Fatty Acyl-Co A: Sphingosine Acyltransferase in Bovine Brain Mitochondria: Its Solubilization and Reconstitution onto the Membrane Lipid Liposomes

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Fatty acyl-Co A: sphingosine acyltransferase (ceramide synthase, EC 2.3.1.24) is mainly localized in the microsomal and mitochondrial membranes. Attempts to isolate the enzyme have failed, largely because there has been little or no detection of the enzyme activity in detergent extracts. In this study, we solubilized the membrane-bound enzyme from bovine brain mitochondria with a Tris–HCl buffer containing 2% Triton X-100 and, after removal of the detergent, reconstituted it with the membrane lipid liposomes. The specific activity of the reconstituted enzyme was approx. 8 times higher than that of the solubilized enzyme. We next examined the lipid dependence of the enzyme, using various phospholipid liposomes. The ability of phospholipids to enhance the activity of solubilized ceramide synthase was specific and structure-related. The most potent stimulator was phosphatidyserine liposomes suggesting an important role of the net negative charges. This paper also describes a highly reproducible high-performance liquid chromatographic (HPLC) procedure for the determination of ceramide synthase activity. Combination of the HPLC method with the reconstituted enzyme system appears to be suitable for elucidating the characteristics of this enzyme.

Key words ceramide synthase; solubilization; reconstitution; liposome; HPLC

Recently, much attention has been paid to the possible involvement of sphingolipid and their breakdown products in various cellular functions such as cell differentiation, cell contact response and cell recognition.1) The basic building block of all sphingolipids is ceramide (N-acyl-d-sphingosine), which is synthesized in microsomess (Ms)2–6) and mitochondria (Mt)7,8) by the condensation of sphingosine with fatty acyl-CoA and then further converted to the diverse sphingolipids. Because of its central role in sphingolipid metabolism, the synthesis of ceramide has been well studied.7–8) Nevertheless, the enzymatic mechanism for ceramide synthesis is still poorly understood, largely because of the resistance of the key enzyme, fatty acyl-CoA: sphingosine acyltransferase (ceramide synthase), to solubilization. Namely, although various detergents, such as Triton X-100, Emlugen 950 and 3-[(β-cholamidopropyl)dimethylammoninio]-1-propanesulfonate (CHAPS), effectively solubilize the membrane proteins from rat liver and brain Ms, little or no ceramide synthase activity has been detected in each supernannant fraction.60

On the other hand, to date, the assay procedures for ceramide synthase have involved the use of 14C-labelled acyl-CoA and non-labelled sphingosine as substrates with the subsequent isolation of the radiolabelled product by thin-layer (TLC)1,3–6) or column7) chromatographies. These procedures take a long time to perform and have some uncertainty in accuracy. During the development of a high-performance liquid chromatographic (HPLC) procedure for the determination of ceramide synthase activity in bovine brain Mt, we found that the enzyme activity in the Triton X-100-solubilized membrane proteins was increased by the addition of the Mt lipids. The finding possibly reflected some regulatory role of the membrane lipids in ceramide synthase activity. This hypothesis was the starting point for the present work.

We describe below some enzymatic properties of the ceramide synthase that was solubilized from bovine brain Mt and reconstituted onto the membrane lipid liposomes. A highly reproducible HPLC method for the determination of ceramide synthase is also presented.

MATERIALS AND METHODS

Reagent The following reagents were commercially obtained: d-sphingosine, dl-erythro-dihydrosphingosine ( sphingamine), bovine brain ceramides, fatty acyl-CoAs, egg yolk l-α-phosphatidylcholine (PC), bovine brain l-α-phosphatidylethanolamine (PE), bovine liver l-α-phosphatidylinositol (PI) and bovine brain l-α-phosphatidyl-t-i-serine (PS) from Sigma; n-octyl-β-D-glucoside, Triton X-100 and Tween 20 from Wako Pure Chemical Co., Osaka; and benzyl chloride from Tokyo Kasei Co., Tokyo.

Preparation of Mt and Ms Fresh bovine brains (322–371 g each) were obtained from a local slaughterhouse, washed with cold saline, and stored at −80 °C until use. A part (206 g) of the frozen tissues was thawed and then homogenized at 4 °C with 3 volumes of 20 mM Tris–HCl buffer (pH 7.4)/0.25 mM sucrose/1 mM EDTA. The homogenate was centrifuged at 1000 × g for 10 min at 4 °C. The supernatant obtained was further centrifuged at 33000 × g for 20 min. The Mt-rich pellet was resuspended in 3 volumes of the same buffer and centrifuged again. The Ms pellet was obtained by centrifugation of these supernatants at 105000 × g for 1 h. Each pellet was stored at −80 °C until use.

Solubilization and Ammonium Sulfate Fractionation of

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Mt Proteins The frozen Mt pellet (22 g, wet weight) was thawed and suspended in 44 ml of 50 mM Tris–HCl buffer (pH 7.4)/0.25 M sucrose/2% Triton X-100. The suspension was stirred at 4°C for 90 min, and then centrifuged at 105000 × g for 45 min at 4°C. To the precipitate was added 22 ml of the same buffer, and the suspension was stirred at 4°C for 45 min and centrifuged. These supernatants were combined and dialyzed against 20 mM Tris–HCl buffer (pH 7.4) for 24 h at 4°C. The dialysate was centrifuged at 27000 × g for 10 min at 4°C to remove small insoluble materials. Solid ammonium sulfate was slowly added to the supernatant with constant stirring, until 30% saturation (176 g/l) was reached. After stirring for 40 min at 4°C, the precipitate was collected by centrifugation at 27000 × g for 10 min at 4°C. The supernatant was brought to 70% saturation (472 g/l) by the further addition of ammonium sulfate, stirred, and centrifuged. These precipitates were dissolved in 10 volumes of 20 mM Tris–HCl buffer (pH 7.4), and the solutions were dialyzed against the same buffer for 24 h at 4°C. The dialysates were stored at −80°C until use.

Preparation of Liposomes Phospholipids were the products of Sigma, and the extraction of Mt lipids was as follows. The frozen Mt pellet (10 g, wet weight) was thawed and extracted with 3 volumes of chloroform/methanol (C/M) (9:1, v/v) at 25°C for 30 min. After centrifugation at 800 × g for 10 min, the lower layer was transferred to another test tube and evaporated to dryness under a nitrogen stream. For the preparation of liposomes, the resulting lipid film or phospholipid (10 mg each) was dissolved with 40 mg of n-octyl-β-D-glucoside in 3.2 ml of C/M (95:5, v/v). The solution was evaporated to dryness under a nitrogen stream. The residue was dissolved in 4 ml of 20 mM Tris–HCl buffer (pH 7.4), and dialyzed 3 times against 31 of the same buffer for 24 h at 4°C. The liposome suspension was stored at 4°C until use.

Enzyme Assay D-Sphingosine (800 nmol) in 100 µl of 20 mM Tris–HCl buffer (pH 7.4)/0.1% Tween 20 and palmitoyl-CoA (400 nmol) in 100 µl of 20 mM Tris–HCl buffer (pH 7.4) were placed in a 12 × 100 mm screw-cap test tube. The suspension was further diluted with 20 mM Tris–HCl buffer (pH 7.4) to a final volume of 450 µl. The enzyme reaction was started by adding 300 µl of Mt suspension or 200 µl of the solubilized enzyme fraction plus 100 µl of liposome suspension (170—200 µg lipid). After an incubation at 37°C for 45 min, the reaction was terminated by adding 3 ml of C/M (2:1, v/v). The mixture was shaken for 10 min at 4°C and centrifuged at 800 × g for 5 min. A 2-ml portion of the lower layer was withdrawn and mixed with C/M (2:1, v/v) and 10% (w/v) ammonium sulfate (1 ml each). The mixture was shaken and centrifuged. A part (1.5 ml) of the lower layer was transferred to another screw-cap test tube and evaporated to dryness under a nitrogen stream. The residue was further dried at 25°C for 1 h over P_2O_5 in vacuo. To the dried sample were added 100 µl of pyridine and 40 µl of benzoyl chloride, and the mixture was allowed to react at 65°C for 30 min. To the reaction mixture was added 800 µl of methanol, and incubation followed at 65°C for 15 min to convert excess benzoyl chloride to volatile methyl benzoate. After evaporation of the solvent under a nitrogen stream, the benzytolated ceramide was recovered by partitioning between 500 µl of a saturated solution of sodium carbonate in methanol and 1 ml of n-hexane. A part (750 µl) of the hexane layer was collected and filtered through a 0.45-µm membrane filter (Ultra-free C3-HV, Millipore Co.).

HPLC Analysis The HPLC system consisted of a Model 6000 A solvent delivery system (Waters, Milford, MA), a Model S-310 A variable-wavelength detector (Soma, Tokyo, Japan) and a Chromatopac C-R4 A chromatographic processor (Shimadzu, Kyoto, Japan). A 10-µl portion of the above filtrate was injected into a 5-µm Spherisorb ODS column (4.6 × 250 mm i.d., Chemco Sciences Co., Osaka, Japan). The benzytolated ceramide was separated from other by-products with 10% (v/v) 2-propanol in n-hexane as the mobile phase. The flow rate was 2.0 ml/min, and the effluent was monitored at 230 nm.

TLC Analysis To identify the benzytolated ceramide peak on HPLC, the eluates corresponding to each peak were collected and evaporated to dryness under a nitrogen stream. The residues were dissolved in 100 µl of n-hexane and spotted on a silica gel TLC plate (20 × 20 cm, Merck) with 10 µl of a standard solution of benzytolated ceramide (400 µg/ml in hexane). The solvent system used was n-hexane/diethyl ether (70:30, v/v), and the spot of benzytolated ceramide was visualized with cupric acetate.

Protein Determination The method of Lowry et al. was used for the determination of proteins in intact Mt and Ms preparations. The protein concentrations of solubilized Mt fractions were determined by the method of ChandraRajan and Klein. Bovine serum albumin was used as the reference.

Enzyme Unit One unit (U) was defined as the amount of enzyme which forms 1 nmol of palmitoylphosphoglycerine per min at 37°C.

RESULTS AND DISCUSSION

HPLC Measurement of Ceramide Synthase Activity in Intact Mt Intact Mt (810 µg protein) was incubated at 37°C for 45 min with palmitoyl-CoA and D-sphingosine, and the reaction products were benzytolated and analyzed by HPLC. Figure 1A shows a typical elution profile of the benzytolated products on HPLC. The peaks for benzytolated derivatives were well separated from each other by isocratic elution with 10% (v/v) 2-propanol in hexane. The separation was finished within 2 min. TLC analysis of the collected eluates revealed that the benzytolated ceramide was eluted as the first peak with a retention time of 1.4 min (TLC data not shown). A standard curve for varying amounts of palmitoylphosphoglycerine is shown in Fig. 1B. The benzytolated ceramide showed a linear relationship between peak height and quantity in the range of 10—200 nmol palmitoylphosphoglycerine in 750 µl of assay mixture (85% recovery). The specific activity of the Mt preparation was 2.6 ± 0.18 U/mg of protein, and it was 1.6 times that of the Ms preparation (Fig. 2). Finally, the sensitivity of this HPLC method was lower than that of the 14C-labeled acyl.
Table 1. Determination of Ceramide Synthase Activity in each Fraction with or without Liposomes

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein (mg)</th>
<th>Liposome addition</th>
<th>Specific activity (U/mg protein)</th>
<th>Total activity (U)</th>
<th>Recovery (%)</th>
</tr>
</thead>
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<tr>
<td>Mt pellet</td>
<td>380</td>
<td>-</td>
<td>2.6</td>
<td>988</td>
<td>100</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>354</td>
<td>+</td>
<td>0.39 (a)</td>
<td>138</td>
<td>14</td>
</tr>
<tr>
<td>extract</td>
<td>+</td>
<td>0.45 (b)</td>
<td></td>
<td>159</td>
<td>16</td>
</tr>
<tr>
<td>Ammonium sulfate precipitation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0—30%</td>
<td>133</td>
<td>-</td>
<td>0.89 (a)</td>
<td>118</td>
<td>12</td>
</tr>
<tr>
<td>+</td>
<td>6.9 (b)</td>
<td></td>
<td></td>
<td>918</td>
<td>93</td>
</tr>
<tr>
<td>30—70%</td>
<td>182</td>
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<td>0.27 (a)</td>
<td>49</td>
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<tr>
<td>+</td>
<td>1.1 (b)</td>
<td></td>
<td></td>
<td>200</td>
<td>20</td>
</tr>
</tbody>
</table>

a) Values obtained in the liposome-noncontaining assay system. b) Values obtained in the liposome-containing assay system. Liposomes were prepared with Mt membrane lipids.

Fig. 1. HPLC Chromatogram for the Benzyolated Reaction Products (A) and Standard Curve for Palmitosylphosphatidic Acid as the Benzyolated Derivative (B)

Assay mixtures containing 10—200 nmol palmitosylphosphatidic acid were extracted, benzyolated, and further partitioned with methanol/hexane. A 10-μl portion of the hexane phase was injected. Each point is the mean of duplicate experiments.

Fig. 2. Ceramide Synthase Activities in Mt and Ms Fractions

Each bar represents the mean ± S.D. of triplicate experiments.

CoA-used method (less than 1 nmol ceramide in 0.5 ml of reaction mixture), but was adequate to determine the in vitro enzyme activity.

Solubilization of Mt Proteins and Their Reconstitution with the Membrane Lipids: Twenty-two grams of the frozen Mt pellet was thawed, treated with detergent, and further fractionated with ammonium sulfate (see Materials and Methods). Table 1 summarizes the results of the determination of ceramide synthase activity in each fraction with or without Mt membrane lipid liposomes. The solubilization of ceramide synthase with Triton X-100 caused an apparent decrease in its specific activity (0.39 U/mg protein versus 2.6 U/mg protein). The following ammonium sulfate fractionation of the Triton X-100 extract indicated that a large part of the enzyme activity was recovered in the 0—30% ammonium sulfate precipitate (total activity, 118 U). The specific activity of ceramide synthase was also increased, from 0.39 to 0.89 U/mg protein, by this procedure, which involves the removal of detergent and inactive proteins. The addition of liposomes to the assay system further increased the specific activity from 0.89 to 6.9 U/mg protein (an approx. 7.8-fold increase). However, the enzyme activity in the Triton X-100 extract was hardly affected by the addition of liposomes (0.45 U/mg protein versus 0.39 U/mg protein). These results suggest that the enzyme activity is enhanced by being anchored on the liposome surface, whereby the enzyme eventually acts as an intrinsic protein. Indeed, the majority of the enzyme activity was recovered in the liposome pellet, when the assay mixture was centrifuged at 230000 × g for 2 h at 4°C (data not shown).

Characterization of the Reconstituted Enzyme: We examined several enzymatic properties of the ceramide synthase in 0—30% ammoniumsulfate precipitate, using the above reconstituted enzyme system. Figure 3A shows the pH dependence of palmitosylphosphatidic acid formation by the reconstituted enzyme. An optimum is observed between pH 7.0 and 7.8. At pH 7.4, the ceramide formation was linear within the protein concentration range from 0.2 to 1.2 mg/ml (Fig. 3B). It was also linear with incubation time (0 to 45 min). There is evidence of ceramide biosynthesis by the reversal of ceramidase (EC 3.5.1.23)12 At pH 4.5, however, the reconstituted enzyme had no
ceramidase activity for palmitoylsphingosine (these data not shown). Figure 4 shows the effect of ionic strength variation on the reconstituted enzyme activity. Within the physiological concentration, the observed salt effect was small. At higher concentrations, NaCl inhibited the ceramidase activity in the reconstitution system.

We next examined the effects of various phospholipid liposomes on the solubilized ceramidase activity. As shown in Table 2, the specific activity of this enzyme increased over a range of 1.6- to 6.7-fold by the presence of liposomes. Phospholipids containing serine and inositol as the polar groups were effective and increased the specific enzyme activity by 6.7- and 5.3-fold, respectively. The addition of PC liposomes resulted in a 4.3-fold increase in the specific activity. The effect of positively charged PE liposomes was small (a 1.6-fold increase). The ability of PS (100%) liposomes to increase the enzyme activity was reduced by mixing it with PE at the ratio of 1:1. These differences in ceramidase activity may reflect the sensitivity of the enzyme to its lipid environment or the altered stability of the enzyme in different lipid environments. When considering the abundance of PE (PE:PS) in Mt membrane lipids, however, not only the phospholipid environment but also other factors present in the Mt lipid liposomes may be important for the enhancement of ceramidase synthetic activity.

The kinetics of ceramide formation by the Mt lipid-reconstituted enzyme were examined using various acyl (C₆ to C₂₄)CoAs as substrates. As shown in Fig. 5, the catalytic efficiency (V_{max}/K_{m}) of the reconstituted enzyme regarding ceramide synthesis changed with a variation in the acyl chain length of the CoA ester. In this experiment, palmitoyl (C_{16:0})- and stearoyl (C_{18:0})-CoAs were good substrates. The presence of one double bond in these acyl chains (palmitoleoyl (C_{16:1})- and oleoyl (C_{18:1})-CoAs) decreased their functions as substrates. The observed substrate specificity is adequate, when considering the majority of palmitoyl- and stearoyl-chains in naturally occurring ceramides. On the other hand, the apparent K_{m} value determined for d-sphingosine was 0.17 mm. The substrate specificity of the reconstituted enzyme was very similar to that of the enzyme in mouse brain Ms. In our reconstituted enzyme system, indeed, the enzyme solubilized from Ms fraction revealed the same specific activity as that of the Mt enzyme for palmitoylsphingosine synthesis (data not shown). However, it still remains to be determined whether or not the ceramidase synthetic activity is due to a protein identical to that present in Mt.

Within the last few years, ceramide has emerged as a novel class of signal-transducing lipids. Ceramide appears to turn on one or more of the three anti-proliferative pathways: the inhibition of cell growth, the induction of cell differentiation and programmed cell death. However, very little is known about how de novo ceramide synthesis is regulated and how the synthesized ceramide contributes to the proposed signal transduction pathway. Thus, ceramide synthase awaits purification and definitive enzymological characterization. In this study, we succeeded in determining the solubilized enzyme activity by supplementing it with membrane lipid liposomes. The use of this lipid-reconstituted enzyme system will allow the accomplishment of such attempts.

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REFERENCES
