Topography of Cerebroside Sulfotransferase in Golgi-enriched Vesicles from Rat Brain

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ABSTRACT Cerebroside sulfotransferase (CST) catalyzes the final step in the synthesis of sulfatide (sulfogalactocerebroside) by transferring the sulfate from 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to galactocerebroside. Orientation of CST was studied in vesicles enriched in this enzyme obtained from 21-d-old rat brain. Several lines of evidence indicate that CST is located on the luminal side of these vesicles. (a) Sulfation of endogenous galactocerebroside occurred in vesicles only in the presence of a detergent to render the membranes permeable to exogenous PAPS. (b) There is a pool of latent enzyme within the vesicle, which is released by Triton X-100. (c) CST is not destroyed by trypsin unless the vesicle membranes are first made permeable by Triton X-100. (d) Glycolipid substrate, when covalently attached to agarose beads, was not sulfated unless the enzyme was solubilized. These results are similar to those obtained with thiamine pyrophosphatase, which is known to be located within the lumen of the vesicles.

This study establishes that an enzyme synthesizing a complex glycolipid is localized within Golgi-enriched vesicles. Since the product of the CST reaction must also be localized to the luminal side of the vesicles, it is most likely that sulfatide is located at the intraperiod line (outer layer) of myelin. The orientation of CST within the vesicle provides a mechanism for the asymmetrical assembly of glycolipids in bilayers.

Sulfatide (galactocerebroside 3-sulfate) is a major glycolipid of the myelin sheath (1). Although clearly an important component, its function in this membrane is uncertain. Based on its ionic interaction with myelin basic protein in vitro, it has been implicated as playing a role in maintaining the integrity of myelin (2–5). Recent studies by Omlin et al. (6) have confirmed the original studies of Herndon et al. (7) that myelin basic protein is located at the cytoplasmic surface (major dense line) of myelin. From this it might be inferred that sulfatide, if it interacts with myelin basic protein, would have a similar location. However, among other postulated roles for sulfatide are that it is part of the Na+/K+ ATPase molecule, where it is involved in transport (8), and that it acts as a receptor for opiates (9–11). These properties would require sulfatide to be on the external surface of the plasma membrane. Immunocytochemical studies with antibodies against both sulfatide and its synthetic enzyme, cerebroside sulfotransferase (CST)[1], located these to the brush border of renal tubules (12, 13). A clearer knowledge of the location of

sulfatide in the membrane would aid in determining its biological role.

Sulfatide is an anionic glycolipid that in biological membranes contains tightly bound water molecules (14), and therefore it is unlikely that it could readily undergo "flip-flop" movement in the lipid bilayer (15, 16). Thus sulfatide should be found in the same location as the enzyme catalyzing the final step in its synthesis. This step, the sulfation of cerebroside, is catalyzed by the enzyme galactocerebroside sulfotransferase (EC 2.8.2.11), which is found in the Golgi-rich fraction of both kidney and central nervous system (17–19). In vesicles prepared from the Golgi apparatus from the liver and kidney, enzymes involved in the synthesis of "complex" carbohydrates have been shown to be oriented towards the luminal (cisternal) side of the vesicles, while other enzymes such as 5'-nucleotidase, fatty acid-CoA ligase, glycerol 3-phosphate acyltransferase, and lysophosphatidic acid acyltransferase are located on the cytoplasmic side of the vesicles (20–23).

In this communication, we describe the use of the strategies employed for the analysis of the orientation of enzymes involved in the formation of phospholipids and complex carbohydrates (20, 22, 23), to show that CST is localized on

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[1] *Abbreviations used in this paper:* CST, cerebroside sulfotransferase; PAPS, 3'-phosphoadenosine 5'-phosphosulfate.

the luminal side of intact vesicles enriched in Golgi enzyme "markers." This suggests that the product sulfatide will also be on the luminal side of the Golgi apparatus and thus it is most likely to be located on the outer half (intraperiod line) of the myelin lipid bilayer.

MATERIALS AND METHODS

Materials

Sprague-Dawley rats (Charles River Farms, Boston, MA) were obtained between 10 and 15 d postnatal. Phosphoadenosine phospho[^{35}S]sulfate was obtained from New England Nuclear (Boston, MA); galactocerebroside was obtained from Supelco, Inc. (Bellefonte, PA), and 2-N-morpholinoethane sulfonic acid, Trizma base, glycine, acetyltrypsin, trypsin, citric acid, and ammonium molybdate were obtained from Sigma Chemical Co. (St. Louis, MO). Thiamine pyrophosphate was obtained from Calbiochem-Behring Corp. (San Diego, CA) and recrystallized from acetone/methanol. Sucrose was obtained from Fisher Scientific Co. (Fair Lawn, NJ). All solvents were reagent grade.

Preparation of Vesicle Fraction from Rat Central Nervous System

Forebrains from 5-10 rats of about 21 d of age were removed, washed, and then minced at 4°C with the use of a razor. This tissue was then homogenized in 4 volumes of 0.32 M sucrose and the homogenate was centrifuged in a Sorvall RC 2B (DuPont Instruments-Sorvall Biomedical Div., Wilmington, DE) at 800 g for 10 min at 4°C. The supernatant fraction was saved and the pellet was rehomogenized in 4 volumes of 0.32 M sucrose and recentrifuged at 800 g for an additional 10 min. The supernatant fractions from both centrifugations were combined, and recentrifuged in a Sorvall RC 2B at 6,000 g for 20 min. The resulting supernatant fraction was placed in a polyallomer tube (1 × 3½ inches) containing a 6.0-ml cushion of 1.6 M sucrose. These tubes were centrifuged for 60 min at 100,000 g in a Beckman ultracentrifuge LS 2B (Beckman Instruments, Inc. Spinco Div., Palo Alto, CA). The material at the interface was collected and used for the subsequent studies (vesicle preparation).

Preparation of the Vesicles for Electron Microscopy (24)

The subcellular fraction was collected by centrifugation in a Beckman microfuge B and resuspended in a cold solution of 1% paraformaldehyde and 1.25% glutaraldehyde in phosphate buffer. After fixation for 24 h at 4°C, the fractions were sedimented by centrifugation, postfixed in osmium tetroxide, dehydrated in graded ethanol solutions, cleared with propylene oxide, and infiltrated with epoxy resin. Thin sections were stained by flotation on uranyl acetate and lead citrate solutions and examined with a Hitachi 600+1 electron-microscope.

Assay for Cerebroside Sulfotransferase (CST) (13)

The incubation mixture contained the following components in a volume of 100 μl: 100 mM 2[N-morpholino]ethane sulfonic acid (pH 6.2), 20 mM MgCl₂, 4 mM dithiothreitol, 2.5 mM ATP, 2.6 × 10^5 dpm of [^{35}S]3'-phosphoadenosine 5'-phosphosulfate (PAPS) (1.0-2.8 mCi/mmol), 8 μl of galactocerebroside (final concentration, 80 μM), and 40 μl of enzyme preparation (containing 100-150 μg of protein). The mixture was incubated at 37°C for the times specified. The reaction was stopped by addition of chloroform/methanol, 1:1 (vol/vol) followed by lipid extraction (11, 25), and the radioactivity in the sulfatide was determined.

Assay for Thiamine Pyrophosphatase

The vesicle fraction was mixed with 20 mM Tris-HCl (pH 8.0) and 11% sucrose (final concentration). The thiamine pyrophosphatase (TPPase) assay was conducted as described by Allen and Slater (26, 27). The incubation mixture contained 33 mM sodium barbital (pH 9.5), 15 mM calcium chloride, and 3.3 mM recrystallized thiamine pyrophosphate. The reaction was started by the addition of 100 μl of the fraction to be analyzed (containing 0.2-0.8 mg of protein), and the mixtures were incubated at 37°C for 5, 10, 15, 20, 45, and 60 min. The reaction was stopped by the addition of trichloroacetic acid (final concentration 5% wt/vol). The protein was centrifuged at 3,000 rpm for 15

min at 4°C in a Sorvall RC 2B and phosphate was determined in the supernatant fraction, as described below.

Phosphate was measured as described by Heinonen and Lahti (28). Stock solutions of 10 mM ammonium molybdate, 1 M citric acid, and 5 N sulfuric acid were prepared. Before each assay a fresh mixture of acid-acetone-molybdate was made (acetone/sulfuric acid/ammonium molybdate 2:1:1 [vol/vol]). The assay mixture contained 0.5 ml of the sample, 100 μl of 10% sodium dodecyl sulfate, 4.0 ml of acid-acetone-molybdate mixture, and 0.4 ml of 1 M citric acid that was added last to complex with unreacted molybdate. The mixture was treated with a Vortex mixer for 15 s and the optical density was read at 355 nm. The standard curve for the phosphate determinations was obtained with monobasic potassium phosphate (phosphate concentrations from 20-1,500 nmol of inorganic phosphate).

Sulfation of Galactosylsphingosine Linked to Agarose Beads

To prepare the derivatized beads, Affi-Gel 10 (Bio-Rad Laboratories, Richmond, CA) was washed four times with 4 volumes of cold isopropanol (4°C) followed by three washings with 3 volumes of dimethyl sulfoxide (DMSO). To this resin was added 25 mg of galactosylsphingosine (Sigma Chemical Co.) containing 3-6 × 10^5 cpm of [^{3}H]galactosylsphingosine (kindly provided by Dr. Yasuo Kishimoto, Kennedy Institute, Johns Hopkins School of Medicine) dissolved in 4.0 ml of DMSO. This mixture was stirred gently for 16-20 h at room temperature. The resin was washed three times with 3 volumes of DMSO. Subsequently, the resin incubated with 2 volumes of 1.0 M 2-aminoethanol, pH 8.0, in water for 4 h at room temperature to block any remaining active sites. The resin was then washed extensively with 0.05 M sodium phosphate buffer, pH 7.0, containing 0.5 M NaCl. Based on the amount of [^{3}H]galactosylsphingosine remaining, the amount bound to the resin was 10-14 μmol/ml of resin.

For the sulfation studies, the glycolipid-coupled resin was first equilibrated in 100 mM 2-N-morpholinoethane sulfonic acid buffer, pH 6.2, containing 0.32 M sucrose, 80 mM MgCl₂, 2 mM mercaptoethanol, and 0.25 mM galactose. To this resin were added 100 μl of incubation buffer, 4 μl of [^{35}S]-PAPS, and 100 μl of an intact vesicle preparation or solubilized enzyme, and the mixtures were incubated for 10, 15, 20, and 45 min. The beads were removed by centrifugation, washed, and the radioactivity incorporated was determined by standard procedures.

Orientation of Thiamine Pyrophosphatase and CST in Isolated Vesicles

Thiamine Pyrophosphatase: The thiamine pyrophosphatase assay described above was carried out in the presence of the nonionic detergent Triton X-100 (0.05-1.0% [wt/vol] final concentration), or an equal volume of 0.32 M sucrose. Activity in the presence of the nonionic detergent as compared with the control gave an indication of the "latent" thiamine pyrophosphatase (22).

For the protease inactivation studies, trypsin or acetylated trypsin was used. The activity of these proteases was determined by their effect on α-N-benzoyl-L-arginine ethyl ester. The trypsin preparation contained 13,200 U/mg while the acetylated trypsin preparation contained 16,800 U/mg. The trypsin and acetylated trypsin were used as 1 mg/ml solutions in 0.9% NaCl. The vesicle fractions (90-400 μl) were incubated in the presence or absence of Triton X-100 (0.15% wt/vol) with 0.1 M Tris-HCl (pH 8.0) and trypsin or acetyltrypsin (ratio of trypsin to protein, 1:40). Incubations were carried out at room temperature, 30°C, and 37°C for 15, 20, 30, and 45 min. The reaction was stopped by the addition of soybean trypsin inhibitor (10 times the amount of trypsin added), after which aliquots were taken for thiamine pyrophosphatase assay.

Orientation of CST: Latency and accessibility of [^{35}S]PAPS to CST were determined by incubating intact vesicles with [^{35}S]PAPS for 15, 30, and 60 min in the absence of exogenous acceptor galactocerebroside. Another set of vesicles was treated similarly but with the addition of Triton X-100 (0.2% wt/vol) to determine sulfation of endogenous substrate. "Latency" was assessed by treatment of the enzyme with Triton X-100 and determining the sulfation of exogenous substrate, as described for TPPase. The susceptibility of CST to protease digestion was also carried out as indicated for TPPase.

Protein Determination

The proteins were assayed by the method of Lowry et al. (29) with bovine serum albumin as standard.
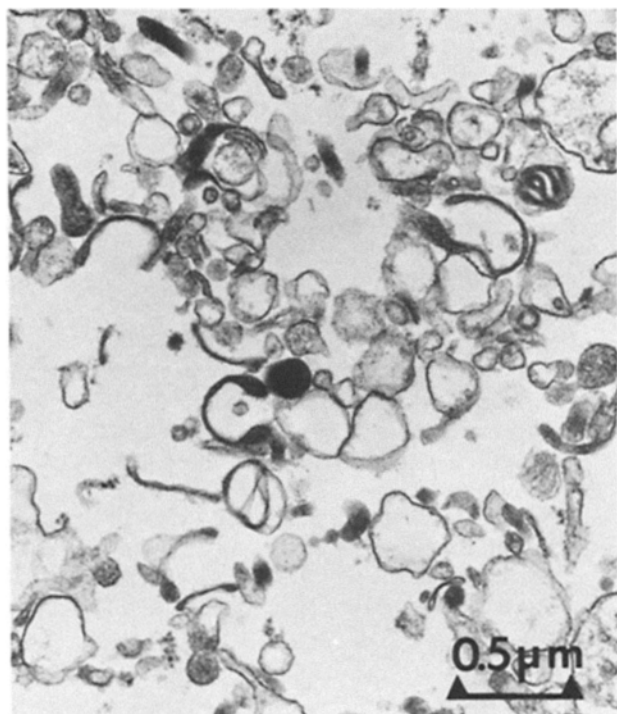
Figure 1   Electron micrograph of Golgi-enriched vesicles obtained from 21-d-old rat brain. The fractions were fixed, dehydrated, and then embedded as described in the text. × 35,000.

RESULTS

Characterization of the Vesicular Fraction

In order to maintain the integrity of the vesicles, the fractions were centrifuged onto a cushion of 1.6 M sucrose. The material that banded at the interface was examined by electron microscopy. Fig. 1 shows a typical example of the fractions examined. Most of the organelles were smooth vesicles measuring 0.1–0.5 μm in diameter with some profiles having a barbell shape. These structures are characteristic of the Golgi apparatus in isolated fractions (30–32). Very few mitochondria, lysosomes, or polysomes were present and no myelin was seen. Quantitation (by counting) showed that almost 80% of these observed structures were smooth vesicles. Furthermore, biochemical characterization (Table I) showed that the specific activity of thiamine pyrophosphatase in the isolated fraction was increased by about twofold over the activity in the homogenate. Similar results were obtained for cerebroside sulfotransferase. Both these enzymes are Golgi markers.[2] Thus the vesicle preparation was enriched in Golgi markers and could be used to study the topography of TPPase and CST.

Determination of Thiamine Pyrophosphatase in the Vesicles

In order to determine the activity of TPPase, a suitable assay for quantitating the amount of phosphate was required. Of the many assays available, the assay described by Heinonen and Lahti (28) was the most suitable for our purposes. Ini-

[2] UDP-galactose:N-acetylglucosamine galactosyltransferase, another Golgi marker, was enriched twofold in our vesicle fraction, but the activity (1.5 nmol/h/mg protein) was too low for meaningful studies on orientation.

tially, the blank values were high, due in part to interfering substances in the substrate thiamine pyrophosphate and to a lesser extent in the vesicle preparation itself. Recrystallizing the thiamine pyrophosphate reduced the blank value by 30%. Turbidity of the supernatant solution after precipitating the proteins with trichloracetic acid was decreased substantially by the addition of 100 μl of 10% sodium dodecyl sulfate. The standard curve obtained was linear from 20 to 1,500 nmol of phosphorous with duplicate samples giving comparable values. Known amounts of phosphorous could be added to our vesicle fractions, and recovered quantitatively.

With use of this assay, TPPase was measured in the vesicle fraction as described under Materials and Methods. The reaction was linear with time for 15 min. All incubations were carried out for 5, 10, 15 and 20 min, but for comparing activity in different preparations, the 10-min value was used. Comparison of activity of TPPase in vesicle fractions in the absence or presence of Triton X-100, showed that samples containing Triton X-100 had two- to threefold increases in specific activity of the enzyme (Table II). According to Hirschberg (22), such an increase in enzyme activity is due to the "latent" enzyme pool. This suggests that the isolated vesicles were not only intact, but also that they had the same orientation as was found in the intact cell.

In order to ensure that this "latent" pool of enzyme was thiamine pyrophosphatase and not nucleoside diphosphatase, we measured activity in the presence of cysteine hydrochloride (10–50 mM), since nucleoside diphosphatase is inhibited by this amino acid (27). In the concentration range used, no inhibition of the reaction was observed (Table II), indicating that we were indeed measuring thiamine pyrophosphatase.

Table I

Thiamine Pyrophosphatase and Cerebroside Sulfotransferase Activities in the Vesicles Obtained from 21-d-old Rat Brain

Preparation	Thiamine pyrophosphatase	Cerebroside sulfotransferase
	nmol/min/mg protein	dpm/min/mg protein
Homogenate	10	67
Vesicles	20	370
Solubilized enzyme (1% Triton X-100)	50	1,500

Enzyme activities were measured as described under Materials and Methods. The values given are the mean of three separate preparations.

Table II

Activity of Thiamine Pyrophosphatase

Assay conditions	Activity of TPPase
	nmol/min/mg protein
Homogenate	
Minus Triton X-100	10
Plus Triton X-100 (0.2%)	20
Vesicle Fraction	
Minus Triton X-100	20
Plus Triton X-100 (0.2%)	50
Plus Triton X-100 (0.2%) and cysteine (10–50 mM)	52

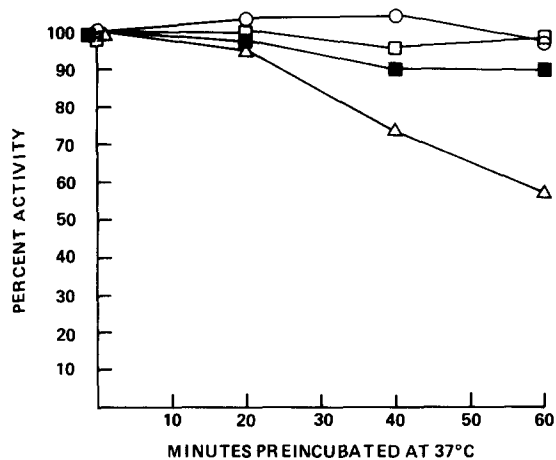Conditions are described in the text. All experiments were performed on freshly prepared vesicles.

FIGURE 2 Golgi-enriched vesicle fractions were either preincubated with trypsin, Triton X-100 only, or trypsin and Triton X-100 at 37°C for the time indicated. Control samples were vesicle fractions preincubated in buffer for the same times. At each time point aliquots were removed, soybean trypsin inhibitor was added, and thiamine pyrophosphatase was determined as described in the text. ○, intact vesicles only; □, intact vesicles + trypsin; ■, intact vesicles + Triton; △, intact vesicles + trypsin + Triton.

To confirm the topography of TPPase, the effect of proteases on these vesicles was determined (Fig. 2). Incubation of intact vesicles with trypsin or acetyltrypsin[3] at room temperature, 30°C, and 37°C did not affect TPPase activity (Fig. 2). To ensure that sufficient trypsin was present, the protein to trypsin ratio was varied from 5:1 to 40:1 and at none of these concentrations of the protease was there any degradation of TPPase. However, if the vesicles were first made permeable with Triton X-100 and trypsin was then added, inactivation of luminal TPPase was observed (Fig. 2). The optimal concentration of Triton X-100 was in the range 0.15% to 0.5%. At 1.0% Triton X-100 there was spontaneous inactivation of TPPase, perhaps due to the release of lysosomal hydrolases. Even in intact vesicles made permeable with Triton X-100, the addition of trypsin did not lead to 100% inactivation. A maximum of 60% inactivation was observed. Moreover, in attempts at solubilizing TPPase with 1% Triton X-100, only 40% of the total activity was measured in the supernatant fraction, while the remainder of the activity was found in the pellet. Our data indicate, therefore, that both soluble and membrane-bound TPPase are inactivated by protease.

Orientation of CST

As the vesicles were intact, sulfation of endogenous galactocerebroside was determined (Table III). The "active" sulfate donor PAPS, like other nucleotides, is unable to cross membranes. In the presence of [35S]PAPS, no sulfation of endogenous acceptor galactocerebroside was detected unless the vesicles were first made permeable with Triton X-100 (Table III). Thus both the acceptor lipid galactocerebroside and the sulfotransferase appear to be localized to the luminal side of the vesicles. This agrees with the previous observation (18) that UDP-galactose:ceramide galactosyltransferase and CST colocalize to the same Golgi fractions.

Further support for this localization of CST was provided by the observation that Triton X-100-solubilized enzyme

[3] Trypsin and acetyltrypsin gave similar results and either could be used for these studies.

showed a 15-fold increase in the sulfation of exogenous galactocerebroside activity (Table II). Thus like TPPase, CST is also "latent." This observation taken in conjunction with the susceptibility of CST to proteases (trypsin or acetyltrypsin) only in the presence of Triton X-100 (Fig. 3), suggests that CST is located in the luminal aspect of the vesicles. Furthermore, trypsin plus Triton X-100 resulted in only partial inactivation of CST.

To determine whether only the solubilized enzyme was inactivated, we examined the effect of various concentrations of Triton X-100 on the vesicles in the presence of a fixed amount of trypsin. We found that both soluble and membrane-bound forms of CST were inactivated in the presence of Triton X-100 at concentrations from 0.05%–2% (at the following concentrations of Triton X-100—0.05, 0.1, and 0.2%—in the presence of trypsin, the inactivation of soluble CST was 35, 51, and 77%, while inactivation of membrane-bound CST was 65, 49, and 23%).

Further evidence for the luminal localization of CST was provided by attaching galactosylsphingosine covalently to agarose beads and examining its sulfation by intact vesicles and by solubilized enzyme. With intact vesicles no sulfation was

TABLE III

Activity of Cerebroside Sulfotransferase in Golgi Vesicles

Conditions	Cerebroside sulfotransferase activity
	dpm/min/mg protein
Vesicles + PAPS	16
Vesicles + PAPS + 50 mM NaF	10
Vesicles + PAPS + 0.2% Triton X-100	100
Vesicles + PAPS + 50 mM NaF + 0.2% Triton X-100	116

Vesicles were prepared as described under Materials and Methods. Mixtures contained the vesicles (about 100 μg of protein) in 0.32 M sucrose, 100 mM imidazole, pH 6.2, 20 mM MgCl₂, 4 mM dithiothreitol, and 47 μM PAPS (specific activity, 1 mCi/mmol) in 100 μl. NaF and/or Triton X-100 were included as indicated. The mixtures were incubated at 37°C for 7 min, since with the nonextracted enzyme the reaction is linear for about the first 10 min. Each value is the mean of three separate determinations. NaF was added to prevent degradation of PAPS by any phosphatases in the vesicle preparation.
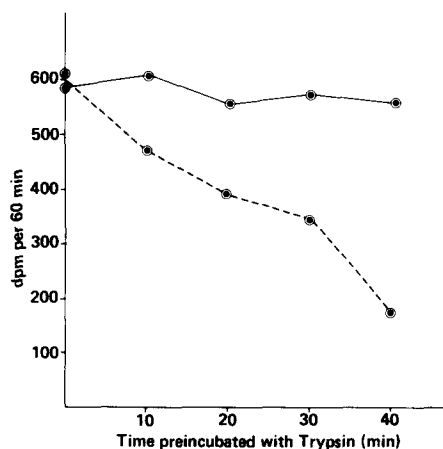

FIGURE 3 Golgi-enriched vesicles were pretreated with trypsin at room temperature for the indicated periods of time, in the presence (○- - -○) or absence (○———○) of Triton X-100. Aliquots were removed, soybean trypsin inhibitor was added, and cerebroside sulfotransferase was determined as described in the text.

detected in the presence of Triton X-100 up to 0.2%. With solubilized enzyme, however, sulfation of psychosine beads was observed (650 dpm/min/mg of protein); with boiled enzyme, sulfation was negligible.

DISCUSSION

Knowledge of the location of sulfatide in myelin and plasma membranes would aid immeasurably in specifying the functions of this complex sulfolipid. In the absence of a suitable marker for sulfatide in the central nervous system, we took advantage of the fact that the synthetic enzyme CST is located in the Golgi-rich fraction in the CNS (18, 19). Previous studies had shown that by analyzing Golgi vesicles for the location of the enzymes involved in the formation of complex carbohydrates, it was possible to predict the orientation of the glycoprotein products in the plasma membrane (21–23, 33). This orientation was subsequently confirmed by other methods. To apply these techniques in our studies it was necessary to prepare intact microsomal vesicles enriched in Golgi markers, such as thiamine pyrophosphatase, and in CST (Table I). The orientation of CST in these vesicles should allow predictions to be made about the location of the sulfatide product within these vesicles, provided that the isolated vesicles were intact and impermeable to hydrolytic enzymes and that the topography of the membrane was the same as that in the intact cell.

Cytochemical studies have shown that the reaction product of thiamine pyrophosphatase is deposited on the luminal side of the Golgi membranes in vivo (34). Based on two lines of evidence, we found the same orientation of thiamine pyrophosphatase activity in our preparation. First, activity was increased about threefold in the presence of the nonionic detergent Triton X-100 (Table II). This activation has been attributed to the fact that much of this enzyme is normally "latent" within the luminal aspect of the Golgi apparatus and becomes available upon treatment with Triton X-100 (22). Second, thiamine pyrophosphatase is resistant to inactivation by external proteases. However, in the presence of Triton X-100, which renders the vesicles permeable to the proteases, the pyrophosphatase is inactivated by trypsin (Fig. 2). As expected, this inactivation is not complete, since a portion of the enzyme remains inaccessible to trypsin. These observations led us to conclude that our vesicles were right-side-out and that they were intact.

With the intact right-side-out vesicle preparation we used three approaches to investigate the orientation of CST. First, we examined the sulfation of endogenous galactocerebroside by 3'-phosphoadenosine 5'-phospho[^{35}S]sulfate in the absence and presence of Triton X-100 (Table III). We found no sulfation of endogenous galactocerebroside unless the vesicles were incubated with PAPS and Triton X-100. Under these conditions PAPS could traverse the permeabilized membrane and sulfatide synthesis was observed (Table III). It is unlikely that the labeled exogenous PAPS will be diluted by endogenous substrate, as the PAPS synthetic enzymes are probably located in the cytoplasm, in common with other nucleotide synthetases (35). Thus, the PAPS so formed, because of its structure, would be unable to cross membranes in the absence of a specific carrier, such as has been found for sugar nucleotides (36, 37). In addition, we showed that CST activity was latent, that the enzyme was resistant to the action of trypsin unless the vesicles were treated with Triton X-100 (Fig. 3)

and, finally, that agarose-linked galactosylsphingosine was not sulfated by intact vesicles. From these results it seems clear that CST, like thiamine pyrophosphatase, is located on the luminal side of the Golgi apparatus, suggesting, in turn, that sulfatide has a similar location and that it will be located in the outer half (intraperiod line) of the myelin bilayer.

How lipids elaborated in the Golgi complex are sorted and then targeted to specific membranes is not known (38). For example, how are the lipid constituents destined for myelin transported to this membrane and maintained in the appropriate conformation? Like other membranes, the lipids in myelin are arranged asymmetrically in the lipid bilayer and appear to be localized in specific domains (39). In this context, it is of interest that galactocerebroside, the precursor of sulfatide, appears to be located on the outer half of the myelin membrane. For instance, the C-6 hydroxyl group of the galactose moiety is accessible to the enzyme galactose oxidase (40); furthermore, Saida et al. (41) showed that when antigalactocerebroside antibody was injected into peripheral nerve or into the dorsal column of the spinal cord, demyelination was induced. We have recently confirmed this observation in both central and peripheral nervous system of rats by using a monoclonal antibody to galactocerebroside. As the antibody cannot penetrate the lipid bilayer, interaction with galactocerebroside would not occur unless this lipid were located in the outer half of the lipid bilayer. This location of the precursor galactocerebroside lends credence to the notion that sulfatide is also found in the outer layer. Moreover, Abrahamsson and co-workers (42) have shown that the sugar moiety of galactocerebroside is arranged parallel to the membrane rather than being oriented perpendicular to the lipid bilayer. Presumably sulfatide would show a similar orientation. In biological membranes, sulfatide is hydrated, so that it is unlikely to display "flip-flop" movement; as shown by Kornberg and McConnell (15), complex lipids in general undergo such movement only very slowly. Because of this arrangement in the lipid bilayer, it is likely that the asymmetrical distribution of sulfatide in the myelin membrane is maintained.

To our knowledge this is the first investigation of the orientation of an enzyme involved in the synthesis of complex glycolipids in intact vesicles enriched in Golgi markers. The presence of CST in the luminal aspect of such vesicles suggests a mechanism whereby sulfatide is asymmetrically assembled in the outer layer of myelin. We plan to establish this orientation unequivocally by use of immunocytochemical studies with antibodies to the enzyme and to sulfatide.

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