Sidedness of ceramide-phosphoethanolamine synthesis on rat liver and brain microsomal membranes

Monique Malgat, Andrée Maurice, and Jacques Baraud[1]

Université de Bordeaux-II, Laboratoire de Biochimie, 146, rue Léo-Saignat, F-33800 Bordeaux cedex, France

Abstract Phosphatidylethanolamine:ceramide-ethanolamine-phosphotransferase catalyzes the synthesis of ceramide-phosphoethanolamine, a sphingomyelin analogue. Its localization was studied in rat liver and brain microsomes. After testing the integrity and the sidedness of microsomal vesicles, trypsin treatment of intact or deoxycholate-disrupted microsomes made it possible to conclude that both the transferase and the ceramide-phosphoethanolamine are located in the cisternal leaflet of the membrane bilayer. Using trinitrobenzenesulfonic acid as a probe, no trace of newly synthesized ceramide-phosphoethanolamine was detectable on the cytoplasmic side of the microsomes. — **Malgat, M., A. Maurice, and J. Baraud.** Sidedness of ceramide-phosphoethanolamine synthesis on rat liver and brain microsomal membranes. *J. Lipid Res.* 1987. **28:** 138–143.

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Sphingomyelin is known to be synthesized by a PC:ceramide-phosphocholine-transferase which translocates phosphocholine from PC to a ceramide (1–4). In previous work (5), we have shown, in rat liver and brain cells, that sphingomyelin is synthesized both by endoplasmic reticulum and by plasma membrane. In that work, we also described the synthesis of ceramide-phosphoethanolamine, a sphingomyelin analogue, for which pulse-chase experiments showed that PE was the direct donor of the phosphoethanolamine moiety.

Ceramide-phosphoethanolamine was identified by incorporation of radioactivity from labeled PE, by its stability in alkaline methanolysis, and by its ability to be methylated by S-adenosylmethionine.

In this report we attempt to study the sidedness of the PE:ceramide-phosphoethanolamine-transferase able to synthesize ceramide-phosphoethanolamine. The transverse membrane orientation that occurs asymmetrically on the cytoplasmic surface of endoplasmic reticulum was studied for enzymes responsible for the biosynthesis of PC, PE, and triacylglycerol (6). To our knowledge, the orientation of the enzymes of sphingomyelin biosynthesis has never been investigated.

Enzyme sidedness was investigated in rat liver and brain microsomes. We did not study the enzyme localization in the plasma membrane, although it was more active than in microsomes (5); this is because fragmentation of the plasma membrane leads to vesicles, many of which are released inside-out. However, it was shown (7) that fragmentation of endoplasmic reticulum produced closed vesicles (microsomes) which maintained proper sidedness. Although this asymmetry has been disputed by Sundler et al. (8), it has been shown in the last decade that lipids and enzymes are asymmetrically distributed between the two leaflets of microsomes (9–14).

Incubation of microsomes with a protease should inhibit the enzymes located on the outer surface, whereas the enzymes of the cisternal leaflet might be accessible after action of a detergent, such as deoxycholate or taurocholate. Such experiments are valid if vesicle integrity is demonstrated and if one examines protease activity with microsomal enzymes, whose transverse localization is well-known (8); this test is eventually performed in the presence of a detergent.

MATERIALS AND METHODS

Materials

[6,6'(n)-^{3}H]Sucrose (12 Ci/mmol) and 1,2-dioleoyl-*sn*-glycero-3-phospho-[2-^{14}C]ethanolamine (49 mCi/mmol) were purchased from Amersham-France. All the un-

Abbreviations: DOC, deoxycholate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; TNBS, trinitrobenzenesulfonic acid; TNP-, trinitrophenyl-.

[1]To whom all correspondence should be sent.

labeled products were obtained from Sigma Chemical Co., except the trypsin inhibitor, which was purchased from Boehringer.

Isolation of subcellular membranes

Microsomes were obtained from rat liver and brain, as described previously (5). All the fractions were lyophilized and stored at −20°C.

Microsomal enzyme assays

NADPH cytochrome C reductase activity was determined (15) as marker of the cytoplasmic surface. Glucose-6-phosphatase was located on the cisternal side and its activity was determined according to Beaufay et al. (15): microsomes (50–250 μg of protein) were incubated with 0.4% sodium taurocholate for 30 min at pH 7.4 and 0°C. Then, 50 mM glucose-6-phosphate, 20 mM histidine–HCl buffer, pH 6.5, and 1 mM EDTA were added, in 200 μl final volume. The mixture was incubated at 37°C for 30 min. The reaction was stopped by addition of 5 ml of 8% trichloracetic acid. Inorganic phosphate was determined according to Napias (16). In the absence of taurocholate, enzyme latency was 94.5%, thus expressing microsomal integrity. Latency was expressed by (1 − A/B) × 100, A in the absence, B in the presence of taurocholate.

Brain microsomal integrity

Microsomes (2 mg of protein/ml) were incubated in 20 mM Tris–HCl buffer, pH 7.4, 15 mM NaCl and 10 mM [^{3}H]sucrose (sp act 1 μCi/mmol) according to Meissner and Allen (17). The mixture was incubated for 3 hr at 23°C. Aliquots (25 μl) were removed and diluted 100-fold into unlabeled medium with rapid mixing. The efflux of sucrose was determined by filtration of 0.5 ml of the diluted sample through 0.45 μm Gelman cellulose triacetate filters, at times ranging from 0.5 to 10 min after mixing. The filters were rinsed three times with the non-radioactive medium, dried, and the radioactivity space was calculated by dividing the radioactivity associated with the filters by the cpm/μl of the undiluted sample and the mg of protein in the filtered aliquot.

Incubation of microsomes with trypsin and/or deoxycholate

Deoxycholate. Microsomes were suspended in 0.05 M Tris–HCl buffer, pH 7.4, 0.25 M sucrose, 0.15 M KCl. They were incubated with 250 μg of DOC per mg of protein, i.e., 0.05% (w/v), for 10 min at 4°C. For further incubations with labeled PE, disrupted microsomes were washed with the buffer in order to eliminate DOC, which would be inhibitory. The microsomes were then recovered by centrifugation for 2 hr at 105,000 *g*.

Trypsin. Intact or deoxycholate-disrupted microsomes were suspended as above and incubated with trypsin (675 μg/mg microsomal protein) for 2 hr at 30°C. The reaction was stopped by trypsin inhibitor (1:2, w/w). Vesicles were washed as described above.

Ceramide-phosphoethanolamine

This sphingomyelin analogue was obtained as previously described (5). Microsomal fractions (250 μg of protein) were suspended in 50 mM Tris–HCl buffer, pH 7.4, and incubated with 130 nmol of labeled PE that had been sonicated in the presence of Triton X-100 (20 μg per ml of incubation medium). Reactions were performed in a final volume of 350 μl, for 3 hr at 37°C. Total lipids were extracted by chloroform–methanol 2:1 and submitted to alkaline methanolysis in 0.3 N NaOH in methanol at 37°C for 1 hr. The chloroform-extractable products (sphingomyelin and ceramide-phosphoethanolamine) were washed with 50 mM KCl, and estimated by measuring the radioactivity.

Reaction of aminophospholipids with TNBS

After incubating microsomes with labeled PE as described above, 100 μl of TNBS in 100 mM $NaHCO_3$ buffer, pH 8.5, was added, and the final TNBS concentration was 0.6 mM, as described by Vale (18). After incubation for 3 hr at 23°C in darkness, the reaction was stopped by addition of 2 ml of chloroform–methanol 2:1. Excess TNBS was eliminated by washing with $NaHCO_3$ buffer. The chloroform was evaporated and the extract was dissolved in chloroform for spotting on thin-layer chromatography plates. The lipids were separated by one-dimensional chromatography in $CHCl_3$–CH_3OH–NH_4OH–H_2O 70:30:4:1 (v/v). The TNP-derivatives were identified by their yellow color and their R_f values: 0.73 for TNP-PE, 0.52 for TNP-PS, and 0.20 for TNP-ceramide-phosphoethanolamine, respectively. These values were determined from standard PE and PS, and from ceramide-phosphoethanolamine purified by alkaline methanolysis.

Protein determination

The protein content of each membrane fraction was determined by the method of Lowry et al. (19), with bovine serum albumin as standard.

RESULTS

Transverse membrane orientation of PE:ceramide-ethanolaminephosphotransferase can be detected by proteolytic inhibition. Although the enzyme is located on the cytoplasmic surface of the endoplasmic reticulum, it can also be found on the external side of the microsomal membrane (7) and is easily affected by protease treatment. However, when the enzyme is located on the other side, it is accessible only after treatment of the microsomes with a detergent such as deoxycholate. These experiments imply that the microsomal membrane is, in

effect, impermeable to trypsin and that the protease may reach the internal side after DOC treatment.

Microsomal integrity

Glucose-6-phosphatase is located on the cisternal side of the microsomes. When intact microsomes were incubated with glucose-6-phosphate, enzyme activity was not detectable. However, after taurocholate treatment, glucose-6-phosphate was hydrolyzed and inorganic phosphate was estimated. **Table 1** shows that for rat liver microsomes, enzyme activity was 26 nmol phosphate/min per mg of microsomal protein, and 473 nmol after previous incubation of microsomes with taurocholate. Latency of enzyme activity was calculated as $(1 - 26/473) \times 100 = 94.5\%$. This demonstrated that the microsomal vesicles were impermeable to exogenous substrate and consequently to trypsin.

Such verification was not possible with rat brain microsomes, in which glucose-6-phosphatase had no measurable activity, even after taurocholate treatment (10). Therefore, microsomal integrity was determined by labeled sucrose efflux measurements, according to Meissner and Allen (17). This procedure involves the labeling of the microsomes with [^{3}H]sucrose and the subsequent dilution of the vesicles in an unlabeled medium. The amount of sucrose remaining in the vesicles is then determined as a function of time after dilution. **Fig. 1** shows that labeled sucrose, confined in the microsomes, was very slowly released, and this was an indication of the microsomal integrity. Of course, treatment with 0.05% DOC impairs this integrity (Fig. 1). Therefore, brain microsomes, like liver microsomes, were impermeable to trypsin.

Effect of trypsin on microsomal enzyme activity

NADPH cytochrome C reductase is located on the external side of the microsomal membrane and is directly accessible to trypsin. Table 1 shows that in liver microsomes enzyme activity was lost after incubation with trypsin. Incubation with DOC did not modify enzyme activity, which, of course, disappeared after subsequent trypsin treatment.

In brain microsomes, the NADPH cytochrome C reductase activity was considerably diminished, but trypsin action was quite similar (Table 1).

For glucose-6-phosphatase, which is located on the internal side, Table 1 shows that its latency in liver microsomes was about 95% and was not modified after trypsin treatment (89%). If the microsomes were to be preincubated with deoxycholate, enzyme activity would be freely expressed, but would be stopped after subsequent trypsin treatment.

These results have enabled us to determine the transmembrane orientation of PE:ceramide-ethanolaminephosphotransferase by incubating microsomes with trypsin (**Table 2**).

Rat liver or brain microsomes were incubated for 3 hr with labeled PE, as described in Materials and Methods. Ceramide-phosphoethanolamine was synthesized. Microsomal preincubation performed for 3 hr at 37°C before addition of PE did not modify the reaction rate; this does not correspond to the observation of Hutson and Higgins (11) for ethanolamine incorporation into microsomal phospholipids by the base-exchange mechanism.

When the microsomes were preincubated with trypsin for 2 hr at 30°C, there was practically no alteration of

TABLE 1. Effect of deoxycholate and trypsin on microsomal enzyme activity

Treatment	Enzyme Activity		
	NADPH Cytochrome C Reductase		Glucose-6-Phosphatase
	Liver Microsomes	Brain Microsomes	Liver Microsomes
	nmol/min per mg microsomal protein		
Intact microsomes			
None	152	7.4	26 (94.5)[a]
+ Trypsin	18.5	0.6	51 (89.2)
Disrupted microsomes			
+ DOC	147	6.0	440 (nil)
+ DOC + trypsin	20	0.0	31 (nil)

Activity of NADPH cytochrome C reductase and glucose-6-phosphatase was estimated for intact microsomes, or after preincubation with trypsin (675 μg/mg of protein), or DOC (250 μg/mg of protein), or both DOC and trypsin, as indicated in Materials and Methods.

[a]Values in parentheses show the percentage latency of glucose-6-phosphatase; this latency is defined as $(1 - A/B) \times 100$, A and B being the activity of the enzyme in the absence and presence of 0.4% sodium taurocholate, respectively. The B value was 473 nmol/min per mg protein.

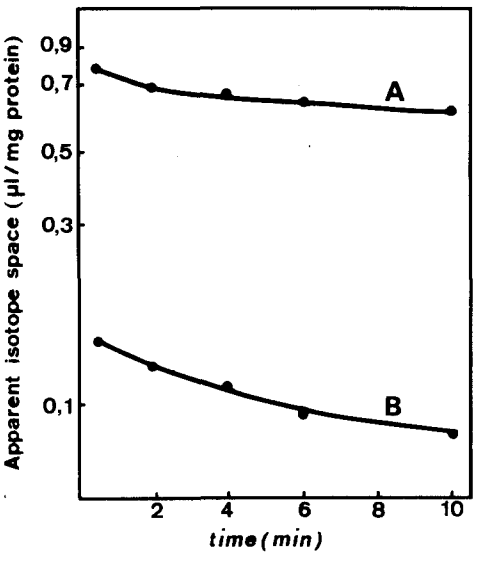

Fig. 1. Integrity of rat brain microsomes. Microsomes were loaded with [^{3}H]sucrose for 3 hr at 23°C. Efflux of sucrose was initiated by 100-fold dilution into unlabeled medium, as indicated in Materials and Methods. A, intact microsomes; B, microsomes disrupted by 0.05% deoxycholate.

transferase activity. When the microsomes were preincubated for 10 min at 4°C in the presence of 0.05% DOC, ceramide-phosphoethanolamine synthesis decreased by 31% for liver and 15% for brain microsomes, respectively. Depierre and Dallner (20) have shown that some enzymes on the internal side of the microsomes are inhibited nearly as much by deoxycholate as by trypsin, and Bernert and Ullman (2) have found that DOC inhibits sphingomyelin synthesis.

When microsomes preincubated with DOC were washed in order to eliminate the detergent, as recommended by Renooij and Synder (12), the activity of ceramide-phosphoethanolamine-transferase was somewhat restored.

Microsomes preincubated with DOC for 10 min at 4°C were incubated with trypsin for 2 hr at 30°C. Then, a trypsin inhibitor was added and the microsomes were washed in order to eliminate the detergent. The disrupted microsomes were incubated with labeled PE and the synthesis of ceramide-phosphoethanolamine represented only 28% for liver and 45% for brain microsomes when compared to synthesis of untreated microsomes.

Labeling of microsomal lipids with TNBS

Rat brain microsomes (250 µg of protein) were incubated with 130 nmol of labeled PE for 3 hr at 37°C and then treated with 0.6 mM TNBS for 3 hr at 23°C, pH 8.5. Then, the lipids were extracted and the extracts of twelve incubations (3 mg of protein) were pooled. TNP-derivatives were separated by chromatography, as described in Materials and Methods.

Chromatography of the intact microsome extracts showed two yellow spots, corresponding to TNP-PE (R_f = 0.73) and TNP-PS (R_f = 0.36), respectively. A very small spot, corresponding to TNP-lysoPE, was also perceptible in some experiments. When the microsomes were disrupted by 0.05% DOC before adding TNBS, chromatography revealed an additional yellow spot corresponding to ceramide-phosphoethanolamine (R_f = 0.20). The lack of such a spot on the first chromatogram was not sufficient to demonstrate the absence of ceramide-phosphoethanolamine on the external side of the microsomes. The TNP-derivative was perceptible only above 4 nmol, i.e., more than a quarter of the total amount expected in these assays (about 14 nmol produced for 3 mg of microsomal protein).

Therefore, the spot of TNP-ceramide-phosphoethanolamine was scraped from the second chromatogram and the same zone was also recovered from the first one; their radioactivity was measured.

For the second chromatogram spot (disrupted microsomes), radioactivity was 128,340 cpm, corresponding to 12.2 nmol of ceramide-phosphoethanolamine. For the first chromatogram (intact microsomes), radioactivity was only 5,520 cpm corresponding to 0.5 nmol, or 4.2% in relation to the first estimation.

TABLE 2. Activity of PE:ceramide-ethanolaminephosphotransferase of rat liver and brain microsomes

Preincubations	Enzyme Activity	
	Liver Microsomes	Brain Microsomes
	nmol ceramide-phosphoethanolamine/mg of protein per hr	
None	2.5 ± 0.15	4.4 ± 0.3
For 2 hr at 30°C without reagent	2.35 ± 0.1	4.35 ± 0.3
+ Trypsin	2.1 ± 0.1	4.5 ± 0.2
+ DOC without washing	1.6 ± 0.2	3.75 ± 0.4
+ DOC + washing	2.0 ± 0.2	4.1 ± 0.3
+ DOC + trypsin	0.7 ± 0.1	2.0 ± 0.1

Intact microsomes were incubated with labeled PE for 3 hr at 37°C. Lipids were extracted and ceramide-phosphoethanolamine was estimated by its radioactivity, after purification by alkaline methanolysis. Microsomes were preincubated with trypsin, deoxycholate, or both DOC + trypsin, as in Table 1. Results are the mean of three assays.

This value did not appear significant and it might be thought that all newly synthesized ceramide-phosphoethanolamine was located on the cisternal side of the microsomal membrane.

DISCUSSION

Rat liver endoplasmic reticulum, broken up into microsomes, maintains a right-side-out orientation (7). In our experiments, the latency of glucose-6-phosphatase was 94.5%, and NADPH cytochrome C reductase was entirely accessible to trypsin, which suggests that the sidedness of the membrane was not disturbed. In brain microsomes, the activity of glucose-6-phosphatase was not measurable and the efflux of [^{3}H]sucrose provided an alternative control of vesicle integrity (17). NADPH cytochrome C reductase activity was much lower, but was inhibited by trypsin, as were liver microsomes.

PE:ceramide-ethanolaminephosphotransferase is a microsomal enzyme of brain and liver cells which leads to the formation of ceramide-phosphoethanolamine (5). When the microsomes were preincubated with trypsin, the rate of synthesis was not affected. Preincubation with DOC strongly inhibited synthesis, but this inhibition was largely reversed by washing microsomes before incubation with PE. Inhibition by DOC was reported for several enzymes located on the cisternal side of the endoplasmic reticulum (20), and for sphingomyelin synthesis (2). It seems that these enzymes require a lipid environment which is destroyed by the detergent.

When the microsomes were preincubated with DOC and then with trypsin, ethanolaminephosphotransferase activity was significantly decreased. When microsomes incubated with labeled PE were immediately treated with TNBS, the reagent allowed the characterization of TNP-PE and TNP-PS, and no labeled ceramide-phosphoethanolamine derivative was detectable. This product was characterized only in DOC-disrupted microsomes before addition of TNBS. It seems that if ceramide-phosphoethanolamine is synthesized in the external leaflet of the microsomal membrane, it would be possible to detect at least a small amount of the newly synthesized product, if not by its yellow color, at least by its radioactivity.

It may be concluded that the transferase responsible for ceramide-phosphoethanolamine synthesis is situated on the cisternal side of the endoplasmic reticulum in rat liver and brain cells, and that the newly synthesized product does not undergo transmembrane movement.

Several previous observations confirm this internal localization.

1. When sphingomyelin synthesis was performed in the presence of exogenous ceramide, the addition of a detergent was required (3). This shows the difficulty for ceramide to reach the site of synthesis. This would be easier if the site was external.

2. A similar situation occurred when ceramide-phosphoethanolamine synthesis was carried out in the presence of exogenous PE: the reaction was slow and linear for at least 4 hr (5). It would be greatly accelerated in the presence of a specific transfer protein incorporating PE into the membrane, as demonstrated (3, 21) for sphingomyelin synthesis in the presence of exogenous PC.

3. Most sphingomyelin (91%) is located in the cisternal leaflet of the endoplasmic reticulum (22), while it was found in the external side of erythrocyte membrane: 80% (23), or 85% (24), or of platelet membrane: 91% (25). It seems natural for sphingomyelin to be synthesized on the side where it accumulates. On the other hand, and as demonstrated in (24), the asymmetric distribution of sphingomyelin did not give rise to a transmembrane orientation, unlike the other membrane lipids.

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