Hydrophobic Labeling, Isolation, and Partial Characterization of the NH₂-terminal Membranous Segment of Sucrase-Isomaltase Complex

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A photogenerated carbene, 3-trifluoromethyl-3-(m-[125I]iodophenyl)carbene (Brunner, J., and Semenza, G. (1981) Biochemistry 20, 7174-7182), was used to label the hydrophobic core of small intestinal brush-border membrane vesicles. Reaction of the carbene with sucrase-isomaltase complex was restricted to a polypeptide segment which is essential for binding the enzyme complex to the native membrane or to liposomes. The same labeling selectivity was obtained when purified sucrase-isomaltase complex was labeled either in Triton X-100 solution or when it was incorporated in egg-lecithin liposomes. During cleavage of sucrase-isomaltase with papain, the radiolabel remained covalently associated with the anchor peptide. It was thus possible to detect easily the polypeptide in the course of subsequent separation and purification operations. The molecular weight of the peptide was determined by gel filtration on Sephadex LH-60 in ethanol-formic acid (Takagaki, Y., Gerber, G. E., Nihei, K., and Khorana, H. G. (1980) J. Biol. Chem. 255, 1536-1541). The figure thereby obtained, 6500, is somewhat lower than that obtained from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (~8000). Circular dichroism of the peptide indicates a secondary structure of high α-helical content. A possible structure of the membranous segment is discussed.

Small intestinal sucrase-isomaltase (sucrose a-D-glucohydrolase, EC 3.2.1.46, and isomaltase, EC 3.2.1.10, respectively) is an integral protein complex of the brush-border membrane which consists of two subunits. Two forms of the enzyme complex can be isolated. Detergent (Triton X-100) extraction of brush-border membrane vesicles yields an amphipathic species whereas solubilization by controlled proteolysis yields a water-soluble form which has lost its capacity to bind to membranes (1-4). Papain solubilization of sucrase-isomaltase does not affect the sucrase subunit (4). In contrast, the apparent molecular weight of the isomaltase subunit is reduced from approximately 160,000 to approximately 140,000. This suggests that the membranous domain "belongs" to the isomaltase polypeptide chain. In fact, based on amino acid analysis of the NH₂ and COOH termini of the subunits derived from papain- or Triton X-100-solubilized enzyme complex, the membrane binding site could be unambiguously located in the NH₂-terminal region of the isomaltase subunit (4).

Previous attempts to isolate and characterize this domain which contains a highly hydrophobic segment (5) were not successful. One main problem was that no useful analytical method was available for detection and quantitation of the peptide in the course of purification operations. In this paper, we describe the selective labeling of the hydrophobic domain of sucrase-isomaltase complex with a carbene photogenerated from [125I]-trifluoromethyl-3-(m-iodophenyl)diazirine (6). This reagent is highly hydrophobic and thus partitions to a very high extent into the lipid core of a membrane. The radiolabel which thereby became covalently bound to the hydrophobic segment was then utilized as a marker of the peptide.

Materials and Methods

Triton X-100 (Sigma) and Na-cholate (Merck) were used without further purification. 2-Chloroethanol (Fluka) was distilled prior to use and diluted with 10% water. Sephadex LH-60 was from Pharmacia, Bio-Gel A-5m was from Bio-Rad, and egg lecithin was from Lipid Products (South Nutfield, Surrey, United Kingdom). Vesicles of rabbit small intestinal brush-border membranes were prepared as described in Ref. 7. Sucrase-isomaltase complex was isolated from membrane vesicles by Triton X-100 solubilization (2). Single bilayer proteoliposomes were prepared from egg lecithin and Triton X-100-solubilized sucrase-isomaltase by the Na-cholate removal method described (3). Sucrase activity was determined by measuring the amount of glucose liberated from sucrose (33 mM) at 37 °C in 33 mM Na-maleate buffer, pH 6.8. D-Glucose was determined using the glucose dehydrogenase kit from Merck (8). Protein was determined according to the Lowry procedure modified by Peterson (9). Phospholipid concentrations were monitored according to Chen et al. (10). NH₂-terminal amino acid analyses were performed by the dansyl method (11). Papain digestion of sucrase-isomaltase complex was carried out according to Ref. 2. [125I]TID was prepared as described in Ref. 6 and was stored as an ethanolic solution (2 to 5 mCi/ml; 10 Ci/mmol) at -20 °C in the dark.

Photolabeling Procedure: Brush-border Membranes—Vesicles (1-5 mg of protein/ml) in 50 mM Na-phosphate, pH 7.5, were flushed with a gentle stream of N₂ for 30 min at 0 °C. [125I]TID (10 to 50 μCi) was added to 1.0 to 1.3 ml of the vesicle dispersion and, after equilibration for 30 min at 4 °C, the sample was photolyzed in a quartz cuvette. A 350-watt medium pressure mercury lamp (Type 350-1008, Illumination Industries, Inc.) was used as a light source. The beam was directed through filters of circulating cold water (30 °C) and a saturated solution of copper sulfate (20 °C) onto the center of the cuvette. The latter filter screens out radiation shorter than 315 nm (12). Labeled brush-border membranes were washed 4 times with Na-phosphate (50 mM, pH 7.5) containing 1% bovine serum albumin and twice with albumin-free buffer. For immunoprecipitation, labeled and washed membrane vesicles were solubilized in buffer (150 mM NaCl, 10 mM Na-phosphate, pH 7.5) containing 2% Triton X-100 for 15 min at 4 °C. Sucrase-isomaltase complex was precipitated from the supernatant (15,000 × g, 10 min) with anti-sucrase-isomaltase antisera from guinea pig (generous gift from Prof. R. Gitzelmann, Kinderspital, Zürich). The immunoprecipitates

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1. The abbreviations and trivial name used are: dansyl, 5-dimethylaminophthalene-1-sulfonyl; TID, 3-trifluoromethyl-3-(m-phenyldiazirine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PC, phosphatidylcholine.
were washed with buffer containing 1% albumin until no further radioactivity was released. The immunoprecipitates were sedimented in an MSE table centrifuge at 3000 rpm for 90 s.

**Sucrase-Isomaltase Egg Lectin Proteoliposomes—**Proteoliposomes (lipid/protein, 5:1, w/w; 1 to 2 mg of protein/ml) were dialyzed against 50 mM Na-phosphate, pH 7.5, were flushed with nitrogen, equilibrated with $[^{125}]$TID, and photolyzed as described above. Labeled sucrase-isomaltase complex was separated from labeled lipid and photolysis products of TID by gel filtration on a column (1.5 x 90 cm) of Bio-Gel A-5m which was equilibrated and eluted with 50 mM Na-phosphate, 2% Na-cholate, pH 7.5. Sucrase-isomaltase (measured by its sucrase activity) was eluted near the void volume of the column, well separated from the bulk (>99%) of the radioactivity applied to the column.

**Sucrase-Isomaltase-Triton X-100 Complex—**Isolated Triton X-100-solubilized sucrase-isomaltase (5 mg of protein/ml) was dialyzed against 50 mM Na-phosphate (pH 7.5) and supplemented with Triton X-100 to give a final detergent concentration of 0.2% (w/v). Labeling and product separation were performed essentially as described for the proteoliposomes.

**Isolation of the Anchor Peptide of Sucrase-Isomaltase—**$[^{125}]$TID-labeled sucrase-isomaltase was reincorporated into egg lectin liposomes and the proteoliposomes digested with papain as described (4). The liposomes containing the hydrophobic peptide were separated from solubilized sucrase-isomaltase by gel filtration on Bio-Gel A-5m (1.5 x 40 cm; flow-rate, 7.2 ml/h) in 50 mM NaCl, 10 mM Tris-HCl, pH 7.5. The pool of the eluted liposomes contained less than 5% of the sucrase activity which was applied to the column. This fraction was due to enzyme complex projecting into the inner, rather than toward the aqueous space of the liposomes and thus not accessible to papain (5). The hydrophobic peptide associated with the liposomes was separated from lipid by either gel filtration (Sephadex LH-60) in organic solvent or using Sephadex G-100 in aqueous buffer containing 1 to 3% Na-cholate.

**Circular Dichroism—**Samples of egg PC containing anchor peptide were prepared as follows. Fractions of peptide which had been separated from lipid by gel filtration in buffer containing Na-cholate as described above and shown in Fig. 3 (pool II) were concentrated by Amicon ultrafiltration (using DM 10 or DM 5 filters) and added to dried egg PC. The peptide-to-lipid molar ratio was 1:50. Reconstitution of peptidoliposomes was performed as described for egg PC-sucrase-isomaltase (3) in 50 mM NaCl, 10 mM Tris-HCl, pH 7.5. Spectra were recorded on a Jasco J-500 A spectropolarimeter equipped with a Jasco Data Processor DP-500 at ambient temperature. A 0.2-mm cell was used. A buffer base-line was recorded and spectra were corrected accordingly. Since pure egg PC liposomes showed no dichroism between 200 and 250 nm, no corrections were made for scattering or absorption. Calculations of peptide concentrations were based on the specific radioactivity of the labeled sucrase-isomaltase using $M_t = 275,000$ (13). To calculate molar ellipticities ($[\theta]$), the molecular weight of the hydrophobic anchor was taken as 6500 (see “Results”) and a mean residue weight of 110 was used (calculated from the partial sequence of the peptide (5)).

**SDS-Polyacrylamide Gel Electrophoresis—**Protein samples were denatured in 2% SDS (2 min, 100 °C) and applied to a discontinuous system (Tris-HCl) as described in Ref. 4. The slab gels were stained in 0.05% Coomassie brilliant blue R-250 or with silver according to the method of Oakley et al. (14). For autoradiography, dried gels were exposed to Kodak X-Omat S films at −80 °C.

**RESULTS**

**Incorporation of $[^{125}]$TID into Brush-bordern Membranes—**Brush-border membranes pre-equilibrated with $[^{125}]$TID covalently incorporated radiolabel when exposed to near ultraviolet irradiation. The time course of the incorporation of radioactivity under standard conditions of irradiation was determined earlier for human erythrocyte ghosts (6) and was shown to be related to the photolytic decay of the diazirine. Photolabeling of brush-border membranes (2-min photolysis) did not increase sucrase or isomaltase activity. Membrane vesicles irradiated in the presence of $[^{125}]$TID for 0, 15, and 120 s were subjected to SDS-PAGE and proteins were stained with Coomassie blue (Fig. 1). The protein patterns were almost identical except for very small amounts of high molecular weight aggregated material which was present in the photolyzed (Fig. 1, B, 15 S; C, 120 s) samples. The autoradiography of the dried gel showed the following features (Fig. 1, a to c). (i) Essentially all of the radioactivity could be removed by albumin extraction from vesicles which had been pre-equilibrated with $[^{125}]$TID but were not photolyzed (lane a). (ii) Most of the radioactivity which upon photolysis remained associated with the brush-border membrane vesicles ran ahead or closely with the tracking dye front. Earlier data obtained from labeled red cell membranes (6) suggest that by far the most of this radioactivity originated from labeled lipid. (iii) The radioactivity along the gel tracks (b and c) gave rise to more or less diffuse bands; some of them correlated with protein bands. (iv) As predicted for a peripheral protein, actin (band A), a component of the cytoplasmatic skeleton (15), was not significantly labeled. (v) Consistent with the protein patterns of the photolyzed samples, traces of radioactivity were found at the top of the gel. However, as indicated by the autoradiography of Fig. 1, the time course of labeling and that of formation of aggregated protein were different. By choosing short irradiation times, substantial protein labeling can be obtained whereas aggregation is negligible (lane b).

**Labeling of Sucrase-Isomaltase Is Confined to the Isomaltase Subunit—**There is compelling evidence that sucrase-isomaltase is bound to the membrane via the isomaltase subunit (4). This mode of anchoring should be reflected in the distribution of radioactivity among proteins of brush-border membranes labeled with $[^{125}]$TID. Brush-border membrane vesicles (2 mg of protein/ml; 50 mM Na-phosphate, pH 7.5) equilibrated with 10 uCi of $[^{125}]$TID (10 Ci/mmol) were irradiated for various lengths of time. Noncovalently bound radioactivity was removed by repeated extraction with buffer containing 1% bovine serum albumin. Samples were boiled in 2% SDS (2 min) and subjected to SDS-PAGE (8.4 x 2.7). To visualize the distribution of label in sucrase-isomaltase complex, labeled brush-border membranes were solubilized in 2% Triton X-100 and the enzyme complex was precipitated with anti-sucrase-isomaltase antibodies. Washed immunoprecipitates were denatured in SDS and run on the same gel. The gel was stained with Coomassie brilliant blue R-250 (lanes A to E) and the dried slab gel was autoradiographed (lanes a to e). Lanes A to C, brush-border membranes exposed to irradiation for 0 s (A), 15 s (B), and 120 s (C). Lane D, immunoprecipitate of sucrase-isomaltase from brush-border membranes labeled in the absence of glutathione. Lane E, immunoprecipitate of sucrase-isomaltase from brush-border membranes labeled in the presence of glutathione (20 mm). Lanes a to e represent the corresponding autoradiographs. The positions of the subunits of sucrase-isomaltase (f, isomaltase; S, sucrase) and A, actin, are indicated by arrows. The Coomassie blue-stained bands in the lower half of D and E originated from the antibodies.

![Fig. 1. Distribution of radiolabel among proteins of brush-border membranes labeled with $[^{125}]$TID. Brush-border membrane vesicles (2 mg of protein/ml; 50 mM Na-phosphate, pH 7.5) equilibrated with 10 uCi of $[^{125}]$TID (10 Ci/mmol) were irradiated for various lengths of time. Noncovalently bound radioactivity was removed by repeated extraction with buffer containing 1% bovine serum albumin. Samples were boiled in 2% SDS (2 min) and subjected to SDS-PAGE (8.4 x 2.7). To visualize the distribution of label in sucrase-isomaltase complex, labeled brush-border membranes were solubilized in 2% Triton X-100 and the enzyme complex was precipitated with anti-sucrase-isomaltase antibodies. Washed immunoprecipitates were denatured in SDS and run on the same gel. The gel was stained with Coomassie brilliant blue R-250 (lanes A to E) and the dried slab gel was autoradiographed (lanes a to e). Lanes A to C, brush-border membranes exposed to irradiation for 0 s (A), 15 s (B), and 120 s (C). Lane D, immunoprecipitate of sucrase-isomaltase from brush-border membranes labeled in the absence of glutathione. Lane E, immunoprecipitate of sucrase-isomaltase from brush-border membranes labeled in the presence of glutathione (20 mm). Lanes a to e represent the corresponding autoradiographs. The positions of the subunits of sucrase-isomaltase (f, isomaltase; S, sucrase) and A, actin, are indicated by arrows. The Coomassie blue-stained bands in the lower half of D and E originated from the antibodies.](http://www.jbc.org/)

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2 As suggested by Hjerten (33), the first figure in the parentheses indicates the total concentration of monomers (gram %, w/w) and the second that of N,N'-methylenebisacrylamide, as percentage of the total monomer concentration (w/w).
distribution of radiolabel among the subunits of sucrase-isomaltase complex which was determined as follows. Labeled brush-border membranes were solubilized in Triton X-100 and sucrase-isomaltase complex precipitated with anti-sucrase-isomaltase antisemum. The immunoprecipitate was denatured in 2% SDS (2 min, 100 °C) and the polypeptide chains were separated by SDS-PAGE. Protein patterns of such samples are shown in Fig. 1 (D and E, Coomassie blue-stained) together with the corresponding autoradiographies (d and e). Evidently, the isomaltase subunit (band I) had incorporated radioactivity whereas no radiolabel could be detected in the sucrase subunit. Some of the radioactivity migrated with the tracking dye front and might be due to labeled lipids that had not been extracted from the immunoprecipitates. In order to establish quantitatively the extent of labeling of sucrase-isomaltase, the corresponding bands were cut out of the Coomassie blue-stained gel and their radioactivity was determined by γ-counting. These data are reported in Table I. They show that labeling of whole brush-border membranes or of sucrase-isomaltase was the same whether or not glutathione had been present in the photolysis medium (see below). During the isolation of sucrase-isomaltase, approximately 0.2% of the radioactivity which was originally present remained associated with the immunoprecipitates. However, SDS denaturation further reduced the amount of radiolabeled associated with sucrase-isomaltase; thus, the isomaltase contained 0.012% of the initial amount of radiolabel and sucrase even less than 0.001%.

Is Labeling of the Isomaltase Subunit Confined to the Nonpolar Membranous Segment?—Evidence for labeling of the brush-border membranes from within the hydrophobic core came from labeling experiments in the presence and absence of glutathione. This water-soluble thiol is predicted to scavenge any carbene in the aqueous environment and hence to compete with peripheral proteins for reactive intermediates of the photolabel. However, as shown in Table I, the presence of glutathione did not reduce the extent of labeling, suggesting that labeling occurred at sites not accessible to the thiol and thus located within the lipid core of the membrane. Other evidence was provided by proteolysis experiments. [125I]TID-labeled brush-border membrane vesicles were subjected to papain digestion and solubilized sucrase-isomaltase complex which demonstrated that the radiolabel in the detergent-solubilized form was confined to the membranous anchor segment.

In both native brush-border membranes and in sucrase-isomaltase-egg PC proteoliposomes (3, 4), the mode of anchoring the enzyme complex to the membrane is very similar. Thus, papain digestion of the proteoliposomes releases more than 95% of enzyme (which is indistinguishable from sucrase-isomaltase solubilized by papain from brush-border membranes) leaving behind in the bilayer the hydrophobic anchor segment. This peptide is presumed to be well protected (shielded) against further proteolytic attack.

When sucrase-isomaltase proteoliposomes were labeled with [125I]TID, the radiolabel was also incorporated exclusively into the isomaltase subunit. That this modification of the protein occurred within the anchor segment was shown as follows. After the photolabeling procedure, the proteoliposomes were subjected to gel filtration on Bio-Gel A-5m in phosphate buffer containing 2% Na-cholate. This step separated the [125I]TID-labeled, enzymatically fully active protein from the bulk of lipid and from more than 99.8% of the radioactivity applied onto the column. The sucrase-isomaltase complex was then reincorporated into egg lecithin liposomes and the resulting proteoliposomes were either subjected to SDS-PAGE or treated with papain followed by a gel filtration in detergent-free buffer. This latter filtration (Bio-Gel A-5m; 50 mM Na-phosphate, pH 7.5) separated the liposomes containing the nonpolar anchor segment from the papain-solubilized sucrase-isomaltase complex. Fig. 2, (B and b) represents an SDS-polyacrylamide slab gel (B, Coomassie blue-stained; b, autoradiography) loaded with samples of undigested proteoliposomes (Fig. 2, T-I and T-S), and of papain-solubilized sucrase-isomaltase (P-I and P-S). Clearly, the radioactivity in the proteoliposomes was associated with the isomaltase subunit (T-I) only and papain treatment led to complete loss of the radiolabel in this subunit. Since papain solubilization removes the NH₂-terminal segment of the isomaltase subunit, the radioactivity in T-I must consequently be located in this anchor peptide.

Isolation of the Nonpolar Peptide—Labeling with [125I]TID of sucrase-isomaltase in its native membrane or, alternatively,

| Table I |
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| **Distribution of radiolabel during isolation of sucrase-isomaltase** |
| **Specific radioactivity in the photolysis sample** performed in the absence of glutathione (20 min). |
| Membranes<sup>a</sup> | 100 (18.7·10<sup>3</sup>)<sup>c</sup> | 100 (20.7·10<sup>3</sup>)<sup>c</sup> |
| Membranes after removal of nonbound radioactivity<sup>d</sup> | 64 (±5; n = 4) | 61 (±4; n = 4) |
| Solubilized sucrase-isomaltase<sup>e</sup> | 90 (±12; n = 4) | 74 (±13; n = 4) |
| Isomaltase in the immunoprecipitate<sup>f</sup> | 0.21 (±0.02; n = 4) | 0.19 (±0.03; n = 4) |
| After SDS-PAGE in the absence of glutathione | <0.001 | <0.001 |
| Sucrase subunit | 0.011 (±0.002; n = 2) | 0.010 (±0.003; n = 3) |
| Isomaltase subunit | 0.001 (±0.002; n = 2) | 0.010 (±0.003; n = 3) |

<sup>a</sup> At each step, the radioactivity was normalized for sucrose activity. The sucrose activity remained unchanged during photolysis and isolation.
<sup>b</sup> The membrane preparation contained approximately 1 mg of protein/ml. [125I]TID was added as an ethanolic solution. The final concentration of ethanol was approximately 1%.
<sup>c</sup> Numbers in parentheses, counts/min/unit.
<sup>d</sup> After photolysis for 60 s, the membranes were washed 4 times with a 10-fold volume of 50 mM Na-phosphate, pH 7.5, containing 1% bovine serum albumin.
<sup>e</sup> Membranes were solubilized in 2% Triton X-100 (30 min; 4 °C) and centrifuged for 10 min at 15,000 × g.
<sup>f</sup> Immunoprecipitates were washed 5 times with 150 mM NaCl, 10 mM Na-phosphate, pH 7.5, 1% bovine serum albumin, and twice with albumin-free saline.
The Membranous Segment of Sucrase-Isomaltase

reacted from within the micelles, all of the radioactivity was confined to the nonpolar segment as demonstrated by reconstitution and papain cleavage experiments (data not shown).

The procedure used to isolate the nonpolar anchor peptide included the following initial steps: (i) selective labeling of sucrose-isomaltase complex as described; (ii) reconstitution of the labeled protein into egg lecithin liposomes; and (iii) papain solubilization of sucrose-isomaltase followed by separation of the liposomes (containing the radioactively labeled anchor peptide) from the solubilized protein. Different methods have been used to separate the peptide from the phospholipid. One of them consisted of gel filtrations on Sephadex G-100 using buffers containing 1 to 3% Na-cholate. Fig. 3 shows radioactivity elution profiles obtained from such gel filtrations and demonstrates the effect of the cholate concentration on the elution behavior of the peptide. From these complex elution profiles, it is evident that the peptide did not exist as a single species but formed populations of particles with different sizes. The prominent peak (eluted at around 80 ml) in 1% Na-cholate decreased with increasing detergent concentration and a new peak at around Vₑ = 120 ml emerged. This suggested the existence of an equilibrium between different species of Na-cholate-peeptide complexes that exhibited distinct Stokes radii. The traces of sucrose-isomaltase eluted with the void volume (~60 ml) of the column (Fig. 3, pool I) and the phospholipids appeared as asymmetric peaks between 105 and 160 ml (indicated as pool III). Intermediate fractions (75 to 105 ml, pool II) contained the pure peptide complexed with Na-cholate. Using a detergent concentration of 1%, the yield of the sucrose- and lipid-free anchor peptide was estimated to be approximately 50% of that applied onto the column.

Alternatively, egg lecithin could be separated quantitatively from the peptide by gel filtration on Sephadex LH-60 using ethanol-formic acid-water as the eluant (16, 17). Liposomes containing the hydrophobic peptide (see above) were lyophilized, dissolved in the organic solvent mixture, and gel filtrated. The elution profile thereby obtained is depicted in Fig. 4. A major peak was eluted centered around an elution volume of 80 ml. When the pooled fractions (from 65 to 90 ml) were concentrated by rotary evaporation and subjected to a second gel filtration, a single, symmetrical peak was obtained again centered at an elution volume of 80 ml. Dried samples of the

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\text{Fig. 2. Distribution of radiolabel in [}^{125}\text{I]}\text{TID-labeled sucrose-isomaltase. TID-labeled and albumin-washed brush-border membrane vesicles were either solubilized by Triton X-100 or treated with papain. After centrifugation (30 min, 100,000 × g), sucrose-isomaltase complex was precipitated with anti-sucrase-isomaltase antisemur and the immunoprecipitates were washed with buffer (0.15 M NaCl, 10 mM Na-phosphate, pH 7.5) containing 1% Triton X-100. The precipitates were subjected to SDS-PAGE (8.4 × 2.7), A (left), immunoprecipitate from Triton X-100-solubilized sucrose-isomaltase (Coomassie blue-stained), A (right), immunoprecipitate from papain-solubilized sucrose-isomaltase (Coomassie blue-stained). b, autoradiography of the dried gel A. B (left), sucrose-isomaltase isolated from [}^{125}\text{I}]\text{TID-labeled proteoliposomes by gel filtration on Sephadex G-100 (corresponds to pool II of Fig. 3). B (right), sucrose-isomaltase solubilized from radiolabeled proteoliposomes by papain and separated from peptidoliposomes and papain by gel filtration on Bio-Gel A-5m (see "Materials and Methods," "Isolation of the Anchor Peptide of Sucrase-isomaltase"). b, autoradiography of the dried gel B. Each gel contained identical amounts (sucrase activity) of detergent- and papain-solubilized sucrose-isomaltase. Positions of the subunits of detergent (T-I and T-S) and papain-solubilized (P-S and P-I) sucrose-isomaltase complex are marked by arrows. TD, tracking dye front. The heavily stained (Coomassie blue) bands in A are due to immunoglobulins. P-S and P-I, the sucrase and isomaltase subunit, respectively, of papain-solubilized sucrose-isomaltase complex; T-S, Triton-sucrase, and T-I, Triton-isomaltase subunits, respectively, as obtained from Triton-solubilized sucrose-isomaltase complex.
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pooled radioactive material formed greasy films on glass walls; the material was insoluble in chloroform but was soluble in ethanol-formic acid, in formic acid alone, in 2-chloroethanol-H₂O (9:1), or in buffers containing SDS. An NH₂-terminal amino acid analysis by the dansylation method gave a single amino acid, alanine. As this amino acid is known to be the NH₂ terminus of the isomaltase subunit (4) and hence of the hydrophobic region (4), this result suggested that papain digestion did not affect the NH₂-terminal segment of the anchor peptide.

**Molecular Weight of the Hydrophobic Peptide**—In an attempt to estimate the molecular weight of the nonpolar peptide, a column of Sephadex LH-60 (1.5 x 95 cm) was calibrated with molecular weight standard peptides (proteins). That a useful correlation exists between the elution volume of a peptide and its molecular weight has been demonstrated recently (17). The calibration curve thereby obtained is shown in Fig. 4. Based on these data, the molecular weight of the peptide was estimated to be approximately 6500. SDS-PAGE (Fig. 5) of the hydrophobic peptide which prior to dissolving in SDS had been passed through a column of Sephadex LH-60 in the organic solvent described above gave \( M_s \approx 8000 \). The peptide, which could hardly be detected by Coomassie blue staining, appeared as a quite homogeneous species as visualized by the silver stain technique (14) or by autoradiography after drying the gel. The peptide did not give rise to aggregates of different molecular weights as was presumably the case during gel filtration in Na-cholate solution (Fig. 3). Since the peptide most likely contains (a) sugar residue(s) at threonine 11 (5), the estimated molecular weight might require a limited correction.

**Fig. 4.** Gel filtration on Sephadex LH-60. \([\text{[}^{125}\text{I}]\)TID-labeled sucrase-isomaltase was reconstituted into egg PC-sucrase-isomaltase proteoliposomes and digested with papain. After separation of the solubilized sucrase-isomaltase, liposomes containing the peptide were gel filtrated on a Sephadex LH-60 column (1.5 x 95 cm; flow rate, 3.6 ml/h) which was equilibrated and eluted with ethanol-formic acid (88%), 7:3 (v/v). The anchor peptide was eluted at around 80 ml (egg lecithin was eluted at around 130 ml). The column was calibrated for molecular weight determinations.

**Fig. 5.** SDS-PAGE of \([\text{[}^{125}\text{I}]\)TID-labeled anchor peptide. Peptide isolated by gel filtration on Sephadex LH-60 in ethanol-formic acid was supplemented with aqueous SDS and the organic solvent was removed by Rotavap evaporation. The final concentration of SDS was adjusted to 2%. Aliquots of the solution (corresponding to approximately 0.2 nmol of peptide) were boiled for 2 min and electrophoresed on a slab gel (15 x 2.7).\(^2\) A, stained with Coomassie blue; B, silver stained; C, autoradiography of A; M, molecular weight standard proteins.

**Fig. 6.** Circular dichroism of the hydrophobic anchor peptide. A, peptide incorporated in bilayers of egg PC liposomes. For comparison, the CD spectrum calculated for 80% a-helix and 20% random coil structure based on data of Greenfield and Fasman (18) is shown (filled circles). B, peptide solubilized in 0.5% (w/v) Na-cholate. C, peptide in 2-chloroethanol-water, 9:1 (v/v). The peptide was transferred from ethanol-formic acid into chloroethanol-water by gel filtration on Sephadex LH-20 equilibrated and eluted with 2-chloroethanol. D, peptide in 2% SDS.
**Circular Dichroism**—Analyses of the spectra using a number of different methods and reference data (18-21) gave consistent results. Accordingly, the content of the α-helix structure ranges between 75 and 85% when the peptide was embedded in egg PC bilayers (Fig. 6A) or in cholate micelles (Fig. 6B). The close similarity of spectra A and B is consistent with the idea that Na-cholate is a mild detergent which does not extensively denature membrane proteins (indeed, it is widely used to solubilize and reconstitute functionally intact membrane proteins) and may indicate that solubilization and reconstitution of the peptide did not substantially affect the native structure of the peptide.

Although the accuracy of our calculations was limited by some uncertainty in the molecular weight determination of the peptide (the figure used was 6500), the predominance of the α-helical structure is unquestionable. It is also supported by the shape of the curves and the crossover wavelength of 201.5 nm.

The spectra of the peptide dissolved in 2-chloroethanol (C) or in 2% SDS (D) show even lower molar ellipticities around 208 nm, suggesting an even higher percentage of α-helix (nearly 100%). This observation agrees well with earlier CD studies demonstrating that 2-chloroethanol and SDS are likely to promote helix structure of proteins (22-24).

**DISCUSSION**

It is generally assumed that the folding of membranous hydrophobic proteins is such that amino acid residues exposed to the lipid core of the bilayer are predominantly hydrophobic. For instance, the membrane-associated segment of sucrose-isomaltase contains a succession (residues 12 to 31 (5)) that is likely to form a highly nonpolar and inert surface. Chemical modification of such areas obviously requires extraordinarily reactive reagents capable of inserting even into C-H bonds of aliphatic residues. Toward this goal, we therefore examined a photoactivatable reagent which generates the highly reactive species, 3-trifluoromethyl-3-(m-iodophenyl)carbene. The stable precursor, TID, is very hydrophobic and thus partitions to a high extent into the lipid core of a membrane (6). This distribution of the precursor as well as the likely absence of any long-lived reactive intermediate during photolysis provides the basis for the selectivity of the reagent in labeling intrinsic versus peripheral proteins.

The present study further characterizes TID as a hydrophobic labeling reagent and demonstrates that labeling of sucrose-isomaltase complex is restricted to that domain which is known from other evidence to be responsible for anchoring the enzyme complex to the membrane. Although it is still not known which sites of the polypeptide chain have been derivatized, our data show that labeling occurred within the segment in contact with the lipid core of the bilayer. In fact, labeling of known peripheral proteins is extremely low and this must be the case also for peptide segments of integral proteins that are exposed to the aqueous environment. Furthermore, addition of reduced glutathione to the buffer did not affect the extent of labeling of either brush-border membrane vesicles or of isomaltase. The present data are consistent with the labeling results obtained earlier on human erythrocyte membranes (6). In that study, it was found that spectrin, the major peripheral protein, incorporated extremely little (less than 0.01%) of the radioactivity which was originally present in the incubation medium. More sensitive measurements (this paper) indicate that the actual labeling of peripheral proteins may even be somewhat lower. Indeed, sucrose, which does not interact directly with the membrane fabric but was insoluble in chloroform-methanol (2:1, v/v) in which other hydrophobic peptides including the anchor segment of small intestinal brush-border aminopeptidase were reported to be soluble (28). Since threonine at position 11 appears to be glycosylated (5), the insolubility of the peptide in chloroform-methanol may not be surprising. Furthermore, recent sequence data have revealed a cluster of positively charged amino acids at the NH2 terminus. The
sequence of the NH2-terminal region of the rabbit intestinal isomaltase having a high degree of homology with that of other species (5) is reproduced below:

CHO

Ala-Lys-Arg-Lys-Phe-Ser-Gly-Leu-Glu-Ile-Thr-Leu-
1
Ile-Val-Leu-Phe-Leu-Ile-Val-Phe-Leu-Ala-Ala-
20
Leu-Ile-Val-Leu-Ala-x-x-Pro-
30
35

Thus, the likely presence of a sugar residue and of a cluster of charged amino acid residues implies a structure of the peptide with a pronounced amphipathic character.

In contrast to gel filtration in organic solvents, the data in Fig. 3 indicate that, in aqueous Na-cholate, the peptide exists as particles of various Stokes radii (mixed micelles). Gel filtration in detergent solutions represents a mild procedure that may be useful for separating amphipathic and hydrophobic peptides. However, as these peptides form complexes with the detergent, separation of peptides on the basis of their size might be less effective than gel filtration in organic solvents.

Information concerning the membranous segment of small intestinal sucrase-isomaltase was derived mainly from NH2-terminal sequence data of the isomaltase subunit (5). The present study provides additional data concerning size and structure of this peptide. The accuracy of molecular weight analyses of small oligopeptides by SDS-PAGE is limited by the finding that the intrinsic charge and shape of small peptides may be more important in determining their mobility in SDS gels than with larger proteins (30). In addition, hydrophobic and amphipathic peptides may be in an aggregated state, or complex formation with SDS may not be complete throughout the entire polypeptide chain. In this work, SDS-PAGE and gel filtration on Sephadex LH-60 was used to estimate the molecular weight of the peptide. Original data from Khorana's laboratory (17) and our own results have demonstrated that a useful correlation exists between the molecular weight of an oligopeptide and its elution volume from a column of Sephadex LH-60. Based on the calibration curve, M ≈ 6500 was determined for the peptide. This corresponds to approximately 60 amino acid residues. The molecular weight derived from electrophoresis was somewhat larger, approximately 8000. Dansylation of the peptide followed by acid hydrolysis gave a single NH2-terminal amino acid, alanine. This suggests that papain digestion did not affect the NH2 terminus of the anchor peptide during cleavage of the isomaltase. It is possible however, that papain cleavage produced a microheterogeneity at the COOH terminus of the anchor. In fact, papain is known not to cleave at a single site, as may be concluded from the heterogeneity of the NH2 terminus of the isomaltase subunit derived from papain-solubilized sucrase-isomaltase (4) (as expected from the broad substrate specificity of this protease).

So far, it has not been possible to degrade the peptide by proteolysis in aqueous Triton X-100 solution (proteases examined were chymotrypsin, trypsin, pronase, and carboxypeptidase Y). Likewise, attempts to determine the amino acid composition of the peptide following acid hydrolysis (6 M HCl; 100 °C, 12 to 72 h) gave results which are not consistent with the amino acid composition derived from the partial sequence known. These results suggest that the peptide is extremely resistant to hydrolysis in aqueous hydrochloric acid, probably due to unsatisfactory solubility and solvation of the peptide.

The most prominent feature of the membranous segment is the continuous hydrophobic stretch that is at least 20 amino acid residues long (residues 12 to 31; Ref. 5). Hydrophobic segments of this size and of high hydrophobicity have been found in various membrane proteins and in a few cases evidence was provided that this stretch occurs in those parts of the molecules known to span the membrane (31, 43). Thus, it is reasonable to suggest that isomaltase, too, is a transmembranous protein. Support of this idea came from circular dichroism of the peptide in a lipid bilayer that suggested that the peptide has a high content of α-helical structure: assuming that the lipid bilayer in a biological membrane is approximately 3.5 nm in thickness, an α-helical chain of 23 amino acids would be needed to traverse the membrane. Apparently, there is good agreement between this model of a transmembrane segment (which does satisfy hydrogen-bonding requirements of the polypeptide backbone in a hydrophobic environment) and the length of the hydrophobic segments in the isomaltase and in various spanning regions of other membrane proteins (31).

Current ideas of the biosynthesis and glycosylation of membrane proteins have led to the conclusion that threonine 11, which in all likelihood is glycosylated, must reside on the extracellular (luminal) side of the membrane (32), and, hence, on the same side as the COOH terminus (4). Consequently, the chain must cross the membrane an even number of times. The present data on the molecular weight of the peptide (corresponding to approximately 60 amino acid residues) imply that the peptide spans the membrane twice. The most probable structure and positioning of the anchor polypeptide chain, accommodating all observations available, is thus the following: threonine 11 is located at or near the luminal surface of the membrane; the hydrophobic stretch (residues 12 to 31) has α-helical conformation and crosses the membrane in an N-out, C-in direction; a relatively short segment (hydrophilic? β-turn?) follows, encompassing the proline residues at position 35 (6) and probably another proline at position 40 (7) and making a loop; finally, one more hydrophobic α-helix (the α-helix content of the peptide is at least 75%) would cross the membrane in the opposite direction (i.e. in a N-in, C-out direction). If sucrase-isomaltase (or rather, pro-sucrase-isomaltase (34–36)) is synthesized and inserted as predicted by the "helical hairpin hypothesis" (Ref. 37; see also Refs. 38–40), this proposed structure of the anchor peptide would correspond to that of two half-hairpins and would imply that two helical hairpins are formed and inserted during biosynthesis (41).}

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