Reactivity of Sphingosine Base in Enzymatic Synthesis of Ceramide

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The reactivity of sphingosine bases as substrates in the enzymatic synthesis of ceramide was in decreasing order of *erythro*-4-sphingenine, *erythro*-sphinganine, *threo*-4-sphingenine and *threo*-sphinganine in the microsomal fraction obtained from chicken liver. Unsaturated base-containing ceramide was enzymatically formed from 4-sphingenine and saturated base-containing ceramide from sphinganine. The formation of unsaturated base-containing ceramide proceeded similar to that of saturated base-containing ceramide. The data explain in part, the fact that 4-sphingenine is generally superior to sphinganine as the constituent sphingosine base in sphingolipids, the biochemical derivatives of ceramide.

Nakano and Fujino¹⁾ demonstrated that 4-sphingenine was the major component, whereas sphinganine was the minor, in the molecule of sphingolipid synthesized by the enzymatic condensation of palmitoyl-CoA with labeled serine with the particulate fraction of rat liver. The finding suggested that 4-sphingenine might be more reactive than sphinganine in the biosynthesis of sphingolipid. The present authors tested in this experiment the reactivity as substrate of major sphingosine base* in enzymatic synthesis of ceramide, N-acyl derivative of sphingosine base. The ceramide is an intermediate in biosynthesis of sphingolipid and known to be biosynthesized in the following equation²⁾:

The resultant data, that, in the synthesis

of ceramide, 4-sphingenine was more active than sphinganine as acceptor and unsaturated ceramide always predominated over saturated ceramide may (metabolically) explain the fact that 4-sphingenine is superior to sphinganine as the constituent sphingosine base of the sphingolipid in nature.

MATERIALS

Sphingosine bases. Substrate sphingosine bases were prepared by hydrolysis of sphingomyelin isolated from beef brain. The sphingomyelin was hydrolyzed with aqueous methanolic 1 N HCl to produce free sphingosine bases according to the procedure of Gaver and Sweeley.³⁾ Two isomers, *erythro*-4-sphingenine and *threo*-4-sphingenine, formed under the conditions used, were separated each other by silicic acid column chromatography with a stepwise-gradient elution technique and thin-layer silicic acid chromatography developed with chloroform-methanol-2 N ammonia water (40:10:1, by vol).⁴⁾ *erythro*-Sphinganine and *threo*-sphinganine were respectively prepared by hydrogenation of the corresponding isomers of 4-sphingenine, according to the procedure of Gatt and Rapport.⁵⁾

* In this paper, the term, sphingosine base, is used as a generic name for sphinganine derivatives such as 4-sphingenine and sphinganine which have been called sphingosine and dihydrosphingosine, respectively, in the literature.

Labeled fatty acid. Palmitoyl-1-¹⁴C and stearoyl-1-¹⁴C were purchased from Daiichi Pure Chemicals Co., Ltd., Tokyo. The specific radioactivity was

determined with the Super scaler Model D-4E of Shimadzu Seisakusho Co., Ltd., Kyoto.

Enzyme solution. This was prepared by following mainly the procedure of Brady, Bladley, Young and Kaller.⁶⁾ The liver tissues obtained from 30-day-old chicken were homogenized with 4 times the volume of 0.25 M sucrose in a Potter-Elvehjem homogenizer. After nuclei, debris and mitochondrial particulates had been removed by centrifugation at $8600 \times g$ for 12 min, the microsomal fraction was sedimented by centrifugation at $105000 \times g$ for 30 min, suspended in 0.2 M phosphate buffer, pH 7.8, at the ratio of 1 ml per gram of the original liver and kept in freezer as the enzyme solution.

Ceramide. Standard ceramide was prepared from beef brain cerebroside by oxidation with periodate followed by reduction with NaBH_4 and by hydrolysis with acid, according to the procedure of Carter, Rothfus and Gigg.⁷⁾ The thin-layer silicic acid chromatogram, infra-red spectrum and melting point ($86 \sim 87^\circ\text{C}$) of the ceramide preparation, agreed well with those of ceramides in the literature.⁸⁾ The preparation consisted of the following four types⁹⁾ of ceramides according to thin-layer 4% borate-impregnated silicic acid chromatography with chloroform-methanol (90:10, by vol): normal acyl-unsaturated dihydroxy base, normal acyl-saturated dihydroxy base, hydroxy acyl-unsaturated dihydroxy base and normal acyl-trihydroxy base.

METHODS

Preparation of incubation mixture. The composition of incubation mixture and the conditions of enzymatic reaction are shown in Table I. The system is basically similar to that used by Sribney.²⁾ The mixture was composed of phosphate buffer, pH 7.4, labeled fatty acid, sphingosine base, a system for acyl CoA formation, enzyme solution and cofactors for the reaction. These were homogeneously mixed together by following the technique of Nakano, Negishi, Ito and Fujino.¹⁰⁾

Separation of ceramide fraction. At the end of the incubation, 3 volumes of methanol were added to the mixture to stop the enzymatic reaction and to extract lipid. The residue was further extracted with chloroform-methanol (2:1, by vol) to get as much lipid as possible. The standard ceramide was added as carrier to the extract, which was washed by the procedure of Folch *et al.*¹¹⁾ The chloroform portion obtained was

TABLE I. INCUBATION MIXTURE FOR BIOSYNTHESIS OF CERAMIDE

Potassium phosphate buffer,		
pH 7.4	100	μmoles
Sphingosine base	4	μmoles
Fatty acid-1- ^{14}C	3	μmoles
Triton X-100	3	mg
Cystein-HCl	20	μmoles
ATP	15	μmoles
CoA	0.5	μmoles
MgCl_2	5	μmoles
KF	50	μmoles
Enzyme solution	0.25	ml
Incubation: 2 hr, 37°C ,	1	ml

condensed and incubated with 4 N KOH in methanol for 2 hr at 37°C to decompose and remove ester lipid, if any. The alkali-stable lipid were applied to silicic acid column chromatography and eluted successively with chloroform-methanol mixture. Ceramide fraction was obtained in the eluate with chloroform-methanol (95:5, by vol).

Assay of radioactive ceramide. The ceramide fraction was subjected to thin-layer silica gel G chromatography developed with chloroform-acetic acid-methanol (94:5:1, by vol),¹²⁾ chloroform-acetic acid (90:10, by vol)¹³⁾ and chloroform-methanol (90:10, by vol). Type of ceramide was analyzed by thin-layer 4% borate-impregnated silica gel G chromatography developed with chloroform-methanol (90:10, by vol)⁹⁾ in reference to the standard ceramide. The location of the spot of lipid was revealed by spraying with 50% H_2SO_4 or by sublimating iodine over the plate and the radioactivity was determined by radio-thin-layer chromatography with the Thin-Layer-Scanner, Aloka JTC-202B of Japan Radio Co., Ltd., Tokyo as well as by autoradiography with the Fuji film of Fuji Photo Film Co., Ltd., Tokyo.

RESULTS AND DISCUSSION

Formation of radioactive ceramide

Enzymatic reaction was carried out with sphingosine base and palmitic acid in the system shown in Table I. After incubation, the ceramide fraction was isolated from the reaction mixture with carrier ceramide and analyzed with thin-layer silicic acid chromato-

FIG. 1. Radio Analysis of Ceramide with Thin-layer Chromatography.

Sphingosine base and palmitic acid-1- ^{14} C were enzymatically reacted in the system shown in Table I. After incubation, ceramide formed was isolated, developed with chloroform-acetic acid (90: 10, by vol) on thin-layer silica gel G plate, detected by iodine vapor and subjected to radio-scanning and radio-printing.

graphy. As seen in Fig. 1, the location of the main spot was equal to that of the standard ceramide on the thin-layer plate, and the spot ceramide had been well labeled according to the radio-thin-layer chromatogram and the autoradiogram. The data show that radioactive ceramide was enzymatically synthesized from the sphingosine base and labeled palmitic acid in the system.

Reactivity of fatty acid

The reactivity of various labeled fatty acids with *erythro*-4-sphingenine in ceramide biosynthesis is expressed in Table II. The rate of incorporation of fatty acids into ceramide was approximately 5: 3: 1 regarding oleic, palmitic and stearic acid. The higher incorporation of oleic acid, which is scarcely found in natural ceramide, may be based on the easier emulsification under the conditions used in the enzymatic reaction.

Sribney²¹ reported that, in the biosynthesis of ceramide with the microsomal fraction obtained from chicken liver, nervonic acid was rather active than palmitic acid as substrate, whereas, in the formation of ceramide with the enzyme preparation obtained from

rat brain, nervonic acid was less active than palmitic acid. Morell and Radin¹³⁾ communicated that, in the synthesis of ceramide with the homogenate obtained from mouse brain, lignoserinic, palmitic and oleic acid were active as substrate in decreasing order. It is presumed from these observation together with the present data that the reactivity of fatty acids as substrate in the enzymatic formation of ceramide may be influenced by the enzymatic system as well as by the reaction conditions employed in the experiment.

TABLE II. REACTIVITY OF LABELED FATTY ACID IN CERAMIDE BIOSYNTHESIS

erythro-4-Sphingenine and various fatty acid-1- ^{14} C were enzymatically reacted in the system shown in Table I. After incubation, ceramide formed was isolated and assayed for radioactivity.

Substrate	Ceramide synthesized
	cpm
Oleic acid-1- ^{14} C	2392
Palmitic acid-1- ^{14} C	1564
Stearic acid-1- ^{14} C	500

Reactivity of sphingosine base

The reactivity of various sphingosine bases

with palmitoyl-1-¹⁴C in ceramide biosynthesis is given in Table III. As seen, 4-sphingenine was more active than sphinganine, that is, about two times more active in case of the *erythro* isomers (natural type) and almost four times more active in the *threo* isomers (unnatural type). The finding implies that sphingosine acyltransferase catalyzing the biosynthesis of ceramide has some substrate specificity toward the sphingosine base, 4-sphingenine and sphinganine. *threo*-Sphingosine base had less activity than *erythro*-sphingosine base in this experiment. *erythro*-4-Sphingenine was rather active than *threo*-4-sphingenine in the enzymatic synthesis of ceramide according to Sribney²⁾ or Ito and Fujino.¹⁴⁾ It has been pointed out that substrate specificity is not always clear-cut toward *erythro*-compound and *threo*-compound in biosynthesis of sphingolipids.^{10,15,16)}

TABLE III. REACTIVITY OF SPHINGOSINE BASE IN CERAMIDE BIOSYNTHESIS

Various sphingosine bases and palmitic acid-1-¹⁴C were enzymatically reacted in the system shown in Table I. After incubation, ceramide was isolated and developed with chloroform-methanol (90: 10, by vol) on thin-layer silica gel G plate, detected by iodine vapor and determined for radioactivity.

Acceptor	Ceramide synthesized
	cpm
<i>erythro</i> -4-Sphingenine	1129
<i>erythro</i> -Sphinganine	620
<i>threo</i> -4-Sphingenine	748
<i>threo</i> -Sphinganine	176
Control (No sphingosine base)	100

Type of radioactive ceramide

Type of ceramide synthesized enzymatically from *erythro*-4-sphingenine and labeled palmitic acid (Experiment A) or from *erythro*-sphinganine and labeled palmitic acid (Experiment B) was assayed. The results are depicted in Fig. 2. In case of Experiment A, the radioactivity was discerned only in a type of ceramide,

normal acyl-unsaturated dihydroxy base (unsaturated base-containing ceramide), while in case of Experiment B, the radioactivity was detected only in a type of ceramide, normal acyl-saturated dihydroxy base (saturated base-containing ceramide). The radioactivity of unsaturated base-containing ceramide was almost twice as much as that of saturated base-containing ceramide. This observation was as well confirmed in the assay by autoradiography. These data show that 4-sphingenine and sphinganine have intactly been incorporated into unsaturated base-containing ceramide and into saturated base-containing ceramide, respectively, in the enzymatic synthesis of ceramide.

FIG. 2. Type of Ceramide Enzymatically Synthesized.

erythro-4-Sphingenine and palmitic acid-1-¹⁴C (Experiment A) and *erythro*-sphinganine and palmitic acid-1-¹⁴C (Experiment B) were enzymatically reacted, respectively, in the system shown in Table I. After incubation, ceramide was isolated with carrier, developed with chloroform-methanol (90: 10, by vol) on thin-layer silica gel G plate impregnated by 4% sodium borate, visualized with iodine vapor and scanned for radioactivity.

1. Normal acyl—saturated dihydroxy base
2. Normal acyl—unsaturated dihydroxy base
3. Hydroxy acyl—unsaturated dihydroxy base
4. Normal acyl—trihydroxy base

Time course of ceramide synthesis

The enzymatic reaction of *erythro*-4-sphingenine with labeled palmitic acid (Experiment I) and of *erythro*-sphinganine with labeled

FIG. 3. Time Course of Ceramide Synthesis.

Enzymatic reaction was carried out in a ten times scale of the system shown in Table I. *erythro*-4-Sphingenine and palmitic acid-1- ^{14} C were used in Experiment I, and *erythro*-sphinganine and palmitic acid-1- ^{14} C in Experiment II. Ceramide was isolated at intervals, developed with chloroform-acetic acid (90:10, by vol) on thin-layer silica gel G plate, located with iodine vapor and counted for radioactivity.

●—● Unsaturated base-containing ceramide (Experiment I)
 ●---● Saturated base-containing ceramide (Experiment II)

palmitic acid (Experiment II) was carried out in a ten times scale of that given in Table I. Ceramide formed was isolated and determined for radioactivity, which is depicted in Fig. 3. As seen here, unsaturated base-containing ceramide in Experiment I was always formed to a greater extent than saturated base-containing ceramide in Experiment B. It was noticed in both the experiments that ceramide formation once decreased on the synthetic way. This might be probably because ceramide initially formed was utilized for

conversion to the ceramide derivatives, such as ceramide phosphoryl choline (sphingomyelin), ceramide glycoside and so on, although the presumption needs a further investigation.

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