Studies on the Mechanism of 3-Ketosphinganine Synthetase*

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The biosynthesis of sphinganine and 4-0-hydroxysphinganine was studied in rat liver microsomes and whole cells of yeast (Hansenula ciferri). It was shown in both cases that the condensation of \[2,3,3-\text{H}_3\text{]serine and palmitic acid yielded long chain bases containing only two deuterium atoms, both of which were located on the terminal (C-1) carbon atom by combined gas-liquid chromatography/mass spectrometry. When the reaction with the liver microsomal system was carried out in \(\text{H}_2\text{O}\) with the protium species of serine, the sphinganine contained a deuterium atom on C-2. These results suggest that the synthesis of 3-ketosphinganine involves the replacement of the \(\alpha\)-hydrogen atom and the carboxyl group of serine by a proton from the medium and a palmitoyl group, rather than a previously proposed mechanism in which the \(\alpha\)-hydrogen of serine is retained. Some stereochimical requirements of 3-ketosphinganine synthetase are discussed.

It is generally accepted that the initial step in the biosynthesis of sphingolipid bases involves the condensation of serine and palmitoyl-CoA (1-3). In this reaction, catalyzed by 3-ketosphinganine synthetase (4), 3-ketosphinganine is formed with the concomitant release of carbon dioxide and CoA (2, 3). The serine is believed to be activated by the formation of a Schiff's base with pyridoxal phosphate in the enzyme-substrate complex; this is a tentative conclusion, based on indirect evidence (2, 5, 6) since definitive studies of the involvement of pyridoxal phosphate at the active site of the purified enzyme have not been made. Assuming that, in fact, pyridoxal phosphate is involved in the reaction, at least two broad mechanisms are possible for the formation of 3-ketosphinganine. By one of these mechanisms, discussed by Braun and Snell (2), the \(\alpha\)-hydrogen atom of serine is retained in a reaction initiated by decarboxylation of the Schiff's base complex, after which 3-ketosphinganine is formed by addition of the palmitoyl group and hydrolysis of the complex. This possibility would appear to be favored by the results obtained

In vivo by Weiss (7), involving subcutaneous injections of serine doubly labeled with tritium and \(^{13}\text{C}\) in the \(\alpha\) and \(\beta\) positions.

Alternatively, the reaction may be initiated by loss of the \(\alpha\)-hydrogen atom of serine to yield a stabilized Schiff's base complex that is further converted to 3-ketosphinganine by addition of the palmitoyl group, followed by decarboxylation. We have used \([2,3,3-\text{H}_3]\text{]serine to examine the mechanism of sphinganine biosynthesis with a crude microsomal system from rat liver, and also with whole yeast cells. The results support the latter mechanism, as the \(\alpha\)-hydrogen atom of serine was totally lost in the reaction. A preliminary report has been made of some of these data (8).

EXPERIMENTAL PROCEDURE

Materials

Sprague-Dawley rats (10 to 14 days old) were obtained from Spartan Research Animals, Haslett, Mich. Deuterium oxide and \([2,3,3-\text{H}_3]\text{]serine were purchased from Merck, Sharp and Dohme of Canada, Ltd. (distributed in the U.S. by Merck and Co., Inc., Rahway, N. J.). Synthetic \(d\)-erythro-sphinganine was purchased from Sigma Chemical Co., St. Louis, Mo. Hexamethyldisilazane and trimethylchlorosilane were obtained from Applied Science Laboratories, State College, Pa. Silica Gel G was obtained from EM Reagent Division, Brinkmann Instruments, Inc., Westbury, N. Y. Yeast extract, malt extract, and peptone were purchased from Difco Laboratories, Detroit, Mich. A slant culture of Hansenula ciferri (mating type F-60-10) was a gift from Dr. C. P. Kurtzman (Northern Regional Research Laboratory, Department of Agriculture, Peoria).

Methods

Gas-Liquid Chromatography—Analyses were carried out on a Hewlett-Packard model 5790A and 5997A gas chromatograph equipped with a flame ionization detector. A U-shaped glass column (6 feet \(\times\) 3 mm) was packed with 3% SE-30 on Supelcoport, 80 to 100 mesh (Supelco, Inc., Bellefonte, Pa.). Nitrogen was the carrier gas. The flash heater and detector were set about 20° above the column temperature, which
was 150° for analyses of serine derivatives and 230° for analysis of N-acetyl Me$_3$Si derivatives of sphingolipid bases.

**Mass Spectrometry**—Mass spectra were obtained with an LKB 9000 combined gas chromatograph/mass spectrometer. Conditions for gas-liquid chromatography were the same as those described above, except that helium was used as the carrier gas. Mass spectra were recorded at 70 eV, with an accelerating voltage of 3.5 kV and trap current of 60 μA. The mass spectrometer was interfaced to a PDP-8/1 minicomputer for on-line, real-time data collection and reduction as described by Brady et al. (11). The deuterium content of labeled compounds was determined by selected ion monitoring as described by Holland et al. (10).

**Incubations with Rat Liver Microsomal Fraction—Microsomes** were prepared from liver of 10 to 14-day-old rats as described by Brady et al. (11). The tissue were homogenized in 5 volumes of 0.25 M sucrose with a Teflon-homogenizer at 2-4°. The homogenate was centrifuged at 8,600 × g for 15 min, and the supernatant fraction was ultracentrifuged at 100,000 × g for 45 min. The homogenomic sediment was suspended in 1 M potassium phosphate buffer (pH 7.5) containing 1 mM pyridoxal phosphate and 1 mM diethiothreitol, equal to 0.2 of the original volume of homogenate.

Typical incubations contained 2 ml of the pure rat liver microsomal fraction in the presence of 20 μM of palmitic acid, 20 μg of Triton X-100, 7.5 μM of CoA, 10 μM of ATP, 1 μM of NADPH, 10 μM of pyridoxal phosphate, 10 μM of diethiothreitol, 25 μM of magnessium chloride, 40 μM of (2,3,3-3H$_3$)serine, and 10 μM of 0.1 M phosphate buffer. The total volume was 12 ml. When incubations were carried out in the presence of 10$^{-4}$M tetrahexylammonium ion, the protium form of serine (40 μM) was added to the mixture; 10 ml of phosphate buffer in 99% D$^2$H$_2$O (pH 7.5) was added instead of buffer prepared in natural water. All incubations were carried out for 1 hour at 37° in a Dubnoff metabolic shaking incubator (Precision Scientific Co., Chicago, Ill.).

**Isolation and Purification of Sphinganine—At the end of an incubation, 1 ml of 1 N sodium hydroxide was added, and the lipids were extracted with three 10-ml portions of diethyl ether. The combined ether extracts were washed once with 10 ml of water, dried over anhydrous sodium sulfate, and evaporated to dryness under a stream of nitrogen. The dry residue was dissolved in a minimal volume of chloroform and then applied on a Silica Gel G thin-layer plate with a sphinganine standard on an adjacent lane. After developing with chloroform/methanol/water (65/25/4, v/v), the plate was exposed to iodine vapor briefly and the area at the same R$_f$ value as that of the standard was recovered. Sphinganine was extracted from the silica gel with three portions (10 ml each) of chloroform/methanol (1/1, v/v). Solvents were evaporated in vacuo.

**Growth of Yeast and Isolation of Tetracetyl-4-hydroxysphinganine—**Astraeus hygrometricus was grown aerobically at 26-28° in liquid media (12) containing 0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1.0% glucose, 0.5% (2,3,3-3H$_3$)serine, and 10 ml of water. Growth was initiated by adding 0.1 ml of yeast previously grown to stationary phase and kept at 4° overnight. Cells were harvested by centrifugation. Tetracetyl-4-hydroxysphinganine and triacetylsphinganine were recovered from the cell paste by extraction with two portions (10 ml each) of acetone, and from the medium with two 10-ml portions of petroleum ether. The extracts were combined and evaporated to dryness in vacuo.

The crude acetylated bases were converted to free long chain base by a modification of the procedure described by Gaver and Sweeley (13). The lipid fraction was dissolved in 10 ml of methanol, and 2 ml of 6 N HCl was then added. The mixture was heated at 80° for 16 hours in a Teflon-lined screw-capped tube. After cooling, chloroform (20 ml) and water (4 ml) were added with mixing, the lower phase was washed once with 10 ml of Folch's upper phase (14), and was then dried in vacuo.

**Preparation of Volatile Derivatives for Gas-Liquid Chromatography—**Sphingolipid bases were N-acetylated by the procedure of Gaver and Sweeley (13) as follows. Methanol (2 ml) was used to dissolve the dry residue of long chain bases, the mixture was sonicated briefly, and acetic anhydride (0.2 ml) was added. The reaction mixture was left at room temperature for 10 min. Chloroform (4 ml) and water (1 ml) were then added, and the two phases were mixed thoroughly and separated by centrifugation. The lower phase was evaporated to dryness under a stream of nitrogen. Trimethylsilylation in a mixture consisting of pyridine/hexamethyldisilazane/trimethylchlorosilane (10/2/1, v/v) was carried out about 10 min prior to analysis by gas-liquid chromatography and mass spectrometry (13).

[2,3,3-3H$_3$]Serine was characterized by mass spectral analysis of the Me$_3$Si-N benzaldehyde derivative, prepared by adding pyridine/hexamethyldisilazane/trimethylchlorosilane (10/2/1, v/v) to the dry solid serine (1 mg/ml). When all the solid had dissolved, benzaldehyde (0.2 ml) was added to the solution, and it was left at room temperature or 30 min before analysis.

**RESULTS**

**Characterization of [2,3,3-3H$_3$]Serine by Mass Spectrometry**—The mass spectrum of the Me$_3$Si benzaldehyde adduct of [2,3,3-3H$_3$]serine is shown in Fig. 1. The molecular weight could be calculated from ions at m/e 325 (m/e 235), which result from loss of a methyl group and Me$_3$SiOH, respectively. The small peak at m/e 249 presumably results from loss of Me$_3$SiOH rather than Me$_3$SiOH but this conclusion has not been verified by measurement of exact mass; it is also possible that it represents a small amount of didideuterioserine in the sample. The latter explanation is supported by the small ion at m/e 324, which is probably derived from loss of a methyl group from one of the Me$_3$Si groups of the didideuterioserine derivative. The intensity at m/e 324 relative to that at m/e 325 indicates that the sample could not contain more than about 20% of the doubly labeled form.

The ion at m/e 237 (m/e 308) may be derived by cleavage of the C-2 and C-3 bond with simultaneous transfer of the Me$_3$Si group on C-3 to the functional group on C-2, accompanied by expulsion of formaldehyde. A similar transfer of a Me$_3$Si group

\[ \text{CD}_3—\text{CD}—\text{COOSiMe}_3 + \text{CD}_3—\text{COO} + \text{Me}_3\text{Si} \]

was carried out about 10 min prior to analysis by gas-liquid chromatography and mass spectrometry (13).

**Incorporation of [2,3,3-3H$_3$]Serine into Sphinganine by Rat Liver Microsomal Fraction** Since 3-ketosphinganine has been reported to be relatively unstable (17), incubations were carried out in the presence of NADPH for reduction of the keto...
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Group with NADPH-dependent 3-ketosphinganine reductase that is present as well in the microsomal fraction (18). Analysis of the Me_3Si-N-acetyl derivatives of the long chain base fraction by gas-liquid chromatography revealed a major peak with the same retention time as that of authentic Me_3Si-N-acetyl-DL-erythro-sphinganine, suggesting that the sphinganine formed in the microsomal preparation had the erythro configuration (19). The major ions in the mass spectrum (Fig. 2A) are consistent with those of Me_3Si-N-acetyl sphinganine previously reported (19), except that some of the ions are shifted to higher masses due to the presence of deuterium atoms in these fragments. The ions located at m/e 472, 247, and 157 in the mass spectrum of the product form are shifted to m/e 474, 249, and 159, respectively, in the product of microsomal conversion of [2,3,3-*H_3]serine to sphinganine, indicating that two deuterium atoms are incorporated into the long chain base. The ion at m/e 474 arises from the molecular ion by loss of a methyl group from one of the Me_3Si groups (20) and cannot be used to locate the deuterium atoms in the molecule. The ions at m/e 249 and 159, on the other hand, are derived from cleavage of the bond between C-2 and C-3 with transfer of MesSi on C-3 to the nitrogen atom by a cyclic transition (15, 20), as shown below. This indicates that both deuterium atoms are on C-1, or that one is on C-1 and one on C-2.

Incorporation of Deuterium from ^2H_2O into Sphinganine by Rat Liver Microsomal Fraction—To determine whether the hydrogen atom lost from serine during sphinganine synthesis can be replaced by a proton from the medium, the rat liver microsomal fraction was incubated with serine in *H_2O. The yield of sphinganine in several experiments averaged about 50% of that observed in natural water, suggesting a possible isotope effect. The mass spectrum of the Me_3Si-N-acetyl derivative is shown in Fig. 2B. The ion at M-15 was located at m/e 473, indicating that the product consisted primarily of a monodeuteriosphinganine. The characteristic ions, in the protium reference sample at m/e 384, 247, and 157, were multiplets in this product, however, and the ion normally at m/e 313 contained appreciable proportions of monodeuterio (about 30%) and dideuterio (about 30%) species. It is difficult to interpret the spectrum completely. Components at m/e 157 and 247 for the protium species undoubtedly reflect the fact that the added deuterium oxide was diluted by natural water in the microsomal fraction to an average of about 80% labeled form. Incorporation into the alkyl chain could be the result of exchange at the stage of 3-ketosphinganine or an equivalent enzyme-bound form. Of key importance are the presence of the bond between C-1 and C-2 with charge retention on the C-2 fragment and liberation of the C-1 fragment as a free radical yields an ion at m/e 384 (20). Since this ion is observed at the same m/e value in the mass spectrum of the proton form of sphinganine (19) it was concluded that the microsomal fraction of rat liver converted [2,3,3-*H_3]serine to sphinganine with complete loss of the deuterium on C-2 of the amino acid and retention of the other two deuterium atoms, which were located on C-1 of the sphinganine.

Homolytic cleavage of the bond between C-2 and C-3 with charge retention on C-3 results in loss of both deuterium atoms and yields an ion at m/e 313, the same mass as that observed with the protium form of sphinganine, which provides additional but indirect evidence that both deuterium atoms are on the fragment containing C-1 and C-2. Homolytic cleavage of the bond between C-1 and C-2 with charge retention on the C-2 fragment and liberation of the C-1 fragment as a free radical yields an ion at m/e 384 (20). Since this ion is observed at the same m/e value in the mass spectrum of the proton form of sphinganine (19) it was concluded that the microsomal fraction of rat liver converted [2,3,3-*H_3]serine to sphinganine with complete loss of the deuterium on C-2 of the amino acid and retention of the other two deuterium atoms, which were located on C-1 of the sphinganine.

Incorporation of Deuterium from *H_2O into Sphinganine by Rat Liver Microsomal Fraction—To determine whether the hydrogen atom lost from serine during sphinganine synthesis can be replaced by a proton from the medium, the rat liver microsomal fraction was incubated with serine in *H_2O. The yield of sphinganine in several experiments averaged about 50% of that observed in natural water, suggesting a possible isotope effect. The mass spectrum of the Me_3Si-N-acetyl derivative is shown in Fig. 2B. The ion at M-15 was located at m/e 473, indicating that the product consisted primarily of a monodeuteriosphinganine. The characteristic ions, in the protium reference sample at m/e 384, 247, and 157, were multiplets in this product, however, and the ion normally at m/e 313 contained appreciable proportions of monodeuterio (about 30%) and dideuterio (about 30%) species. It is difficult to interpret the spectrum completely. Components at m/e 157 and 247 for the protium species undoubtedly reflect the fact that the added deuterium oxide was diluted by natural water in the microsomal fraction to an average of about 80% labeled form. Incorporation into the alkyl chain could be the result of exchange at the stage of 3-ketosphinganine or an equivalent enzyme-bound form. Of key importance are the presence

Fig. 1. Mass spectrum of the benzaldehyde Schiff's base of 1,3-di-O-trimethylsilyl-[2,3,3-*H_3]serine.

Fig. 2. Mass spectra of 1,3-di-O-trimethylsilyl-N-acetyl sphinganine recovered after incubation of rat liver microsomal fraction with [2,3,3-*H_3]serine (A) and *H_2O (B). Details of the experimental conditions are described under "Experimental Procedure."
of m/e 158 and m/e 248, the lack of label in the C-1 fragment ion at m/e 103, and the location of the predominant fraction of the (M-a) + ion at m/e 385, which together indicate significant incorporation of deuterium at C-2 in the sphinganine and none at C-1. There were several ions in the region of M-15 in addition to m/e 472, but their intensities were lower.

Incorporation of [2,3,3-H3]Serine into Sphingolipid Bases by Yeast—The yeast, Hansenula ciferri, was shown by Stodola et al. (21) to produce a relatively large amount of acetylated 4-hydroxysphinganine and a small amount of acetylated sphinganine. To determine whether this strain of yeast synthesizes sphingolipid bases from serine by the same pathway as that observed in the mammalian system, regarding the loss or retention of the α-hydrogen atom of the serine, [2,3,3-H3]serine was incubated with yeast cells, and the pattern of deuterium labeling in the isolated sphingolipid bases was studied. Mass spectra of Me3Si-N-acetylsphinganine and Me3Si N-acetyl 4-hydroxysphinganine from the yeast are shown in Fig. 3. The mass spectrum of the sphinganine derivative was not exactly identical with that of a reference sample of the base or that from the rat liver microsomal fraction after incubation with the labeled serine, probably because of some minor contamination of the peak from the gas chromatograph. Nevertheless, it could be concluded that the α-deuterium atom of the serine was largely lost during conversion of the serine to sphinganine in vivo by the yeast. The location of major fragmentation and rearrangement ions at m/e 159, 249, 384, and 474 establishes that the principal sphinganine product contained two deuterium atoms on C-1, as argued earlier. Using the intensities of these masses and those 1 mass unit higher, the ratios of peaks that could have been derived from trideuteriosphinganine to those from the dideuteriosphinganine were 0.21, 0.25, 0.43, and 0.47, respectively, for m/e 160/159, 250/249, 385/384, and 475/474. The normal isotopic abundance in a reference sample of sphinganine gave ratios of 0.14, 0.26, 0.21, and 0.32 for the corresponding ion pairs 2 mass units lower. The differences indicated some excess abundance at m/e 475 and 385, which was of the same magnitude as that observed for the alkyl chain and C-3 at m/e 314/313 (0.23 in control and 0.43 for labeled product). The yeast cells were apparently able to convert some of the labeled serine to labeled palmitate, which was incorporated into the alkyl chain of the long chain base. Certainly there was no evidence from the mass spectrum for significant synthesis of sphinganine containing all three deuterium atoms of the labeled serine.

In the mass spectrum of Me3Si-N-acetyl 4-hydroxysphinganine, the ion at M-15 was shifted from m/e 560, where it occurs in the mass spectrum of the protium form (22), to m/e 562, indicating the presence of two deuterium atoms in the molecule. Losses of fragments c, b, and a (Fig. 3) yielded ions at the same m/e values as observed in a reference sample; thus, both deuterium atoms must be located on C-1. This finding is consistent with the observation that sphinganine from yeast also contained two deuterium atoms on C-1 and with the proposed pathway of 4-hydroxysphinganine synthesis (23, 24).

DISCUSSION

Loss of deuterium from C-2 of [2,3,3-H3]serine in the synthesis of sphinganine and 4-hydroxysphinganine has been observed with a rat liver microsomal system and whole cells of yeast. Our findings are not in agreement with those of Weiss (7), who had concluded from experiments carried out in vivo with suckling rats that the α-hydrogen atom of serine is retained. Examination of his data indicates, however, that in one-half of the experiments using serine labeled on C-2 and C-3 with tritium, label on C-2 of the sphingosine isolated from brain was relatively much less than that incorporated on C-1. Furthermore, incorporation of tritium oxide into sphingosine in vivo was always greatest in position C-2. Other problems associated with these experiments, such as an extremely low yield of formic acid from C-2 of sphingosine, the indirect method used to determine the distribution of label in the serine substrate, and the recovery of 14C in C-2 of sphingosine after administration of [3-14C]serine to the rats, make it difficult to interpret the data that Weiss presented.

Assuming that pyridoxal phosphate is involved in the mechanism of 3-ketosphinganine synthetase, we have concluded that activation of the serine as the Schiff's base complex III or a stabilized form of III, as shown in Fig. 4 (Pathway B), is more appropriate than direct decarboxylation of complex I or a stabilized carbanion form of this complex (Pathway A). Nonenzymatic loss of deuterium from position α of the

![Fig. 3. Mass spectra of 1,3-di-tri-methylsilyl-N-acetysphinganine (A) and 1,3,4-tri-O-trimethylsilyl-N-acetyl-4-hydroxysphinganine (B) recovered after incubation of Hansenula ciferri with [2,3,3-H3]serine as described under "Experimental Procedure."](https://example.com/fig3.png)
mechanism of 3-ketosphinganine synthetase

**Fig. 4.** Alternative mechanisms for the biosynthesis of 3-ketosphinganine from serine and palmitoyl-CoA. Functional groups on the heterocyclic ring of pyridoxal phosphate have been omitted for simplicity. Pathway A, the α-hydrogen atom of serine is retained in 3-ketosphinganine. Pathway B, loss of the α-hydrogen atom of serine precedes decarboxylation and addition of the palmitoyl group.

The synthesis of long chain sphingolipid bases by Pathway B implies that 3-ketosphinganine synthetase should be classified, according to the scheme proposed by Braunstein (28), as a type involving initial labilization of the α-hydrogen atom, with decarboxylation resulting subsequently because of the formation of an unstable 2-imino-3-oxo acid. The reaction is not to be confused with enzymatic decarboxylation of amino acids, in which the α-hydrogen atom is not lost (29). Biosynthesis by this proposed mechanism is consistent with the report of DiMari et al. (30) that decarboxylation of serine in sphingolipid base synthesis is dependent on added palmitoyl-CoA.

When conclusive evidence has been obtained for the participation of pyridoxal phosphate in 3-ketosphinganine synthetase, further examination of the stereochemistry of the reaction will be justified. Since the chiral carbon atom in serine has the same absolute configuration (S) as at C-2 in sphinganine, it can be predicted that the two key steps in the reaction, substitution of the α-hydrogen atom by a palmitoyl group and substitution of the carboxyl group by a proton from the medium, will proceed by a combination of an inversion and a retention of configuration. Other possibilities, involving either inversion or retention of configuration at both steps, are ruled out because they would lead to the wrong stereoisomer of sphinganine.

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