Purification of the Serine Palmitoyltransferase Complex Responsible for Sphingoid Base Synthesis by Using Affinity Peptide Chromatography Techniques*

(Received for publication, October 18, 1999, and in revised form, December 20, 1999)

Kentaro Hanada‡§§, Tomoko Hara‡§, and Masahiro Nishijima‡

From the §Department of Biochemistry and Cell Biology and ¶CREST, Japan Science and Technology Corp., National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

Serine palmitoyltransferase (SPT), a membrane-bound enzyme of the endoplasmic reticulum, catalyzes the condensation of palmitoyl coenzyme A (CoA) and L-serine to produce 3-ketodihydrosphingosine. This enzyme contains at least two different subunits, named the LCB1 and LCB2 proteins. In the present study, we expressed a FLAG- and His6 peptide-tagged version of the hamster LCB1 protein in a Chinese hamster ovary cell mutant strain lacking the endogenous LCB1 subunit and purified SPT from the cells near to homogeneity by affinity peptide chromatography. The endogenous LCB2 protein was co-purified with the tagged LCB1 protein in purification of SPT. In various aspects, including optimum pH, acyl-CoA specificity, and sphingofungin sensitivity, the activity of purified SPT was consistent with the activity detected in lysates of wild-type Chinese hamster ovary cells. The optimum concentration of palmitoyl-CoA for 3-ketodihydrosphingosine formation by purified SPT was ~25 μM, and the apparent Kₘ of L-serine was 0.28 mM. Competition analysis of the SPT reaction with various serine analogs showed that all of the amino, carboxyl, and hydroxyl groups of L-serine were responsible for the substrate recognition of the enzyme. SDS-polyacrylamide gel electrophoretic analysis of purified SPT, together with immunoprecipitation analysis of metabolically labeled LCB proteins, strongly suggested that the SPT enzyme consisted of the LCB1 and LCB2 proteins with a stoichiometry of 1:1.

Sphingolipids are ubiquitous constituents of membrane lipids in mammalian cells and are also distributed widely in other animals, plants, and microbes (1). It has been demonstrated that sphingolipids play essential roles in the growth of both mammalian and yeast cells (2, 3). Sphingoid bases, ceramide, and sphingosine-1-phosphate modulate various cellular events, including proliferation, differentiation, senescence, apoptosis, and inflammatory responses (for reviews, see Refs. 4–6). Moreover, sphingomyelin and glycosphingolipids, which are major components of the exoplasmic layer of the plasma membrane lipid bilayer, comprise cholesterol-enriched and detergent-resistant membrane subdomains (7–12), where various signaling events occur (13, 14).

Sphingolipid biosynthesis is initiated by condensation of L-serine with palmitoyl coenzyme A (CoA)¹ to generate 3-ketodihydrosphingosine (KDS), a reaction catalyzed by serine palmitoyltransferase (SPT) (EC 2.3.1.50) (see Ref. 15 for a review of sphingolipid biosynthesis). SPT is suggested to be a key enzyme for regulation of the sphingolipid level in cells, because regulation of sphingolipid synthesis at the SPT step prevents a harmful accumulation of metabolic sphingolipid intermediates, including sphingoid bases and ceramide, whereas repression of other anabolic steps in the sphingolipid synthetic pathway may cause the intermediates to accumulate. SPT is likely to be a membrane-bound enzyme of the endoplasmic reticulum, with its catalytic site protruding into the cytosol (16, 17). Genetic and biochemical studies have revealed that at least two different genes, LCB1 and LCB2, are required for expression of SPT activity (2, 18–21) and that both the LCB1 and LCB2 proteins are subunits of the SPT enzyme (22). However, important properties of this enzyme, including the entire subunit composition, remain undetermined, largely because purification of SPT has not been achieved.

The nature of the SPT enzyme is in itself an obstacle to its purification. First, only a few types of detergents are suitable for solubilization of this membrane-bound enzyme without inactivation, narrowing conventional chromatographic methods applicable to the purification of SPT. Second, no affinity ligands useful for purification of active SPT are so far known. Chen et al. (23) have recently demonstrated that solubilized SPT activity was efficiently adsorbed by a matrix conjugated with a potent SPT inhibitor, but they failed to elute the SPT enzyme from the matrix without inactivation. Third, because SPT consists of different subunits, overproduction of one subunit type alone does not facilitate purification of this enzyme complex.

In the present paper, we show that a FLAG- and hexahistidine (His6)-tagged version of the hamster LCB1 protein functionally substitutes for the wild-type LCB1 protein in Chinese hamster ovary (CHO) cells. We purified active SPT from the CHO cells expressing the doubly tagged LCB1 protein near to homogeneity by affinity peptide chromatography techniques and characterize the purified SPT enzyme complex consisting of the tagged LCB1 protein and the endogenous LCB2 protein.

EXPERIMENTAL PROCEDURES

Materials—FLAG peptide, anti-FLAG M2 affinity gel, various acyl-CoAs, and L-serine and its analogs were purchased from Sigma; nickel-nitrotriacetic acid (Ni-NTA) agarose was from Qiagen GmbH (Hilden, Germany); egg phosphatidylcholine was from Avanti Polar Lipids Inc.

¹ The abbreviations used are: CoA, coenzyme A; KDS, 3-ketodihydrosphingosine; SPT, serine palmitoyltransferase; CHO, Chinese hamster ovary; Ni-NTA, nickel-nitrotriacetic acid; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
Precipitated membranes (20 mg of protein) were suspended in 4 ml of containing 1 mM EDTA and homogenized with a 40-ml Dounce tissue and then eluted with 4.5 ml of Buffer A containing 120 to a column. The gel in the column was washed with 15 ml of Buffer A concentration of 0.25 M, the resultant sample was centrifuged (104 rate in 800 mg of protein/ml with a syringe equipped with a 24-gauge needle and stored unless otherwise noted. After the labeled cells had been harvested by scraping, the membranes were prepared from the cells as described previously (25). The radioactivity extracted from enzyme-negative controls was regarded as a background control.

SPT—Plasmids and CHO Cell Strains—pSV-cLCB1 is a recombinant plasmid for expression of the hamster wild-type LCB1 protein, and the plasmid pSV-HTcLCB1 encodes an NH2-terminally His6-tagged LCB1 protein (20). For double tagging of the LCB1 protein with the FLAG and positions showing recovery of SPT activity, one clone designated as LY-B/FHcLCB1 strain was chosen for purification of the SPT enzyme complex.

pMKTNeo, a mammalian expression vector having the SRα promoter, was a gift from Dr. Kazuo Maruyama (Tokyo Medical and Dental University, School of Medicine, Tokyo, Japan). After deletion of the 3'-untranslated region of the hamster LCB2 cDNA (20), the LCB2 open-reading frame was inserted in the expression cloning site of pMKTNeo, and the recombinant plasmid was named pMKTNeo-cLCB2.

Preparation of Membranes from CHO Cells—LY-B/FHcLCB1 cells were cultivated in spinner bottles containing 1 liter of ES medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 2 mM L-glutamine, NaHCO3 (1 g/l), 10 mM Hepes-NaOH (pH 7.4), and 5% (v/v) fetal calf serum at 37 °C. Thereafter, all manipulations were done at 4 °C or on ice. Cells from 5-liter cultures were harvested by centrifugation (600 x g for 5 min), and washed with 250 ml of phosphate-buffered saline. The washed cells were suspended in 30 ml of 10 mM sucrose containing 1 mM EDTA and homogenized with a 40-mI Dounce tissue grinder (pH type A, Wheaton, Millville, NJ) by 70 strokes. The homogenate was centrifuged (1000 x g for 15 min) to remove cell debris and 20% (v/v) Tween 20. After addition of 2 ml to the supernatant, a final concentration of 0.25 M, the resultant sample was centrifuged (10 000 x g for 1 h). The precipitate, as intact membranes, was suspended in 10 mM Hepes-NaOH buffer (pH 7.5) containing 0.25 M sucrose at ~10 mg of protein/ml with a syringe equipped with a 24-gauge needle and stored at ~70 °C until use.

Purification of SPT—A stock suspension of egg phosphatidylcholine (5 mg/ml of deionized water) was prepared by sonication at room temperature. Hereafter, all manipulations were done at 4 °C or on ice. The membrane suspension was centrifuged (10 000 x g for 30 min) and the precipitated membranes (~20 mg of protein) were suspended in 4 ml of 125 mM sodium phosphate buffer (pH 8.0) containing 188 mM NaCl. After addition of 0.5 ml of 5 mg/ml phosphatidylcholine and 0.5 ml of 20% (v/v) sucrose monolaurate to the supernatant, the mixture was incubated for 10 min. After centrifugation (10 000 x g, 30 min) of the mixture, the supernatant fluid was recovered as the solubilized membrane fraction. The solubilized membrane fraction was incubated with 0.5 ml of anti-FLAG M2 affinity gel, which had been equilibrated with Buffer A (0.1 mM sodium phosphate buffer (pH 8.0) containing 50 mM NaCl, 0.1 mg/ml phosphatidylcholine, and 0.1% sucrose monolaurate), for 30 min with gentle shaking. After centrifugation (10 000 x g for 20 s), the precipitated gel was suspended in 0.5 ml of Buffer A and transferred to a column. The gel in the column was washed with 15 ml of Buffer A and then eluted with 4.5 ml of Buffer A containing 120 mM NaCl/FLAG peptide. After addition of imidazole to the elution fraction at a final concentration of 10 mM, this sample was mixed with 0.5 ml of Ni-NTA agarose. The mixture had been equilibrated with Buffer B (0.1 M sodium phosphate buffer (pH 8.0) containing 0.1 M NaCl, 0.1 mg/ml phosphatidylcholine, 0.1% sucrose monolaurate, and 10 mM imidazole), for 1 h with gentle shaking. After centrifugation (1000 x g for 1 min), the precipitated matrix was suspended in 10 ml of Buffer B and precipitated for washing. After a repeat of this washing step twice, the precipitated matrix was suspended with 5 ml of 0.1 M sodium phosphate buffer (pH 8.0) containing 0.1 M NaCl, 0.1 mg/ml phosphatidylcholine, 0.1% sucrose monolaurate, and 250 mM imidazole and incubated for 10 min. After precipitation of the matrix by centrifugation (1000 x g and 1 min), the supernatant fraction was collected as the elution fraction of His6 affinity chromatography. For removal of imidazole, the elution fraction was diluted 5-fold with 10 mM Hepes-NaOH buffer (pH 7.5) containing 0.25 mM sucrose and 0.1% sucrose monolaurate and concentrated to ~2 ml by ultrafiltration with Ultrafree-MC (Millipore, Bedford, MA). After repeating these dilution and concentration steps twice, the purified SPT fraction was divided and stored at ~70 °C until use. The anti-FLAG M2 affinity gel used was regenerated by washing with glycine-HCl buffer (pH 3.5) and subsequent neutralization.

Assay of SPT Activity—The enzyme source was incubated in 200 ml of a standard SPT reaction buffer (50 mM Hepes-NaOH buffer (pH 7.5) containing 0.25 M sucrose monolaurate, 10% (v/v) glycerol, and 13 mM L-threonine) at 37 °C for 10 min. After stopping the reaction, lipids were extracted, and the radioactivity of the [3H]KDS that formed was measured as described previously (25). The radioactivity extracted from enzyme-negative controls was regarded as a background control.

SDS-Polyacrylamide Gel Electrophoresis (PAGE), Silver Stain, and Western Blot—SDS-PAGE was carried out by a modification of the method of Laemmli (26). Samples for SDS-PAGE were incubated in a SDS-sample buffer (1.0 M Tris-Cl buffer (pH 6.8) containing 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, and 13 mM L-threonine) at 37 °C for 15 min. If necessary, acrylamide gel supplemented with 3 M urea was used. Molecular mass standards were purchased from Bio-Rad. Proteins separated on gel were stained with a silver staining kit (Wako, Osaka, Japan). For Western blot analysis, proteins separated by SDS-PAGE were transferred to a polyvinylidene difluoride membrane (Bio-Rad). After the blot membrane was blocked with ~0.1% skim milk in phosphate-buffered saline containing 0.1% Tween 20, the LCB1 and LCB2 proteins on the membrane were detected by using an anti-hamster LCB1 antibody and anti-hamster LCB2 antibody (0.5 μg/ml), respectively, as the primary antibody and a horse-radish peroxidase-conjugated goat anti-ribbit IgG (Bio-Rad; 1:2500 dilution) as the secondary antibody with an enhanced chemiluminescence reagent (Amersham Pharmacia Biotech). The anti-hamster LCB1 (anti-cLCB1 73/890) and anti-hamster LCB2 (anti-cLCB2 26/45) antibodies were routinely used were raised by immunization of rabbits with the peptides corresponding to the 73–90 amino acid residues of the hamster LCB1 protein and to the 26–45 amino acid residues of the hamster LCB2 protein, respectively, and purified by affinity chromatography with the antigen peptide coupled matrices.

Co-Immunoprecipitation of the FHcLCB1 or LCB1 Protein with the LCB1 Protein after Metabolite—LY-B/FHcLCB1 and CHO-K1 (4 x 106) cells were seeded in 25 ml of Ham’s F-12 medium containing 10% (v/v) newborn calf serum and cultured at 37 °C for 24 h. After a wash with Cys/Met-free RPMI 1640 medium (Dainippon Seiyaku Co., Osaka, Japan) supplemented with 2 mM l-glutamine, the monolayers were incubated in 10 ml of Cys/Met-free RPMI 1640 medium supplemented with 2 mM l-glutamine, 10% (v/v) fetal calf serum, 1% Ham’s-f-12 medium, and 0.5 mM of a (35S)Met/Cys protein labeling mix (ICN-EasyTagII, 1175 Ci/mmol, NEN Life Science Products, Inc.) at 37 °C for 18 h. Hereafter, all manipulations were done at 4 °C or on ice unless otherwise noted. After the labeled cells had been harvested by scraping, the membranes were prepared from the cells as described previously (8). Membranes were solubilized with 1% sucrose monolaurate in 800 μl of Buffer C (0.1 mM sodium phosphate buffer (pH 8.0) containing 0.3 mM NaCl and 0.1 mM sucrose). After centrifugation (10 000 x g, 30 min), solubilized supernatant fluid was incubated with protein A Sepharose (a 50-μl bed volume [Amersham Pharmacia Biotech]), which had been equilibrated with Buffer C containing 0.1% sucrose monolaurate, for 1 h. After precipitation of the resin (10 000 x g for 10 s) to remove proteins nonspecifically bound to protein A-Sepharose, 250 μl of the supernatant fluid was mixed with anti-hamster LCB2 antibody-coupled or preimmune IgG-coupled protein A-Sepharose (a 10-μl bed volume) and incubated for 2 h with shaking as described previously. Then, the resin was washed seven times with 1 ml of Buffer C containing 0.1% sucrose monolaurate and incubated in 80 μl of the SDS-sample buffer at 37 °C for 30 min. After precipitation of the resin, the supernatant fluid was subjected to SDS-PAGE. Radioactive images of proteins on gel were analyzed with a BAS2000 Image Analyzer (Fuji Film Co., Tokyo, Japan).

Protein Determination—Protein concentrations were determined with the Pierce BCA protein assay kit using bovine serum albumin as the standard.
The solubilized membrane fraction was incubated with an anti-FLAG antibody-coupled matrix, and proteins bound to the matrix were eluted with a buffer containing the FLAG peptide. This step was very effective for purification of SPT: ~1000-fold enrichment of the activity with a ~30% recovery yield (Table I). The eluent was then incubated with Ni-NTA agarose as a His₆ affinity matrix, and proteins bound to the matrix were eluted with a buffer containing 250 mM imidazole, resulting in a further 2.5-fold enrichment of the activity with an ~60% recovery yield (Table I). Imidazole in the elution fraction was removed by ultrafiltration, and the resultant fraction was used for characterization of highly purified SPT.

Silver Staining and Western Blotting of Purified SPT—Protein patterns of the purification fractions were analyzed by SDS-PAGE with 10–20% (w/v) gradient acrylamide gel, which is capable of separating proteins ranging from 6.5 to 200 kDa. Most of the proteins of the solubilized membrane fraction did not bind to the FLAG affinity matrix (Fig. 2A, lanes 2 and 3), and the elution fraction of this chromatography displayed four visible bands, a major band at Mr 58,000 and minor bands at Mr 47,000, 31,000, and 26,000, by silver stain (Fig. 2A, lane 4). After further fractionation by His₆ affinity chromatography, only one band at Mr 58,000 was detected in the elution fraction (Fig. 2A, lanes 5 and 6). The observation that the highly purified SPT fraction displayed only one visible band on silver stain analysis apparently contradicted our previous conclusion that the SPT enzyme complex contains at least two subunit types, the LCB1 and LCB2 proteins. Western blot analysis with anti-hamster LCB1 and LCB2 antibodies revealed that the FH-LCB1 protein overlapped the LCB2 protein in the SDS-PAGE gel (data not shown; see also Figs. 1C and 3C). However, SDS-PAGE with 7.5% (w/v) acrylamide gel containing 3 M urea well separated the FH-LCB1 and LCB2 proteins, allowing us to display two bands (Mr 58,000 and 54,000) in the highly purified SPT fraction (Fig. 2B).

Based on Western blot analysis, the Mr 58,000 protein in the urea-containing SDS-PAGE gel was identified as the FH-LCB1 protein, and the Mr 54,000 protein was identified as the LCB2 protein (Fig. 3A). We checked specificity of the antibodies used as follows. When the membrane fraction of untransfected CHO-K1 cells was blotted, the anti-LCB2 antibody specifically recognized a Mr 54,000 protein in the acrylamide gel containing 3 M urea (Fig. 3B, lane 1) or a Mr 58,000 protein in the gel containing no urea (Fig. 3C, lane 1). Transfection of cells with the hamster LCB2 cDNA-expression vector but not an empty vector caused overexpression of the specifically recognized protein (Fig. 3, B and C), indicating that the specific protein was the LCB2 protein. In addition, Western blotting of membrane fractions from wild-type, LY-B, and LY-B/FH-LCB1 cells proved the anti-hamster LCB1 antibody to be specific (Fig. 1C).

Molecular mass values deduced from the cDNAs of the hamster LCB1, LCB2, and FH-LCB1 proteins were 52,519, 62,882, and 54,468 Da, respectively (20). It is currently unknown why the Mr values in SDS-PAGE and the deduced molecular mass values of these proteins differ.

Enzymatic Characterization of Purified SPT—The time course of KDS formation by purified SPT was almost linear for at least 10 min (Fig. 4A), and this activity was also proportional to the amount of the purified enzyme up to 25 ng (Fig. 4B). The optimum pH for the activity was found to be between pH 7.5 and 8.5 (Fig. 4C), and purified SPT was highly sensitive to sphingofungin B, a potent inhibitor of SPT (27), with an ID₅₀ of 23 nM (Fig. 4D). These enzymatic properties of purified SPT were well consistent with those of the SPT activity previously characterized in cell homogenates or membranes from wild-type CHO cells (24, 25).
When the palmitoyl-CoA dependence of KDS formation by purified SPT was examined, the maximum activity was observed at about 25 μM (Fig. 4E). The double reciprocal plots of KDS formation versus L-serine in the presence of 25 μM palmitoyl-CoA showed that the apparent Kₘ for L-serine was 0.28 mM and that the apparent Vₘₐₓ was 660 nmol of KDS/mg of protein/min (Fig. 4F). Based on this apparent Vₘₐₓ value and the assumption that the one-to-one complex of the FHcLCB1 and LCB2 proteins had one catalytic site, the apparent catalytic number of purified SPT was estimated to be ~80/min. Due to the inhibitory effect of palmitoyl-CoA at higher concentrations (Fig. 4E), the Kₘ for L-serine and Vₘₐₓ values could not be determined under excess concentrations of palmitoyl-CoA.

An analysis of the acyl-CoA substrate specificity of purified SPT indicated that palmitoyl-CoA was the best substrate among various acyl-CoAs examined (Table II). Pentadecanoyl-CoA and heptadecanoyl-CoAs were the next most effective, whereas myristoyl- and stearoyl-CoAs and palmitoleoyl-CoA (an unsaturated form of palmitoyl-CoA) were very poor substrates. Myristoyl- and stearoyl-CoAs and palmitoleoyl-CoA (an unsaturated form of palmitoyl-CoA) were very poor substrates. Among various CoA esters examined (Table II), 11β-hydroxysterol-CoA was the best substrate of purified SPT, followed by 11β-hydroxysterol-CoA, 17α-hydroxysterol-CoA, and 5α-dihydrotestosterone-CoA.

A, Western blot analysis with anti-hamster LCB1 and LCB2 antibodies. A, Western blotting of purified SPT. Purified SPT (1 μg) was separated by SDS-PAGE with 7.5% (w/v) acrylamide gel containing 3 M urea and stained by silver staining. B, elution fraction (20 ng of protein), respectively, of anti-FLAG M2 affinity gel. C, elution fraction (90 ng of protein) of Ni-NTA agarose chromatography, respectively, of Ni-NTA agarose chromatography. D, solubilized membranes (920 ng of protein) was separated by SDS-PAGE with 7.5% (w/v) acrylamide gel containing 3 M urea and stained by silver staining.
the activity applied to the control matrix was not adsorbed (Fig. 5A). An anti-hamster LCB2 antibody (α2) and a preimmune IgG (PI) was covalently conjugated to Hi-Trap NHS-activated Sepharose® (Amersham Pharmacia Biotech). Purified SPT (15 ng of protein) was incubated with the anti-hamster LCB2 antibody-conjugated or preimmune IgG-conjugated Sepharose in 100 μl of Buffer D (0.1 M sodium phosphate buffer (pH 8.0) containing 0.3 M NaCl, 0.1 M sucrose, 0.1% sucrose monolaurate, and 0.1 mg/ml phosphatidylcholine) at 4 °C for 4 h. The matrix was precipitated by centrifugation, and the supernatant was recovered as the unadsorbed fraction. After washing with Buffer D, the matrix was incubated with 100 μl of a SDS-sample buffer devoid of 2-mercaptoethanol at 37 °C for 30 min, and the supernatant after precipitation of the matrix was recovered as the adsorbed fraction. A, SDS-PAGE and silver staining of the fractions. Each sample was incubated with a SDS-sample buffer containing 50 mM dithiothreitol, 5 mM pyridoxal phosphate, 0.1 mM L-[3H]serine, and 25 μM of various acyl CoAs at 37 °C for 10 min, and the radioactivity of the [3H]KDS that formed was measured as described under “Experimental Procedures.” The data from three experiments are shown as percentage of the activity applied to the antibody-conjugated matrix.

FIG. 4. Enzymatic characterization of purified SPT. A, time course of the activity. Purified SPT (25 ng of protein) was incubated in the standard SPT reaction buffer at 37 °C for various periods, and the radioactivity of the [3H]KDS that formed was measured as described under “Experimental Procedures.” B, protein dependence of the activity. Various amounts of purified SPT were incubated in the standard SPT reaction buffer at 37 °C for 10 min. C, pH dependence of the activity. Purified SPT (25 ng of protein) was incubated in 0.1 M 1,3-bis(tris(hydroxymethyl)methylamino)propane-HCl buffers (pH 6.5–9.3) containing 5 mM EDTA, 5 mM dithiothreitol, 50 mM pyridoxal phosphate, 25 μM palmitoyl-CoA, and 0.1 mM L-[3H]serine at 37 °C for 10 min. D, sphingofungin B sensitivity of the activity. Purified SPT (25 ng of protein) was incubated in the SPT reaction buffer containing various concentrations of sphingofungin B at 37 °C for 10 min. The data are shown as percentage of the activity applied to the antibody-conjugated matrix. E, palmitoyl-CoA dependence of the activity. Purified SPT (25 ng of protein) was incubated in the SPT reaction buffer containing various concentrations of palmitoyl-CoA at 37 °C for 10 min. F, L-serine dependence of the activity. Purified SPT (25 ng of protein) was incubated in the SPT reaction buffer containing various concentrations of L-serine at 37 °C for 10 min. The data obtained are shown as double reciprocal plots.

TABLE II

| Acyl CoA (chain length (No. of C)) | KDS formed (%) |
|----------------------------------|----------------|
| Myristoyl (14)                   | 13.0 ± 1.4     |
| Pentadecanoyl (15)               | 73.1 ± 1.1     |
| Palmitoyl (16)                   | 100 ± 4        |
| Heptadecanoyl (17)               | 61.9 ± 5.4     |
| Stearoyl (18)                    | 11.1 ± 0.8     |
| Arachidoyl (20)                  | −2             |
| Palmitoleoyl (16)                | 15.6 ± 3.9     |

a cis-9-Hexadecenoyl CoA.

5B). These results suggested that SPT was a complex of the LCB1 and LCB2 proteins with a stoichiometry of 1:1. It might be inappropriate to conclude the subunit stoichiometry from the silver staining patterns alone, because it remained undetermined whether the stained density per protein molecule was equal between the different subunits. Therefore, we also employed another measure, the radioactivity of metabolically labeled LCB proteins. LV-B/FHLCB1 cells were incubated with a [35S]Met/Cys protein labeling mix for 18 h, and membranes were prepared from the cells. After solubilization of membranes under conditions in which SPT sustained the activity, the solubilized membrane fraction was subjected to
**FIG. 6.** Co-immunoprecipitation of the FHcLCB1 or LCB1 protein with the LCB2 protein after metabolic labeling. LY-B/FHcLCB1 and CHO-K1 cells were incubated with [35S]EasylTag for 18 h. Membranes prepared from the labeled cells were solubilized with 1% (w/v) sucrose monolaurate, and the solubilized membrane fraction was incubated with protein A-Sepharose for 1 h. After precipitation of the resin, the supernatant fluid was incubated with anti-hamster LCB2 antibody-coupled (α2) or preimmune IgG-coupled (PI) protein A-Sepharose for 2 h. The resin was washed, and then, proteins immunoadsorbed by the resin were eluted by incubation in the SDS-sample buffer at 37 °C for 30 min. After precipitation of the resin, the supernatant fluid was subjected to SDS-PAGE with 7.5% (w/v) acrylamide gel containing 3 M urea in the gel, but one explanation is that the conformation of the LCB2 protein changes depending on the existence of 3 M urea (Fig. 6A). Because the ratio of the radioactivity of the FHcLCB1 protein to that of the LCB2 protein in the specific immunoprecipitate was determined to be 1:1.75 ± 0.65 (n = 3) by image analysis, the molecular ratio of the FHcLCB1 protein to the LCB2 protein in the immunoprecipitate was estimated to be 1:1.1 ± 0.4 by taking account of the numbers of Cys plus Met residues in these proteins. A nearly identical value (1:1.1 ± 0.3, n = 3) was also estimated for the ratio of the endogenous LCB1 protein to the LCB2 protein, when immunoprecipitation analysis was performed with wild-type CHO cells instead of LY-B/FHcLCB1 cells (Fig. 6B, lanes 1 and 2). We previously showed that under similar conditions of immunoprecipitation, the anti-hamster LCB2 protein immunoprecipitated ∼80% of SPT activity along with most of the LCB1 and LCB2 proteins from the solubilized membrane fraction of wild-type CHO cells (22), suggesting that the complex of the LCB1 and LCB2 proteins in the immunoprecipitate was relevant to the active SPT enzyme.

Note that the Mr values of the wild-type LCB1 and LCB2 proteins in 3 M urea-containing gel of SDS-PAGE were 53,000 and 54,000, respectively (Fig. 6A, lane 2; see also Figs. 1C and 3B), whereas the Mr values of the LCBI and LCB2 proteins in gel containing no urea were 53,000 and 58,000, respectively (Fig. 6B, lane 2; see also Figs. 1C and 3C). Therefore, we used the latter gel conditions for separation of the two wild-type LCB proteins by SDS-PAGE. It remains unknown why the Mr of the LCB2 protein changes depending on the existence of 3 M urea in the gel, but one explanation is that the conformation of the LCB2 protein is affected by urea in SDS-PAGE.

**DISCUSSION**

Purification of enzymes is a crucial step in elucidating the molecular mechanisms of metabolism. SPT has been regarded as a key enzyme for sphingolipid metabolism since Braun and Snell (28) and Stoffel et al. (29) initially demonstrated more than 30 years ago that sphingoid base synthesis proceeds through condensation of L-serine and palmitoyl-CoA to produce KDS in a particulate fraction of yeast, _Hansenula ciferri_, cells. However, the purification of SPT has not been attained so far, most likely due to technical obstacles. Nevertheless, by somatic genetic and molecular biological approaches, we have isolated CHO cell mutants defective in SPT (22, 30) and also obtained cDNAs encoding the hamster LCB1 and LCB2 proteins, both of which are subunits of SPT (20). We therefore decided to express the FHcLCB1 protein, a doubly affinity peptide-tagged version of the hamster LCB1 protein, in CHO mutant LY-B cells lacking the endogenous LCB1 subunit and purify an active SPT complex from the cells by affinity peptide chromatography. This method proved to be very effective, allowing us to purify the SPT enzyme complex near to homogeneity with a reasonably high yield (Table I and Fig. 2). The activity of the purified SPT enzyme was consistent in various aspects (i.e., the optimum pH, the acyl-CoA specificity, and sphingofugin B sensitivity) with the SPT activity detected in lysates and membranes of wild-type CHO cells (Fig. 4 and Table II; see also Refs. 24 and 25), indicating that these enzymatic characteristics of SPT were not affected or were little affected by the affinity peptide sequences linked to the LCB1 subunit.

Neither L-alanine, L-serinamide, d,L-serinol, nor L-serine methylester served as strong competitors of the [3H]KDS formation from L-[3H]serine (Table III), indicating that all of the hydroxyl, amino, and carboxyl groups of L-serine are responsible for the recognition of the amino acid substrate by the SPT enzyme. For production of KDS metabolites from acyl-CoA and L-serine by purified SPT, palmitoyl-CoA was the best substrate among the various acyl-CoAs (Table II). Pentadecanoyl- and heptadecanoyl-CoAs were also effective, whereas myristoyl-, stearoyl-, palmitoleoyl-, and arachidoyl-CoA were far less effective. The observed acyl-CoA specificity of purified SPT was well consistent with the acyl-CoA specificity of the SPT activity previously characterized in cell lysates or membranes (16, 25). Palmitoyl-CoA is one of the most abundant acyl-CoA types in mammalian cells, whereas the levels of pentadecanoyl- and heptadecanoyl-CoAs are negligible (31). Therefore, palmitoyl-CoA is likely to be the predominant acyl-CoA substrate of SPT in _vivo_, and for this reason, the chain length of most sphingoid bases from mammalian cells is 18.

The optimum concentration of palmitoyl-CoA for KDS production by purified SPT was determined to be around 25 μM (Fig. 4E), whereas that of palmitoyl-CoA for SPT activity in CHO cell lysates was 0.2 mM or higher (data not shown; see also Ref. 25). The difference in the optimum palmitoyl-CoA concentration between cell lysates and purified SPT might be partly attributable to the removal of acyl-CoA hydrolase activity, which could quench palmitoyl-CoA (16), during the purification processes.

In SDS-PAGE and silver stain or Western blot analysis, the highly purified SPT fraction displayed only two protein types, the FHcLCB1 and endogenous LCB2 proteins (Figs. 2 and 3). No other proteins were detected in the resolution range of 6.5–200 kDa by SDS-PAGE (Fig. 2A). These results strongly suggest that the SPT enzyme consists of the LCB1 and LCB2 proteins, although we do not deny the possibility that other proteins play accessory roles in the SPT reaction _in vivo_ by transient association with the LCB1/LCB2 complex.

For elucidation of the subunit stoichiometry of the SPT complex, the purified SPT fraction we obtained had a shortcoming. Some FHcLCB1 protein molecules not associated with the LCB2 protein were also accumulated during the affinity pep-
tide-mediated purification process. To overcome this problem, we applied the purified SPT fraction to an anti-LCB2 antibody-conjugated matrix and analyzed the adsorbed fraction by silver staining. The density of the FHcLCB1 protein band in the adsorbed fraction was similar to that of the LCB2 protein band (Fig. 5A). In addition, co-immunoprecipitation experiments after metabolic labeling of the LCB proteins showed that the molar ratio of the FHcLCB1 protein to the LCB2 protein in the anti-hamster LCB2 antibody-dependent immunoprecipitate was about 1:1 (Fig. 6A). A similar ratio was obtained for the molar ratio of the FHcLCB1 protein to the LCB2 protein in the anti-mouse LCB2 antibody-dependent immunoprecipitate (Fig. 6B). These results suggest that the SPT enzyme consists of LCB1 and LCB2 subunits with a stoichiometry of 1:1, although it remains to be determined whether the SPT complex is a heterodimer, heterotetramer, or larger oligomer of these two subunits. We could not estimate the molecular mass of the purified SPT by gel filtration chromatography, because the purified SPT lost its activity during replacement of the detergent from sucrose monolaurate, which formed a large micelle, to octylglucoside and CHAPS, which formed relatively small micelles (data not shown). However, the rational method for purification of SPT described in this study will allow researchers to analyze in detail this enzyme chemically and physically, including elucidation of its molecular mass.

REFERENCES

1. Karlsson, K.-A. (1970) Chem. Phys. Lipids 5, 6–43
2. Buede, R., Rinker-Schaffer, C., Pinto, W. J., Lester, R. L., and Dickson, R. C. (1991) J. Bacteriol. 173, 4325–4332
3. Hanada, K., Nishijima, M., Kiso, M., Hasegawa, A., Fujita, S., Ogawa, T., and Akamatsu, Y. (1992) J. Biol. Chem. 267, 23527–23533
4. Hannun, Y. A. (1996) Science 274, 1855–1859
5. Spiegel, S., and Merrill, A. H., Jr. (1996) FASEB J. 10, 1388–1397
6. Mathias, S., Peña, L. A., and Kolesnick, R. N. (1998) Biochem. J. 35, 465–480
7. Hanada, K., Itazawa, K., Nishijima, M., and Akamatsu, Y. (1993) J. Biol. Chem. 268, 13820–13823
8. Hanada, K., Nishijima, M., Akamatsu, Y., and Pagano, R. E. (1995) J. Biol. Chem. 270, 6254–6260
9. Stevens, V. L., and Tang, J. (1997) J. Biol. Chem. 272, 18020–18025
10. Schroeder, R. J., Ahmed, S. N., Zhu, Y., London, E., and Brown, D. A. (1998) J. Biol. Chem. 273, 1150–1157
11. Ahmed, S. N., Brown, D. A., and London, E. (1997) Biochemistry 36, 10944–10953
12. Naslavsky, N., Shmeeda, H., Friedelander, G., Yanai, A., Futerman, A. H., Barenholz, Y., and Traboulos, A. (1999) J. Biol. Chem. 274, 20763–20771
13. Simons, K., and Ikonen, E. (1997) Nature 387, 569–572
14. Anderson, R. G. (1998) Annu. Rev. Biochem. 67, 199–225
15. Merrill, A. H., Jr., and Sweetley, C. C. (1996) in Biochemistry of Lipids, Lipoproteins and Membranes (Vance, D. E., and Vance, J., eds) pp. 309–339, Elsevier Science B. V., Amsterdam, The Netherlands
16. Williams, R. D., Wang, E., and Merrill, A. H., Jr. (1984) Arch. Biochem. Biophys. 228, 282–291
17. Mandon, R., Ehoes, I., Rothor, J., van Echten, G., and Sandhoff, K. (1992) J. Biol. Chem. 267, 11144–11148
18. Nagiec, M. M., Baltisberger, J. A., Wells, G. B., Lester, R. L., and Dickson, R. C. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 7899–7902
19. Zhao, C., Beeler, T., and Dunn, T. (1994) J. Biol. Chem. 269, 21480–21488
20. Hanada, K., Hara, T., Nishijima, M., Kuge, O., Dickson, R. C., and Nagiec, M. M. (1997) J. Biol. Chem. 272, 32108–32114
21. Weiss, B., and Stoffel, W. (1997) Eur. J. Biochem. 249, 239–247
22. Hanada, K., Hara, T., Fukasawa, M., Yamaji, A., Umeda, M., and Nishijima, M. (1998) J. Biol. Chem. 273, 3757–37594
23. Chen, J.-K., Lane, W. S., and Schreiber, S. L. (1999) Chem. Biol. 6, 221–235
24. Kobayashi, S., Furuta, T., Hayashi, T., Nishijima, M., and Hanada, K. (1998) J. Biol. Chem. 273, 1150–1157
25. Acero, E., and Schreiber, S. L. (1998) J. Biol. Chem. 273, 22244–22250
26. Laemmli, U. K. (1970) Nature 227, 680–685
27. Zweerink, M. M., Edison, A. M., Wells, G. B., Pinto, W., and Lester, R. L. (1992) J. Biol. Chem. 267, 32108–32114
28. Braun, P. E., and Snell, E. E. (1968) J. Biol. Chem. 243, 3775–3783
29. Stoffel, W., LeKim, D., and Sticht, G. (1968) Hoppe-Seyler’s Z. Physiol. Chem. 349, 664–670
30. Hanada, K., Nishijima, M., and Akamatsu, Y. (1999) J. Biol. Chem. 265, 22137–22142
31. Tardi, P. G., Mukherjee, J. J., and Choy, P. C. (1992) J. Biol. Chem. 267, 23527–23533
Purification of the Serine Palmitoyltransferase Complex Responsible for Sphingoid Base Synthesis by Using Affinity Peptide Chromatography Techniques
Kentaro Hanada, Tomoko Hara and Masahiro Nishijima

J. Biol. Chem. 2000, 275:8409-8415.
doi: 10.1074/jbc.275.12.8409

Access the most updated version of this article at http://www.jbc.org/content/275/12/8409

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 30 references, 16 of which can be accessed free at http://www.jbc.org/content/275/12/8409.full.html#ref-list-1