A Water-Soluble Homodimeric Serine Palmitoyltransferase from Sphingomonas paucimobilis EY2395T Strain: Purification, Characterization, Cloning, and Overproduction*

Hiroko Ikushiro, Hideyuki Hayashi and Hiroyuki Kagamiyama¶

Department of Biochemistry, Osaka Medical College, Takatsuki, Osaka 569-8686, Japan.

Running Title:
Water-Soluble Homodimeric Serine Palmitoyltransferase
SUMMARY

Serine palmitoyltransferase (SPT, EC 2.3.1.50) is a key enzyme in sphingolipid biosynthesis and catalyzes the decarboxylative condensation of L-serine and palmitoyl coenzyme A to 3-ketodihydrosphingosine. We found that the gram-negative obligatory aerobic bacteria *Sphingomonas paucimobilis* EY2395T have significant SPT activity and purified SPT to homogeneity. This enzyme is a water-soluble homodimeric protein unlike eukaryotic enzymes, known as heterodimers composed of tightly membrane-bound subunits, named LCB1 and LCB2. The purified SPT shows an absorption spectrum characteristic of a pyridoxal 5’-phosphate-dependent enzyme. The substrate specificity of the *Sphingomonas* SPT is less strict than the SPT complex from Chinese hamster ovary cells. We isolated the SPT gene encoding 420 amino acid residues (M, 45,041) and succeeded in overproducing the SPT protein in *Escherichia coli*, in which the product amounted to about 10–20% of the total protein of the cell extract. *Sphingomonas* SPT shows about 30% homology with the enzymes of the α-oxamine synthase family, and amino acid residues supposed to be involved in catalysis are conserved. The recombinant SPT was catalytically and spectrophotometrically indistinguishable from the native enzyme. This is the first successful overproduction of an active enzyme in the sphingolipid biosynthetic pathway. *Sphingomonas* SPT is a prototype of the eukaryotic enzyme and would be a useful model to elucidate the reaction mechanism of SPT.
Sphingolipids are ubiquitous membrane components having a backbone structure called long-chain base (LCB) which is N-fatty acylated and linked to various polar head groups. In eukaryotes, sphingolipids such as sphingosine, sphingosine-1-phosphate, and ceramide are known to play important roles as second messengers in various cellular events including proliferation, differentiation, senescence, apoptosis, and immune response (1).

Serine palmitoyltransferase (SPT: EC 2.3.1.50) catalyzes the pyridoxal 5′-phosphate (PLP)-dependent condensation reaction of L-serine with palmitoyl coenzyme A (CoA) to generate 3-ketodihydrosphingosine (KDS). This reaction is the first committed step in sphingolipid biosynthesis, utilizing substrates that are shared by other metabolic pathways, and has an activity lower than those of other enzymes involved in sphingolipid biosynthesis. Therefore, SPT is thought to be a rate-determining enzyme in the sphingolipid synthetic pathway and, accordingly, a key enzyme regulating the cellular sphingolipid content (2).

Eukaryotic SPTs have been known as membrane-bound proteins, enriched in the endoplasmic reticulum (ER) with their catalytic sites facing the cytosol (3). Genetic studies have shown that two different genes, \(\text{LCB1}\) and \(\text{LCB2}\), are required for SPT activity in the yeast \textit{Saccharomyces cerevisiae} (4–6). Subsequently, mammalian homologues of the \(\text{LCB}\) genes from mouse, human and Chinese hamster ovary (CHO) cells have also been reported (7–9). The biochemical studies using the CHO cell mutants demonstrated that both the LCB1 and LCB2 proteins are subunits of SPT (10, 11). There is a high sequence homology between LCB1 and LCB2, and they are classified as new members of the PLP-dependent \(\alpha\)-oxamine synthase subfamily (6). Based on the finding that LCB1 does not have a PLP-binding motif while LCB2 carries a lysine residue expected to form a Schiff base with PLP, LCB1 and LCB2 have been speculated as a regulatory unit and a catalytic unit, respectively (6, 10, 11).
is, however, no biochemical explanation for the regulation mechanism of the SPT reaction at present. The purification of the native form SPT from any eukaryotes has not been successful because of the extremely low content and instability of this enzyme. We, too, tried to construct the expression system of the mouse SPT complex in *E. coli* (12). Affinity-tagged forms of mouse LCB proteins lacking the membrane-anchor regions were co-expressed in *E. coli* as partially soluble proteins, but the purified SPT complex did not show enzymatic activity. As the only achievement, Hanada *et al.* obtained an active SPT complex from the CHO cell mutants expressing a hexahistidine-tagged LCB1 protein (11). However, it is very difficult to obtain a sufficient amount of the active enzyme for detailed enzymological analysis from such purification sources.

Although sphingolipids are not typical membrane constituents in prokaryotes, there are some exceptions. In strict anaerobes such as the genera *Bacteroides*, *Porphyromonas* and *Prevotella*, high levels of sphingolipids were found; in some species their contents in the total extractable lipid came to 70% (13, 14). It has been reported that *Bacteroides melaninogenicus* contains a water-soluble SPT, but the purification of this enzyme was not successful (15). The gram-negative obligatory aerobic bacteria *Sphingomonas* and *Sphingobacterium* are the genera whose lipid composition and structure of their sphingolipids have been investigated most extensively (16, 17). In cells of *Sphingomonas paucimobilis*, the lipopolysaccharide in their outer membrane is completely substituted by sphingoglycolipid, and its proposed structure is 1-\(\text{O-D-glucuronosyl-2-N-2'}\)-hydroxymyristoyldihydrosphingosine (glucuronosyl ceramide) (18, 19).

We found that cells of *S. paucimobilis* EY2395\textsuperscript{T} and *Sphingobacterium spritivorum* EY3101\textsuperscript{T} contain significant SPT activity and report here the purification to homogeneity and characterization of SPT from *S. paucimobilis* EY2395\textsuperscript{T}. The primary structure of the enzyme has been deduced from the cloned gene, and the SPT protein was successfully
overproduced in *E. coli*. The results show that SPT from *S. paucimobilis* is a prototype of eukaryotic enzymes.

**MATERIALS AND METHODS**

*Chemicals.* L-Serine and other natural L-amino acids were obtained from Nacalai Tesque (Kyoto, Japan). Palmitoyl CoA and lauroyl CoA were from Funakoshi (Tokyo, Japan). Myristoyl CoA, *n*-heptadecanoyl CoA, stearoyl CoA, arachidoyl CoA, palmitoleoyl CoA, oleoyl CoA, *O*-phospho-L-serine, α-methyl-D,L-serine, and, L-serine methylester were from Sigma. Serinol and serinamide were from Aldrich. 3-Hydroxypropionic acid was from Tokyo Kasei Kogyo (Tokyo, Japan). 4-(2-Aminoethyl)-benzenesulfonyl fluoride (AEBSF) was from Roche Molecular Biochemicals. The LMW gel filtration calibration kit, gel filtration calibration kit, Phenyl Sepharose CL-4B, PD-10, Superose™ 12, and MonoQ™ HR 5/5 were from Amersham Pharmacia BioTech. DEAE-Toyopearl 650M, Butyl Toyopearl 650M were from Tosoh (Tokyo, Japan). The CHT-II Econo-Pac® cartridge was from Bio-Rad Laboratories. Competent *E. coli* JM109 was purchased from Nippon Gene (Tokyo, Japan). *E. coli* BL21(DE3) pLysS and plasmid pET21b were from Novagen. A plasmid pUC118 was from TaKaRa (Kyoto, Japan). All other chemicals were of the highest grade commercially available.

*Bacterial Strains and Growth Conditions.* *S. paucimobilis* EY2395<sup>T</sup> and *S. spiritivorum* EY3101<sup>T</sup> were gifts from Dr. Eiko Yabuuchi, Aichi Medical University, Aichi, Japan. Each strain was grown in 1 liter of LB medium in 5-liter flasks at 30°C and 90 rpm. Cells were harvested in the late exponential growth phase (after 8–9 h) and stored at −20°C before use.
**Assay of the Enzyme Activity.** In the purification steps, the SPT activity was measured according to the methods of Williams et al. (20) with minor modifications. The enzyme solution was incubated in 100 µl of a standard SPT reaction buffer (100 mM HEPES-NaOH buffer (pH 7.5) containing 0.1 mM EDTA, 5 mM dithiothreitol, 10 µM PLP, 0.4 mM palmitoyl CoA, and 2 mM L-[³H] Ser (37 kBq/mmol)) at 37 °C for 10 min. The reaction was terminated by addition of 100 µl of 2 N NH₄OH. The lipids were extracted with CHCl₃/CH₃OH (2:1 (v/v)), and the radioactivity of the [³H]-KDS that formed was measured. The radioactivity extracted from enzyme-negative control was regarded as a background. For steady-state kinetic analysis, [¹⁴C]-KDS was added to the extraction solvent as the internal standard to estimate recoveries throughout the entire procedure. [¹⁴C]-KDS was enzymatically synthesized from [¹⁴C]-serine using purified SPT. For TLC analysis, [¹⁴C]-serine (10 kBq/mmol) or [¹⁴C]-palmitoyl CoA (10 kBq/mmol) was used as the substrate. The lipids were extracted and separated on TLC plates (Silica Gel 60, Merck) with a solvent of chloroform, methanol, and 2N NH₄OH (40:10:1 v/v). [¹⁴C]-dipalmitoylphosphatidylcholine (DPPC) was added to the extraction solvent as the internal standard. Radioactive lipids on the TLC plates were visualized and their relative radioactivity was determined using a BAS2000 Image Analyzer™ (Fujifilm, Tokyo, Japan).

**Purification of SPT.** All purification procedures were performed at 4°C. The buffer of 20 mM potassium phosphate (pH 6.5) containing 0.1 mM EDTA, 5 mM DTT, 0.1 mM AEBSF (protease inhibitor) and 20 µM PLP was used in all the following procedures except for the FPLC steps. The cells (30 g wet weight) were suspended in 300 ml of the buffer and disrupted sonically (Branson Sonic Power, Sonifier model 450) at 20 kHz for 9 min. The intact cells and debris were removed by centrifugation (10,000 × g, 40 min). After the addition of (NH₄)₂SO₄ (final concentration, 30% saturation) and centrifugation (100,000 × g, 60 min), the supernatant solution was applied to a Phenyl Sepharose CL-4B
column (2.5 × 20 cm) equilibrated with the buffer containing 30%-saturated (NH₄)₂SO₄. The enzyme activity was eluted with 1 liter of linearly decreasing (NH₄)₂SO₄ concentrations (30 to 0%). After the addition of (NH₄)₂SO₄ (final concentration, 30% saturation) again, the active fractions were applied to a Butyl-Toyopearl 650M column (2.5 × 20 cm) equilibrated with the buffer containing 30%-saturated (NH₄)₂SO₄. The enzyme activity was eluted with 1 liter of linearly decreasing (NH₄)₂SO₄ concentrations (30 to 0%). The pooled active fractions were dialyzed against 2 liters of the buffer. The dialysate was then applied to a DEAE-Toyopearl 650M column (2.5 × 20 cm) equilibrated with the buffer. The enzymes were eluted with 1 liter of linear gradient from 0 to 500 mM NaCl. The pooled active fractions were concentrated and dialyzed against 1 liter of 10 mM potassium phosphate buffer (pH 6.5) containing 0.1 mM EDTA, 5 mM DTT, 0.1 mM AEBSF and 20 μM PLP. The dialysate was applied to a CHT-II column (1.35 × 3.5 cm), which had been connected to an FPLC system, equilibrated with the same buffer. The enzyme was eluted with a 50-ml linear gradient from 10 to 200 mM potassium phosphate (pH 6.5). The eluted enzyme was dialyzed against 1 liter of 50 mM Tris-HCl buffer (pH 7.5) and then applied to a Mono-Q™HR column (0.55 × 5 cm) equilibrated with the same buffer. The column was washed with 50 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl and eluted with a 25-ml linear gradient from 150 to 200 mM NaCl (pH 7.5). The active fractions were combined, concentrated to 1 ml, filtered, and stored at 4°C. When 30 g of cells were used as the starting material, 350 μg of a pure preparation of SPT was obtained.

**Amino Acid Sequencing.** The purified enzyme (8.9 nmol) was carboxymethylated, desalted and digested at 37°C for 30 min with lysyl endopeptidase (33 pmol). The digested peptides were isolated by reversed-phase high performance liquid chromatography on a Cosmosil 5C18 AR-II column (2.0 × 150 mm) with a linear gradient from 0 to 60% acetonitrile containing 0.05% trifluoroacetic acid. The amino acid sequences were
determined using a Hewlett Packard G1005A protein sequencing system.

Isolation and Sequencing of Genomic DNA Clones Encoding SPT. Based on the amino acid sequences of the SPT peptides, we synthesized degenerate oligonucleotides to obtain partial DNA fragments encoding the SPT gene by PCR against genomic DNA from S. paucimobilis. The oligonucleotides were 5' - GA(TC)GC(TCAG)CC(TCAG)GA(TC)AT(TCA)GCICC-3' and 5' - GC(TCAG)GT(TG)AA(TGA)AT(AG)TA(TCAG)GG-3' corresponding to the amino acid sequences DAPDIAP and PYIFTA, respectively (Fig. 5, dashed lines). The genomic DNA of S. paucimobilis was prepared by a standard method (21). PCR was performed using LA Taq DNA polymerase (TaKaRa, Kyoto, Japan) under the following conditions: 30 cycles of 94˚C for 30 s, (40+ t)˚C for 30 s, and 72˚C for 1 min, then 72˚C for 10 min, where t denotes that the annealing temperature was successively increased by 0.25˚C at each cycle. The PCR product was directly cloned into a pCRII vector (Invitrogen, Netherlands) and sequenced by the “Dye-Terminator Cycle Sequencing” kit and an ABI 373A DNA sequencer (Perkin-Elmer). To obtain the full-length SPT gene, a genomic DNA library (1 × 10^6 recombinants) was screened with the ^32P-labeled PCR product (846 bp) as a probe. The library was constructed as follows: A genomic DNA from S. paucimobilis was partially digested by Sau3AI, 2.5- to 3.5-kb fragments were agarose-gel purified and ligated into BamHI-digested pUC118, and these constructs were used to transform E. coli JM109. Labeling of the probe and detection of hybridized fragments were performed using the BcaBEST™ labeling kit (TaKaRa, Kyoto, Japan) and Quick-Hyb® hybridization solution (Stratagene), respectively. Twelve positive clones of the first screening were isolated. Restriction mapping and partial sequencing revealed that all 12 clones were derived from the same gene. The complete DNA sequence was determined for both strands of the three longest clones.
**Gel filtration.** The enzymes were applied to a Superose™ 12 and fractionated at a flow rate of 0.5 ml/min with an FPLC system. Bovine pancreas ribonuclease A ($M_r$ 13,700), bovine pancreas chymotrypsinogen A ($M_r$ 25,000), hen egg ovalbumin ($M_r$ 43,000), bovine serum albumin ($M_r$ 67,000), rabbit muscle aldolase ($M_r$ 158,000), bovine liver catalase ($M_r$ 232,000), horse spleen ferritin ($M_r$ 440,000), bovine thyroid thyroglobulin ($M_r$ 669,000), *E. coli* aspartate aminotransferase ($M_r$ 43,573 × 2), and *E. coli* branched-chain amino acid aminotransferase ($M_r$ 33,962 × 6) were used as standard proteins.

**Spectrophotometric measurement.** The absorption spectra of SPT were recorded with a Hitachi spectrophotometer U-3300 at 25°C. The buffer solution for the absorption measurements contained 50 mM HEPES-NaOH (pH 7.5) and 0.1 mM EDTA. SPT was equilibrated with the above buffer by gel filtration using a PD-10 (Sephadex G-25) column (Amersham Pharmacia Biotech) prior to the measurement.

**Other Methods.** Protein concentration during the purification procedure was determined with a BCA protein assay kit (Pierce Chemical) using bovine serum albumin as a standard. The protein concentration of purified SPT was determined spectrophotometrically using a molar extinction coefficient of $2.83 \times 10^4$ M$^{-1}$ cm$^{-1}$ at 280 nm for the PLP form of the enzyme. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with an SDS-Tris system using 10% polyacrylamide gel according to the procedure described by Laemmli (22).

**RESULTS**

*SPT activity in S. paucimobilis and S. spiritivorum.* *S. paucimobilis* and *S. spiritivorum* contain a large amount of sphingolipids as their cell component (16,17), and
thus sphingolipid biosynthetic enzymes have been expected to exist in these bacteria. Cell-free extracts (100,000 \times g supernatants) prepared by sonication of cells of these strains were examined for SPT activity by incubation with \[^{14}C\] -serine and unlabeled palmitoyl CoA. The lipids were extracted and subjected to TLC analysis. Some radioactive products were detected for each extract (Fig. 1). One of these products was indistinguishable in migration from KDS formed by mouse liver microsomes. Cell-fractionation experiment indicates that the SPTs of these bacteria are water-soluble enzymes (Fig. 1). Because the extracts of \textit{S. paucimobilis} showed higher SPT activity than that of \textit{S. spiritivorum}, \textit{S. paucimobilis} was selected as the starting material for the purification of SPT.

\textbf{Purification of SPT from \textit{S. paucimobilis}.} The enzyme was purified to homogeneity by five steps of column chromatography. As shown in Fig. 2, the purified SPT showed a single protein band with an apparent \textit{M}_r of about 50,000 on SDS-PAGE. Cell-free extracts of \textit{S. paucimobilis} contain a large amount of yellow pigment, which was very difficult to remove by the initial two hydrophobic column chromatographies. Table I summarizes the purification of SPT from the \textit{S. paucimobilis} extract. About 350 \mu g of the protein could be obtained from 6 liters of \textit{S. paucimobilis} culture. The purification yield was reproducibly over 100% probably because some coexisting inhibitory materials were removed as the purification proceeded. The purified enzyme could be stored at 4°C in sterile capped vials for up to 2 months in a 20 mM Tris-HCl buffer (pH 7.5) without loss of activity.

\textbf{Physical Characterizations.} The \textit{M}_r of the purified enzyme was estimated to be about 90,000 by gel filtration. Electrospray ionization mass spectrometry (ESI-MS) gave a signal at \textit{m/z} 44,916. These results show that the native SPT from \textit{S. paucimobilis} has a dimer structure composed of two identical subunits. The purified SPT had an absorption spectrum with two peaks at 338 and 426 nm other than the protein absorption peak at 278
nm in 50 mM HEPES-NaOH (pH 7.5) containing 0.1 mM EDTA (Fig. 3). These absorption peaks are characteristic of PLP enzymes, which contain the cofactor bound to the ε-amino group of a lysine residue in the active site (23). The addition of serine to the enzyme gave rise to an intense absorption band at 426 nm and a less intense band at 338 nm, indicates that 338 nm absorption represents the active species and that the external aldimine complex was formed.

_Catalytic Properties and Substrate Specificity._ The time course of KDS formation by the purified SPT was almost linear for at least 20 min, and this activity was proportional to the amount of the purified enzyme up to 100 ng under the reaction conditions mentioned above. The optimum pH for KDS formation was 7.5–8.0. SPT from _S. paucimobilis_ was not inhibited by halide ions. No inhibition of SPT activity was found with relatively high concentrations of palmitoyl CoA (up to 10 mM).

The substrate specificity is summarized in Tables II and III. To assess the specificity for amino acids, we determined the production of [14C]-KDS derivatives from [14C]-palmitoyl CoA with 20 natural amino acids or various serine analogs. Purified SPT (50 ng) was incubated with 0.8 mM [14C]-palmitoyl CoA and 4 mM various amino as the substrate. The activity was detected only for serine among the natural amino acids examined (data not shown). Serine methylester and O-phosphoserine were converted to KDS at lower rates (5% and 3% of the mean levels of KDS formed with serine, respectively). However, at present, we cannot preclude the possibility that serine derived from serine methylester by hydrolysis was metabolized to KDS. We also examined the inhibition effect of excess amounts of nonradioactive competitors on the [3H]-KDS production from 0.1 mM L-[3H]-serine (Table II). [3H]-KDS production was inhibited about 80% by 4mM nonradioactive L-serine under this assay condition. The inhibition of the [3H]-KDS production by 4 mM each of other natural amino acids except for cysteine.
was 40% or less. The effect of cysteine can be ascribed to the thiazolidine formation of
cysteine with PLP (23). O-Phosphoserine was the most effective, and α-methy-DL-serine,
3-hydroxypropionate, and serine methyester followed in this order. Seriamide and serinol
were essentially inert. Among various acyl CoAs examined, palmitoyl CoA was the best
substrate (Table III). The unsaturated bond of the acyl chain of CoA analogs did not
significantly influence the SPT activity. The activity-chain length profile showed a bell-
shaped pattern, which peaked around C\textsubscript{16}.

**Kinetic Parameters of Native SPT.** Steady-state analysis of the purified SPT was
carried out. The kinetic parameters for SPT were determined with respect to serine and
palmitoyl CoA. As shown in Fig. 4, the experimental data were analyzed according to the
Ordered Bi Bi mechanism (24). The $K_m$ values for serine and palmitoyl CoA were 4.2 mM
and 0.87 mM, respectively, and the $k_{cat}$ value was 140 min$^{-1}$ (Table IV).

**Cloning of the SPT Gene.** The nucleotide sequence of one of the three clones
sequenced, \textit{SPT1} (GenBank accession no. AB055142), is shown in Fig. 5. \textit{SPT1} contains a
1260-bp open reading frame (65% GC content) encoding a protein of 420 amino acid
residues. The amino-terminal protein sequence of purified SPT was Thr-Glu-Ala-Ala-Ala-,
indicating that the first methionine of purified SPT had been cleaved by processing. The
deduced amino acid sequence of SPT is also shown in Fig. 5. The molecular weight of
44,916 obtained by ESI-MS is in good agreement with the value of 44,910 calculated from
the deduced amino acid sequence of SPT without the first methionine within experimental
class error.

**Sequence Comparisons.** The non-redundant databases at the National Center for
Biotechnology Information were scanned for amino acid sequences similar to the \textit{S.}
\textit{paucimobilis} SPT sequence using the BLAST algorithm (25). The predicted \textit{S.}
\textit{paucimobilis} SPT protein is related to proteins grouped as the α-oxamine synthase family.
This gene family includes eukaryotic SPT subunits, 5-aminolevulinic acid synthase (ALAS) in heme biosynthesis, 8-amino-7-oxononanoate synthase (AONS) in biotin biosynthesis, and 2-amino-3-ketobutyrate CoA ligase (KBL) in the threonine utilization pathway, all of which catalyze chemically similar reactions using the cofactor PLP (26-30). The amino acid sequence alignment of *S. paucimobilis* SPT and mouse LCB1 and LCB2 proteins is shown in Fig. 6. Overall sequence homology is found between these proteins except for the N-terminal transmembrane region of mouse LCB proteins. SPT has 27% identity and 48% similarity with mouse LCB1 and 31% identity and 49% similarity with mouse LCB2. Fig. 7 shows the amino acid sequence alignment of *S. paucimobilis* SPT and three prokaryotic enzymes in the α-oxamine synthase family. There are 33% amino acid identity and 56% similarity between SPT and AONS of *Bacillus sphaerious*, 33% identity and 54% similarity between SPT and KBL of *Bacillus subtilis*, and 36% identity and 55% similarity between SPT and ALAS of *Agrobacterium radiobacter*.

**Expression of the SPT Gene in E. coli.** In order to construct the expression system for *S. paucimobilis* SPT in *E. coli*, the internal *Nde* I restriction site (↑↑↑↑ATGCAT) of *SPT1* was changed to ATGCAC without changing the codons by site-directed mutagenesis, and the new restriction sites, *Nde* I and *Hin*dIII, were introduced to *SPT1* at the translation initiation and termination sites, respectively, by PCR. The modified *SPT1* was ligated into a pET21b vector, and the recombinant plasmid was used to transform *E. coli* BL21 (DE3) pLysS cells. The SPT produced was functional, and the product amounted to about 10–20% of the total protein in the crude extract of *E. coli*. Because of the overproduction of the protein, the expressed SPT would be partially in the apo form, but it could be converted to the holoenzyme by addition of PLP to the cell lysate. The recombinant enzyme was purified to homogeneity using DEAE-Toyopearl, Butyl-Toyopearl, and hydroxyapatite column chromatographies. The recombinant SPT provided a 50-kDa band on SDS-PAGE.
The N-terminal sequence of the purified enzyme, Thr-Glu-Ala-Ala-Ala-, agreed with that of the native enzyme. Thus, the first methionine of the recombinant SPT was similarly removed by processing. The purified SPT showed a peak with $m/z = 44,899$ on ESI-MS, which is in agreement with the native SPT within experimental error. The catalytic properties of the recombinant SPT was the same as that of the native SPT; there is no significant difference between the two enzymes in their steady-state kinetic parameters (Table IV).

**DISCUSSION**

Because large scale cultivation of strict anaerobic bacteria is difficult and the unsuccessful purification of the *B. melaninogenicus* SPT has already been reported (15), we searched for aerobic bacteria containing sphingolipids and chose *S. paucimobilis* as an alternative purification source. *S. paucimobilis* contains sphingolipids which form more than 30% of the total extractable lipid (16, 19). There is a report that $^{14}$C-labeled fatty acids or amino acids were incorporated into the sphingolipids of *S. paucimobilis* (31). These findings suggest the possibility for this bacterium to contain SPT.

The most important finding is that the *S. paucimobilis* SPT is water-soluble and is a dimer composed of two identical subunits. All the eukaryotic enzymes examined so far are heterodimers, and both of the subunits are membrane-bound proteins. Membrane localization of eukaryotic SPT complexes seems reasonable because the product of this enzyme is a hydrophobic lipid incorporated into membranes. The relationship between cellular localization and the mechanism of the product release of bacterial SPT is the next subject of research; How is the reaction product, KDS, transferred to the membrane? Does
SPT interact with membrane *in vivo*? Do other sphingolipid biosynthetic enzymes also exist as water-soluble form in *S. paucimobilis*? As for the subunit composition, we can reasonably consider that bacterial SPTs are homodimers. Judging from the high sequence homology between LCB1 and LCB2, ancestral SPT would have been a homodimer, and the gene was duplicated at some point early in the evolution of eukaryotes. The functional benefit for the heterodimerization, or the role of the LCB1 subunit, however, remains unknown.

The purified enzyme showed an absorption spectrum characteristic of the PLP enzyme. The ratio in the peak height of the PLP-derived absorption bands (338 and 426 nm) to the protein-derived band (278 nm) indicates that SPT binds two PLP molecules per dimer. *S. paucimobilis* SPT is not inhibited by halide ions, although SPT activity of *B. melaninogenicus* has been reported to be significantly inhibited (15). The inhibition by high concentrations of palmitoyl CoA, which has been observed for the eukaryotic enzymes, was not detected in *S. paucimobilis* SPT (Fig. 4) as well as in the *B. melaninogenicus* enzyme (11, 15, 32).

The substrate recognition of *S. paucimobilis* SPT was not so strict, especially for the acyl CoA substrate, compared to the eukaryotic enzymes (11, 32). This observation might reflect the difference in the biological functions of sphingolipids between prokaryote and eukaryote. In eukaryotes, because sphingolipid metabolites take part in the intra- and intercellular signal transduction pathways, it would be necessary to regulate strictly their chemical structures and amounts by the synthetic enzymes. On the other hand, such physiological functions are not known for bacterial sphingolipids.

As shown in Table II, *O*-phosphoserine inhibited the [*H]-KDS formation from L-*[H]-serine as potently as the L-serine. This is consistent with the finding that *O*-phosphoserine was converted to a KDS derivative. Both serinamide and serinol,
derivatives in which the carboxyl group of serine is modified, are not potent competitors of
\[^{3}H\]-serine in the above reaction. The inhibitory effects of 3-hydroxypropionate and \(\alpha\)-
methyl-DL-serine agreed with the belief that they can form complexes with SPT, which
mimic the Michaelis complex and the external aldimine, respectively. These results
indicate that the carboxyl group of serine is essential for the recognition of the amino acid
substrates by SPT.

Sequence comparison between the \(S.\ paucimobilis\) SPT and other prokaryotic
enzymes of the \(\alpha\)-oxamine synthase family shows that conserved amino acids are
distributed throughout the entire sequence (Fig. 7). In addition to Lys 267 that forms a
Schiff base linkage with PLP, catalytically important residues identified by X-ray
crystallography on AONS from \(E.\ coli\) are completely conserved at the corresponding
positions in SPT, such as His 159, Asp 231, and His 234 (Figures 6 and 7, \textit{asterisk} and
\textit{triangles}) (33). These residues interact directly with PLP, and His 159 and Asp 231 are
also conserved in other PLP-dependent enzymes such as aspartate aminotransferases from
various organisms.

We have succeeded in construction of the overproduction system of SPT in \(E.\ coli\). The growth rate of \(E.\ coli\) was not inhibited even after the expression was induced, and the
SPT overproduced was catalytically active. Until now, it has been thought that the toxicity
of KDS, the reaction product of SPT, is one of the reasons why the expression system of
SPT in \(E.\ coli\) cannot be constructed. However, the present results imply that KDS is not
toxic to the \(E.\ coli\) host, or it is rapidly metabolized. The recombinant SPT was
catalytically and spectrophotometrically indistinguishable from the native protein. There
has been little success in the overproduction of the enzymes in the sphingolipid biosynthesis
pathway, and almost no detailed research exists concerning the enzymatic characterization
of these enzymes. This work permitted to give enough amount of SPT for the three-
dimensional structural analysis of this protein, which is essential for elucidation of the reaction mechanism of this enzyme. We are now attempting crystallization and X-ray diffraction studies of the S. paucimobilis SPT. Information obtained from the Sphingomonas enzyme would provide us with insight into the more complex eukaryotic homologue.

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Footnotes

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¶To whom correspondence should be addressed. Tel.: +81-726-83-1221, ext 2645; FAX: +81-726-84-6516; E-mail: med001@art.osaka-med.ac.jp

1 Abbreviations: EY, Eiko Yabuuchi, Aichi Medical University; T, Nomenclatural type strain for the species; PLP, pyridoxal 5'-phosphate; LCB, long chain base; SPT, serine palmitoyltransferase; CoA, coenzyme A; KDS, 3-ketodihydrosphingosine; ER, endoplasmic reticulum; CHO, Chinese hamster ovary; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethansulfonic acid; DPPC, dipalmitoylphosphatidylcholine; AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; ESI-MS, electrospray ionization mass spectrometry; ALAS, 5-aminolevulinic acid synthase; AONS, 8-amino-7-oxononanoate synthase; KBL, 2-amino-3-ketobutyrate CoA ligase
### Table I. Purification of SPT from *S. paucimobilis*

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<tr>
<th>Steps</th>
<th>Total Activity (nmol/min)</th>
<th>Total Protein (mg)</th>
<th>Specific Activity (nmol/min/mg)</th>
<th>Yield (%)</th>
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Table II  Effects of various amino acids on the formation of \([^{3}\text{H}]\)-KDS from L-[\(^{3}\text{H}\)]-serine.

Purified SPT (50 ng) was incubated with 0.1 mM L-[\(^{3}\text{H}\)]-serine, 0.8 mM palmitoyl CoA and 4 mM of each nonradioactive competitor indicated. The levels of radioactivity of \([^{3}\text{H}]\)-KDS that formed are shown as a percentage of the mean level of \([^{3}\text{H}]\)-KDS formed in the absence of the competitors. The other natural amino acids were inert as competitors. Each value varied ±10% between experiments.

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<td>(O)-Phosphoserine</td>
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Table III  Acyl CoA specificity of purified SPT

Purified SPT (50 ng) was used. The levels of KDS are shown as a percentage of the mean level of KDS formed with palmitoyl CoA. Each value varied ±10% between experiments.

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<th>Substrate</th>
<th>Relative Activity</th>
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<td>Lauroyl CoA (C12:0)</td>
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<td>Myristoyl CoA (C14:0)</td>
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<td>Palmitoleoyl CoA (C16:1)</td>
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<td>Oleoyl CoA (C18:1) (^a)</td>
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<tr>
<td>Elaidoyl CoA (C18:1) (^b)</td>
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</table>

\(^a\) *cis*-9-octadecenoyl CoA

\(^b\) *trans*-9-octadecenoyl CoA
Table IV  Kinetic parameters of purified SPT

The data for the native SPT are from Fig.4. The data for the recombinant SPT were obtained by same assay.

<table>
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<tr>
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<th>$K_m$ (Ser)</th>
<th>$K_m$ (PalmitoylCoA)</th>
<th>$k_{cat}$</th>
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<td>Native SPT</td>
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<td>0.87 mM</td>
<td>140 min$^{-1}$</td>
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<tr>
<td>Recombinant SPT</td>
<td>10.6 mM</td>
<td>0.87 mM</td>
<td>181 min$^{-1}$</td>
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FIGURE LEGENDS

Fig 1: Thin-layer chromatography of radio-labeled products obtained by assays of SPT of mouse liver microsomes, *S. paucimobilis* and *S. spiritivorum*. *Lanes 1* and *4*, mouse liver microsomes as a reference; *lane 2*, crude extract after sonication of *S. paucimobilis*; *lane 3*, the supernatant after centrifugation at 100,000 × *g* of *S. paucimobilis* crude extract; *lane 5*, crude extract after sonication of *S. spiritivorum*; *lane 6*, the supernatant after centrifugation at 100,000 × *g* of *S. spiritivorum* crude extract.

Fig 2: SDS-polyacrylamide gel electrophoresis at various steps of SPT purification. *Lane 1*, crude extract after sonication and centrifugation at 100,000 × *g* (20 µg of protein); *lane 2*, Phenyl Sepharose CL-4B column (10 µg of protein); *lane 3*, Butyl-Toyopearl column (5 µg of protein); *lane 4*, DEAE-Toyopearl column (5 µg of protein); *lane 5*, CHT-II (hydroxyapatite) column (1 µg of protein); *lane 6*, Mono-Q column (1 µg of protein). The samples (10 µl) were analyzed by SDS-polyacrylamide gel electrophoresis on a 10% gel and stained with the Coomassie Brilliant Blue R-250. Prestained Protein Marker, Broad Range, from New England Biolabs was used as the molecular mass standard.

Fig 3: Absorption Spectrum of purified SPT. The conditions were as follows: 50 mM HEPES-NaOH buffer, 0.1 mM EDTA, pH 7.5, 25°C, 0.35 mg/ml of a enzyme.

Fig 4: Kinetic characterization of native SPT from *S. paucimobilis*. The enzyme assay was performed as described under “MATERIALS AND METHODS”. [³H]-serine as the substrate and [¹⁴C]-KDS as the internal standard were used. *Panel A*, the apparent rate constants (*k*<sub>app</sub>) for the KDS formation were plotted as a function of palmitoyl CoA.
concentration. Each solid line represents the theoretical curve according to the initial velocity equation on the Ordered Bi Bi mechanism using the kinetic parameters summarized in Table IV. **Panel B**, determination of $K_m^{\text{Ser}}$, $K_m^{\text{Pal}}$ and $k_{\text{cat}}$. Primary plot of $[\text{Palmitoyl CoA}]/k_{\text{app}}$ versus $[\text{Palmitoyl CoA}]$ at various serine concentrations. The secondary substrate (serine) concentrations were 1 mM (closed circle), 2 mM (open circle), 4 mM (closed square), 10 mM (open square), 20 mM (closed triangle), 50 mM (open triangle). **Inset**, the secondary plot of $[\text{Palmitoyl CoA}]/k_{\text{app}}$–ordinate intercept replot versus $1/[\text{Ser}]$.

**Fig 5**: Nucleotide and deduced amino acid sequences of *S. paucimobilis* SPT gene. The deduced amino acid sequence is given below the nucleotide sequence. The putative Shine-Dalgarno (SD) sequence is indicated by the double underline. The internal amino acid sequence of SPT determined by Edman degradation is indicated by the underline. The annealing sites of the oligonucleotides for the degenerate PCR cloning are indicated by the dashed underline. An asterisk marks the termination codon. An open circle marks the lysine residue predicted to bind PLP.

**Fig 6**: Comparison of SPT protein sequences. The deduced amino acid sequence of SPT protein from *S. paucimobilis* SPT is compared to those of both LCB1 and LCB2 proteins from mouse. Alignment analysis was performed with GENETYX (Software Development Co., Fukuoka, Japan). Residues conserved among all proteins are fulltone-inverted and those conserved between the two of these members are halftone-inverted. An asterisk marks the lysine residue predicted to bind PLP. Triangles mark the residues corresponding to identified residues in the active site of AONS from *E. coli* (35).

**Fig 7**: Comparison of protein sequences of four PLP-dependent acyl CoA transferases.
The deduced amino acid sequence of SPT protein from *S. paucimobilis* SPT is compared to those of four of the PLP-dependent acyl CoA transferases. Alignment analysis was performed with GENETYX. SPT, *S. paucimobilis* SPT; AONS, *Bacillus sphaericus* 8-amino-7-oxononanoate synthase; KBL, *Bacillus subtilis* 2-amino-3-ketobutyrate CoA ligase; ALAS, *Agrobacterium radiobacter* 5-aminolevulinic acid synthase. Residues conserved among all proteins are fulltone-inverted and those conserved among the three of those proteins are halftone-inverted. An *asterisk* marks the lysine residue predicted to bind PLP. *Triangles* mark the residues corresponding to identified residues in the active site of *E. coli* AONS.
Figure 1
Ikushiro et al
(M1:01550)
Figure 2
Ikushiro et al.
(M1:01550)
Figure 3
Ikushiro et al.
(M1:01550)
Figure 4
Ikushiro et al.
(M1:01550)
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A water-soluble homodimeric serine palmitoyltransferase from Sphingomonas paucimobilis EY2395T strain: purification, characterization, cloning, and overproduction
Hiroko Ikushiro, Hideyuki Hayashi and Hiroyuki Kagamiyama

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