume material quantitatively partitions into the aqueous phase of every binary system including that containing 1-butanol, the least hydrophobic aliphatic alcohol that is still immiscible with water. These results indicate that the material recovered in the retention-volume fraction of the Sepharose 4B column is hydrophilic. In contrast, peptide I partitions preferentially into the organic phase composed of the various alcohols. Peptide I is apparently amphipathic in nature, since the amount which partitions into the organic phase decreases with increasing hydrophobicity of the alcohols.

The lipophilic character of the two fractions was judged by determining the ability of the isolated peptides to reconstitute into vesicles. The $^3$H-labeled peptides were incubated with cholate and [$^{14}$C]phosphatidylcholine, and the mixture was chromatographed on Sephadex G-50 column. The [$^{14}$C]-phosphatidylcholine vesicles which are formed by the removal of cholate, elute in the void volume of the column. The $^3$H-labeled peptide I coelutes with the $^{14}$C-labeled vesicles, suggesting that the peptide is reconstituted into the lipid bilayer. In contrast, the $^3$H-labeled material which is recovered in the retention-volume fractions of the Sepharose 4B column does not coelute with the $^{14}$C-labeled vesicles but is included in the Sephadex G-50 column. Thus, peptide III must lack sequences necessary for membrane association.

The void-volume fractions of the Sephadex G-50 column containing peptide I were pooled and concentrated, and the material was rechromatographed on a Sepharose 4B column. The $^3$H-labeled peptide I again coelutes with the [$^{14}$C]phosphatidylcholine vesicles, estiablishing that the isolated peptide is reconstituted into the bilayer. The reconstituted peptide cannot be dissociated from the vesicles by treatment with 1 M NaCl. This indicates that ionic forces are not responsible for the association of the peptide with the bilayer. In addition, papain treatment of the reconstituted peptide does not result in a significant release of labeled material from the vesicles. Therefore, no additional papain-sensitive sequences are exposed on the external surface of the bilayer as a result of reconstitution. Consequently, peptide I must associate with the bilayer with an orientation which is similar to the original orientation of the reconstituted transpeptidase.

## Discussion

Reductive methylation (Means, 1977; Tack & Wilder, 1981) has proven to be a useful technique for detecting the

papain-generated peptides derived from the membrane binding domain of γ-glutamyltranspeptidase. The identification of these peptides as $NH_2$-terminal or as lysine-containing peptides depends upon the specificity of the labeling technique (Means & Feeney, 1968). Under the conditions used in the current study, $NaBH_4$ selectively reduces only aldehydes, ketones (Chaiken & Brown, 1949), and Schiff bases (Fisher et al., 1958). Since these functional groups are not normally found in proteins, nonspecific reduction by $NaBH_4$ should not occur. Incubation of γ-glutamyltranspeptidase with $NaB^3H_4$ without prior exposure to formaldehyde or galactose oxidase results in no appreciable incorporation of tritium (data not shown). Further specificity results from the fact that formaldehyde forms a Schiff base with only the $NH_2$-terminal amino group and the ε-amino group of lysine. Reductive methylation results in the formation of *N*-monomethyl and *N*-dimethyl derivatives (Kallen & Jencks, 1966). The properties of proteins are not significantly altered by this modification since the differences in the $pK_a$'s of the modified amino groups are too small to significantly alter electrostatic interactions at physiological pH (Bradbury & Brown, 1973). In the present study, no decrease in the enzymatic activity of γ-glutamyltranspeptidase was observed nor were reconstitution or papain proteolysis affected by reductive methylation.

Oxidation of galactose residues by galactose oxidase followed by $NaB^3H_4$ reduction of the resulting aldehyde has been utilized for the specific labeling of ceruloplasmin (Morell & Ashwell, 1972) and the glycoproteins and glycolipids of the erythrocyte membrane (Morell et al., 1966; Gahmberg & Hakomori, 1973). Galactose oxidase will oxidize galactose and *N*-acetylgalactosamine residues which are present as monosaccharides or as part of a larger oligosaccharide (Avigad et al., 1962). The enzyme exhibits no activity toward other hexoses. In the present study, hydrolysates of all protein and peptide fractions labeled by galactose oxidase/$NaB^3H_4$ were characterized by paper chromatography as containing only [$^3$H]galactose.

Reconstituted γ-glutamyltranspeptidase and the enzyme associated with isolated brush border membrane vesicles both exhibit a similar asymmetry of orientation (Tsao & Curthoys, 1980), a similar sensitivity to papain proteolysis, and a similar temperature dependence in their energies of activation (Hughey et al., 1979). The detergent dialysis method of reconstitution also results in the asymmetric orientation of aminopeptidase N (Hussain et al., 1981), aminopeptidase M (Curthoys et al., 1980), and sucrase–isomaltase (Brunner et al., 1978) on the external surface of the vesicle bilayer. The mechanism of membrane association of γ-glutamyltranspeptidase with unilamellar vesicles is therefore likely to approximate the mechanism of membrane association with the native membrane.

Reductive methylation and galactose oxidase/$NaB^3H_4$ treatment of γ-glutamyltranspeptidase result in significant incorporation of label into only the larger of the two hydrophobic peptides generated by papain proteolysis. Therefore, in addition to a hydrophobic sequence, peptide I contains both primary amino groups and galactose residues. The presence of primary amino groups which are charged at neutral pH and of a hydrophilic oligosaccharide side chain suggests that peptide I is amphipathic. This conclusion is consistent with the observed partitioning characteristics of peptide I (Table I). The lysine and galactose residues of peptide I probably reside within a hydrophilic sequence which loops out of the membrane but is contiguous with the hydrophobic sequence that is integrated into the bilayer. This model is supported by the amino acid sequence analyses of several membrane-associated proteins. Lysine residues separate distinctly hydrophilic, peripheral sequences from the hydrophobic domains responsible for the membrane association of these proteins (Black & Coon, 1982; Feracci et al., 1982; Spiess et al., 1982). The galactose-containing oligosaccharide of peptide I could also be localized in such a peripheral sequence as has been proposed for the $NH_2$-terminus of the membrane binding domain of the sucrase–isomaltase complex (Frank et al., 1982). Proteolysis of a sensitive residue within this loop would cause the cleavage of the membrane binding domain to produce two distinct peptides, each possessing sequences that are integrated into the lipid bilayer. If only one of the two peptides retained the labeled primary amino groups and the oligosaccharide chain, this would explain why peptide I is labeled by all three techniques, whereas peptide II is labeled only by [$^{125}$I]TID.

Peptides III and IV are released by papain proteolysis of the reconstitued transpeptidase, suggesting that these peptides are peripheral sequences which reside on the exterior surface of the vesicle bilayer. The partitioning behavior of peptide IV (Table I), its inability to undergo reconstitution, and the finding that peptide III is a glycopeptide all indicate that these peptides are hydrolphilic. Peptides III and IV could be derived from a loop structure at the membrane surface which connects the two hydrophobic sequences which are integrated into the bilayer. The peptides could be derived from portions of the exposed $NH_2$-terminal sequence of the large subunit or from a portion of the connecting sequence between the hydrolphilic domain of the large subunit and the hydrophobic membrane binding domain.

**Registry No.** PγGT, 9046-27-9.

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# Synthesis and Characterization of a Fluorescence Probe of the Phase Transition and Dynamic Properties of Membranes†

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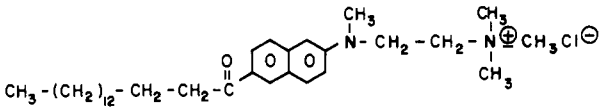
ABSTRACT: We describe the synthesis and characterization of a new fluorescence probe whose emission spectra, anisotropies, and wavelength-dependent decay times are highly sensitive to the phase state of phospholipid vesicles. This probe is 6-palmitoyl-2-[[2-(trimethylammonio)ethyl]methylamino]naphthalene chloride (Patman). The emission maximum of Patman shifts from 425 to 470 nm at the bilayer transition temperatures. The spectral properties of Patman reveal nanosecond time-dependent spectral shifts, which are the result of membrane relaxation around the excited state of Patman. The apparent fluorescence lifetimes of Patman are strongly dependent upon the emission wavelength, and the fluorescence phase and modulation data prove that the spectral shifts are due to an excited-state process, and not ground-state heterogeneity. As expected, the fluorescence anisotropies reflect the phase transitions of the bilayers. In addition, the anisotropies are dependent upon the emission wavelength because the duration of the excited state varies across the emission spectrum. The different apparent lifetimes across the emission spectrum allow the relaxed and unrelaxed emission spectra to be resolved by phase-sensitive detection of fluorescence. Also, the emission spectra of Patman show marked shifts to longer wavelengths as the excitation wavelength is increased. These red-edge excitation shifts are sensitive to the temperature and phase state of the bilayers.

Fluorescence probes are widely used to study the physical properties of proteins and membranes. Generally, such probes are selected on the basis of their sensitivity to the phenomenon of interest. For instance, probes such as 1,6-diphenyl-1,3,5-hexatriene (DPH)[1] are highly sensitive to the phase state of lipid bilayers, as revealed by its fluorescence anisotropy (Shinitzky & Barenholz, 1974; Lentz et al., 1976a,b). This sensitivity originates with its rodlike structure, the motions of which are highly sensitive to the dynamic and order properties of the acyl side chain regions of membranes (Kawato et al., 1977; Lakowicz et al., 1979). Other probes, such as the widely used naphthylaminesulfonic acids, are sensitive to contact with water and to the polarity of the surrounding environment (Weber, 1952; Slivak, 1982).

Scheme I



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[1] Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; Patman, 6-palmitoyl-2-[[2-(trimethylammonio)ethyl]methylamino]naphthalene chloride; DOPC, DMPC, and DPPC, dioleoyl-, dimyristoyl-, and dipalmitoyl-L-α-phosphatidylcholine, respectively; DPPG, dipalmitoyl-L-α-phosphatidylglycerol; CTABr, cetyltrimethylammonium bromide; SDS, sodium dodecyl sulfate; fwhm, full width at half-maximum intensity of a steady-state emission spectrum; Prodan, 6-propionyl-2-(dimethylamino)naphthalene; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane.

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