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Characterization of Enzymatic Synthesis of Sphingolipid Long-Chain Bases in *Saccharomyces cerevisiae*: Mutant Strains Exhibiting Long-Chain-Base Auxotrophy Are Deficient in Serine Palmitoyltransferase Activity

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We have begun a biochemical-genetic analysis of the synthesis of sphingolipid long-chain bases in *Saccharomyces cerevisiae* and found evidence for the occurrence of serine palmitoyltransferase (SPT) and 3-ketosphinganine reductase, enzymes that catalyze the initial steps of the pathway in other organisms. SPT activity was demonstrated in vitro with crude membrane preparations from *S. cerevisiae* as judged by the formation of radiolabeled 3-ketosphinganine from the condensation of palmitoyl-coenzyme A (CoA) with radiolabeled serine. Shorter (C_{12} and C_{14}) and longer (C_{18}) acyl-CoAs sustain significant SPT activity, a result consistent with the finding of both C_{18} and C_{20} long-chain bases in the organism. Three products of the long-chain-base synthetic pathway, 3-ketosphinganine, erythrosphinganine, and phytosphingosine, neither directly inhibited the reaction in vitro nor affected the specific activity of the enzyme when these bases were included in the culture medium of wild-type cells. Thus, no evidence for either feedback inhibition or repression of enzyme synthesis could be found with these putative effectors. Mutant strains of *S. cerevisiae* that require a sphingolipid long-chain base for growth fall into two genetic complementation groups, *LCB1* and *LCB2*. Membrane preparations from both *lcb1* and *lcb2* mutant strains exhibited negligible SPT activity when tested in vitro. Step 2 of the long-chain-base synthetic pathway was demonstrated by the stereospecific NADPH-dependent reduction of 3-ketosphinganine to erythrosphinganine. Membranes isolated from wild-type cells and from an *lcb1* mutant exhibited substantial 3-ketosphinganine reductase activity. We conclude that the Lcb$^-$ phenotype of these mutants results from a missing or defective SPT, an activity controlled by both the *LCB1* and *LCB2* genes. These results and earlier work from this laboratory establish that SPT plays an essential role in sphingolipid synthesis in *S. cerevisiae*.

Fungi and plants contain sphingolipids that are distinguished from animal sphingolipids; they contain phosphoinositol as part of their polar head groups with phytosphingosine (PHS) as the major long-chain-base (LCB) component (1, 8). Such sphingolipids have been shown to be highly localized in the plasma membrane of *Saccharomyces cerevisiae* (17). To study the metabolism and function of sphingolipids in *S. cerevisiae*, we isolated mutant strains, termed Lcb$^-$, auxotrophic for sphingolipid LCBs. The Lcb$^-$ strains fell into two genetic complementation groups, termed *LCB1* and *LCB2* (18). Without an appropriate LCB, these strains were unable to grow and synthesize sphingolipid (18, 27) and rapidly lost viability (18); these data suggested one or more vital roles for the yeast sphingolipids.

No information is available concerning the initial steps of sphingolipid LCB synthesis in *S. cerevisiae*. We have characterized the first two steps of LCB synthesis (Fig. 1) as catalyzed by isolated membranes from *S. cerevisiae*. These in vitro enzymatic studies indicate that step 1, carried out by serine palmitoyltransferase (SPT), is lacking in both *lcb1* and *lcb2* mutant strains.

(Some of this work has previously appeared in abstract form [19].)

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MATERIALS AND METHODS

Abbreviations. The following abbreviations are used in this report: DHS, DL-sphinganine or DL-dihydrosphingosine; 3-KDS, DL-3-ketosphinganine; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

Materials. L-[G-^{3}H]serine (25 mCi/mmol) and L-[3-^{14}C]serine (55 mCi/mmol) were from ICN Radiochemicals. D-[1-^{3}H]glucose (15 mCi/mmol) was from American Radiolabeled Chemicals, Inc. Fatty acyl coenzymes A (CoAs), DL-erythro-DHS, hexokinase (type C-300), and glucose 6-phosphate dehydrogenase (type VII) were from Sigma Chemical Co. 3-KDS was prepared as described by Gaver and Sweeley (6) and Shapiro et al. (20). DL-Threo-DHS was separated from erythro-DHS in a mixture of the two isomers obtained from Sigma Chemical Co. as the biphenylcarbonyl derivatives and separated by high-performance liquid chromatography (HPLC) as previously described (18). Tetra-acetyl PHS, a gift of R. F. Vesonder (Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, Ill.), was converted to PHS as previously described (18).

Strains, culture media, and conditions. The strains of *S. cerevisiae* used in this study are listed in Table 1. Strains 1D and 31A were obtained by mutagenesis of strain MC6A as described by Wells and Lester (27). Some of the following strains were obtained from dissected tetrads (parental strains are in parentheses: 1B2D (31A × W303-1B) and X45A, X45B, X45C, and X45D (1B2D × W303-1B). The following strains were derived from random spores (18): X19D3 (24C

TABLE 1. Genotypes and origins of the yeast strains used in these studies

Strain	Genotype	Source or reference
MC6A	*MAT***a** *ino1-13 ino4-8*	4
SJ21R	*MAT***a** *ura3-52 leu2-3,112 ade1 MEL1*	12
W303-1B	MATα *ade2-1 can1-100 ura3-1 his3-11,15 leu2-3,112 trp1-1*	R. Rothstein
31A	*MAT***a** *lcb1 ino1-13 ino4-8*	18
1D	*MAT***a** *lcb2 ino1-13 ino4-8*	18
1B2D	*MAT***a** *lcb1 ino*	18
X45A	*MAT***a** *lcb1 ade2-1 ura3-1 trp1-1 ino*	18
X45B	*ino*	18
X45C	ade2-1 leu2-3,*112 his3-11,15*	18
X45D	MATα *lcb1 ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11,15*	18
X19D3	MATα *lcb2 ade2-1 ura3-1 trp1-1 leu2-3,112*	18
X23D3	MATα *lcb1 ura3-1 leu2-3,112*	18

× W303-1B) and 23D3 (15CS × W303-1B). Culture media and growth condition were as previously described (18).

Preparation of a membrane fraction. Cells were grown to mid-logarithmic phase (A_{650}, 4 to 6), washed twice with 50 mM potassium phosphate buffer (pH 7.0), and disrupted with 0.3 to 0.5-mm-diameter glass beads in 50 mM potassium phosphate buffer (pH 7.0) containing dithiothreitol (5 mM) and phenylmethylsulfonyl fluoride (1 mM), using a bead beater, for 8 to 10 1-min treatments interspaced with cooling periods. The homogenate was centrifuged at 4,000 × *g* for 10 min to remove unbroken cells and debris. The supernatant was centrifuged at 100,000 × *g* for 1 h at 4°C. The pellet was homogenized in the same buffer by using a Potter-Elvehjem homogenizer, and this suspension was centrifuged at 100,000 × *g* for 1 h. The pellet obtained was homogenized and suspended in the buffer described above containing 20 to 30% glycerol and stored at −20°C. Protein was measured by using the biuret method with bovine serum albumin as the standard (7).

SPT assay method I. The SPT assay method I procedure was essentially that of Williams et al. (28). Each reaction mixture contained the following components in a volume of 0.2 ml: 0.1 M HEPES (pH 8.3), 5 mM dithiothreitol, 2.5 mM EDTA, 50 μM pyridoxal phosphate, 40 μM palmitoyl CoA, 5 mM L-serine, L-[G-^{3}H]serine (usually 5 μCi), and 0.2 mg of membrane protein. After incubation at 30°C for 20 min with shaking, the reaction was terminated with 0.5 ml of 0.5 N NH_4OH containing 5 μmol of L-serine. The labeled product was extracted by adding the following and mixing vigorously: 3.0 ml of $CHCl_3$-CH_3OH (1:2), 50 μg of 3-KDS or DHS in 50 μl of 95% ethanol, 2 ml of $CHCl_3$, and 4 ml of 0.5 N NH_4OH. The tubes were centrifuged for 10 min in a clinical centrifuge, the upper aqueous phase was removed by aspiration, and the lower phase was washed three times with several volumes of water to remove all traces of unreacted [^{3}H]serine. An aliquot of the $CHCl_3$ layer in a scintillation vial was evaporated to dryness over a steam bath, and scintillation cocktail of the following composition was added: 0.4% 2-(4′-*t*-butylphenyl)-5-(4′-biphenyl)-1,3,4-oxidazole–0.03% 2-(4′-biphenyl)-6-phenylbenzoxazole in Triton X-100–toluene–water (1:2:0.35). The radioactivity was measured on a Packard PRIAS scintillation counter. Radioactivity in the control without enzyme was subtracted from all of the assays to calculate the specific activity.

SPT assay method II. A slightly different assay procedure was developed and used for inhibition studies with LCBs because of nonenzymatic reactions obtained with tritiated serine in the presence of certain LCBs, viz., 3-KDS and PHS. The radioactive artifact migrates to the solvent front when run on Whatman SG 81 paper developed with $CHCl_3$-CH_3OH-2 N NH_4OH (40:10:1). It was found subsequently that the basic pH of the assay procedure promoted artifact formation so that it became necessary to develop an assay and extraction procedure under essentially neutral or acidic conditions. In the modified assay procedure, the only changes were 0.1 M HEPES buffer (pH 8.3) replaced with 0.1 M potassium phosphate buffer (pH 7). The LCB (in 10 μl of 95% ethanol) was added prior to membrane addition. The reaction was terminated by addition of 0.4 ml of 0.5 N acetic acid, followed by additions of 0.1 ml of 0.1% lauryl sulfate, 1.2 ml of water, 0.1 ml of 50 mM L-serine, and 0.05 ml of DHS (1 mg/ml in 95% ethanol). After mixing, 2 ml of ethyl acetate was added and the mixture was vortexed vigorously. Finally, 2 ml of chloroform was added, the tubes were vortexed, and the two layers were separated after centrifugation in a clinical centrifuge for 5 to 10 min. The aqueous layer was removed by aspiration, and the chloroform layer was washed three times with approximately 4 ml of water before removal of an aliquot of the organic layer for scintillation counting.

3-KDS reductase assay. 3-KDS reductase was assayed by the method of Stoffel et al. (25). The incubation mixture contained the following components in a volume of 2.0 ml: 0.2 mmol of Tris-Cl (pH 7.8), 0.1 μmol of $MgCl_2$, 0.4 μmol of NADP, 7 μmol of ATP, 0.15 μmol of D-[1-^{3}H]glucose (230 dpm/pmol), 20 μg of hexokinase, 4 μg of glucose 6-phosphate dehydrogenase, 0.2 μmol of 3-KDS in 20 μl of 95% ethanol, 1 mg of Triton X-100, and 0.1 to 0.8 mg of membrane protein. The mixture (without 3-ketosphinganine and membrane protein) was incubated at 30°C for 30 min to allow for generation of [^{3}H]NADPH. 3-KDS and membranes were then added, and the mixture was incubated for another 30 min. The reaction was terminated, and the radiolabeled $CHCl_3$-soluble DHS formed was extracted and counted as in SPT assay method I, described above.

Identification of chloroform-soluble enzymatic products. Paper chromatography of the radiolabeled $CHCl_3$-soluble products was carried out on silicic acid-impregnated paper (Whatman SG 81) treated with EDTA (23) by using $CHCl_3$-CH_3OH-2 N NH_4OH (40:10:1) as the developing solvent. Zones with unlabeled LCB standards were visualized by spraying with 0.2% ninhydrin in 95% ethanol. Lanes with radioactivity were cut into 1-cm sections and placed in scintillation vials, and the radioactivity was measured by scintillation counting with 4 ml of the above-described scintillation cocktail.

Liquid chromatography of the UV-absorbing *N*-biphenylcarbonyl derivatives of LCBs was performed on an Altex 5 μ Ultrasphere octyldecyl silane column (0.46 by 25 cm) eluted with CH_3OH-H_2O (90:10) at a flow rate of 1.5 ml/min and monitored at 280 nm. The derivatives of the radioactive $CHCl_3$-soluble enzymatic products with added unlabeled LCB standards were prepared as previously described (5). Samples of 0.75 ml were collected and evaporated to dryness over a steam bath, scintillation cocktail was added, and the radioactivity was measured by scintillation counting.

RESULTS

Identification of enzymatic products formed in the SPT assay with membranes from wild-type *S. cerevisiae*. Crude

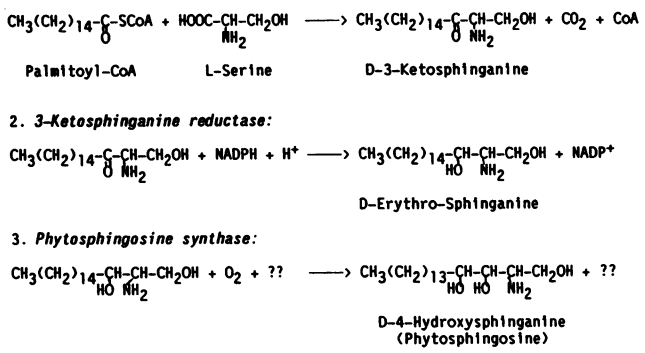

FIG. 1. Pathway of sphingolipid LCB synthesis.

membranes of strain MC6A exhibited SPT (Fig. 1) activity as assayed by conversion of labeled serine to a labeled $CHCl_3$-soluble product, putatively 3-KDS; the activity was absolutely dependent on the presence of palmitoyl CoA (Table 2, experiment 1). Chromatography of the radiolabeled $CHCl_3$-soluble extract on silica gel-impregnated paper (see Materials and Methods) revealed one radioactive zone that migrated with the unlabeled 3-KDS standard; after treatment of the extract with sodium borohydride, the putative 3-KDS disappeared and a single product that migrated with the DHS standard as expected appeared (data not shown). Finally, the radiolabeled $CHCl_3$-soluble product was converted to an *N*-biphenylcarbonyl derivative which exhibited the same retention time on reverse-phase liquid chromatography as the standard *N*-biphenylcarbonyl-3-KDS (Fig. 2). We therefore conclude that under the in vitro assay conditions used, the enzymatic product was 3-KDS.

TABLE 2. SPT activity in crude membranes from strain MC6A[a]

Changes in assay conditions	Enzyme activity (%)[b]
Expt 1	
None	100
Minus palmitoyl CoA	3
Minus dithiothreitol	67
Minus EDTA	69
Minus pyridoxal 5-phosphate	62
Minus carrier 3-KDS[c]	43
Expt 2	
None	100
Plus 0.10 mM 3-KDS	84
Plus 1.00 mM 3-KDS	96
Plus 0.10 mM erythro-DHS	90
Plus 1.00 mM erythro-DHS	68
Plus 0.10 mM PHS	89
Plus 1.00 mM PHS	113
Expt 3	
None	100
Plus 0.1 mM L-cycloserine	85
Plus 1.0 mM L-cycloserine	38
Plus 10 mM D-cycloserine	74
Plus 50 mM D-cycloserine	9

[a] SPT assays were carried out with method I (experiments 1 and 3) or 2 (experiment 2).
[b] Basal activities: 103 (experiment 1), 102 (experiment 2) and 104 (experiment 3) pmol/min/mg of protein.
[c] Carrier LCB added after termination of the enzyme reaction.

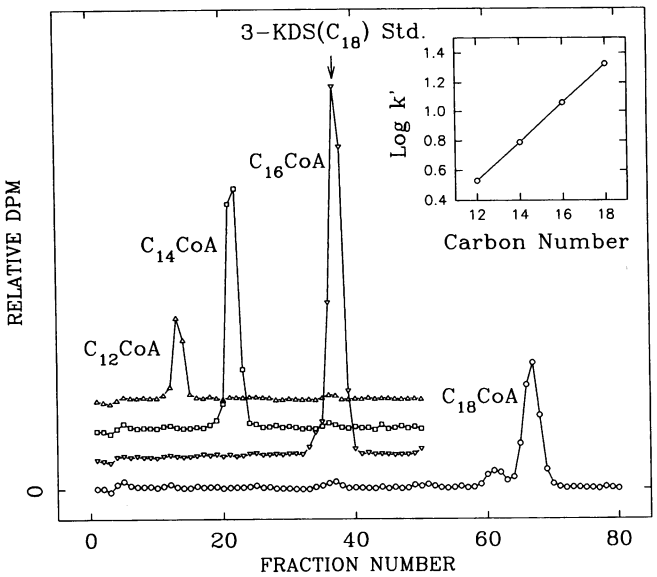

FIG. 2. HPLC of SPT assay products carried out with C_{12} to C_{18} acyl CoAs. The labeled $CHCl_3$-soluble products obtained in the SPT assay with strain MC6A membranes (method I) starting with C_{12} to C_{18} acyl CoAs were converted to *N*-biphenylcarbonyl derivatives and subjected to reverse-phase HPLC (see Materials and Methods), and each fraction was evaluated for tritium. Unlabeled 3-KDS, added to the $CHCl_3$ extracts before derivatization, was monitored at 280 nm. The data for each experiment were displaced vertically for convenience; the tritium levels in the main peaks were 5,737 (C_{12}), 19,518 (C_{14}), 33,484 (C_{16}), and 14,466 (C_{18}) dpm. Insert: logarithm of the capacity factor, $(V - V_0)/V_0$, for the major peak in each experiment plotted against the carbon number of the acyl CoA used.

In addition to palmitoyl CoA, four other fatty acyl CoAs of various chain lengths were tested as substrates in the SPT assay; substantial activity was observed with C_{12}, C_{14}, and C_{18} CoAs, and negligible activity was observed with C_{20} CoA (Table 3). *N*-Biphenylcarbonyl derivatives of the products obtained with the different fatty acyl CoAs were prepared and subjected to reverse-phase HPLC. In each case, one major product was observed (Fig. 2). Standards for the putative C_{14}, C_{16}, and C_{20} 3-keto products were unavailable; however, a plot of the log of their capacity factors versus carbon number along with that of the available standard C_{18} derivative exhibited the linear relationship expected for the presumed homologous products (Fig. 2, insert). The existence of both C_{18} and C_{20} LCBs in *Saccharomyces* sphingolipids (21) is compatible with the in vitro acyl CoA specificity exhibited by SPT (Table 3).

Properties of *S. cerevisiae* SPT activity. SPT activity was

TABLE 3. Specificity of SPT with respect to chain length of acyl CoA[a]

Fatty acyl CoA chain length	Mean enzyme activity (pmol/min/mg of protein) ± SD (no. of expts)
C_{12}	35 ± 5 (6)
C_{14}	85 ± 13 (6)
C_{16}	106 ± 12 (6)
C_{18}	78 ± 16 (6)
C_{20}	2 ± 1 (2)

[a] SPT was assayed by method I (see Materials and Methods) with membranes from strain MC6A at 1 mg/ml and with fatty acyl CoAs at 40 μM.

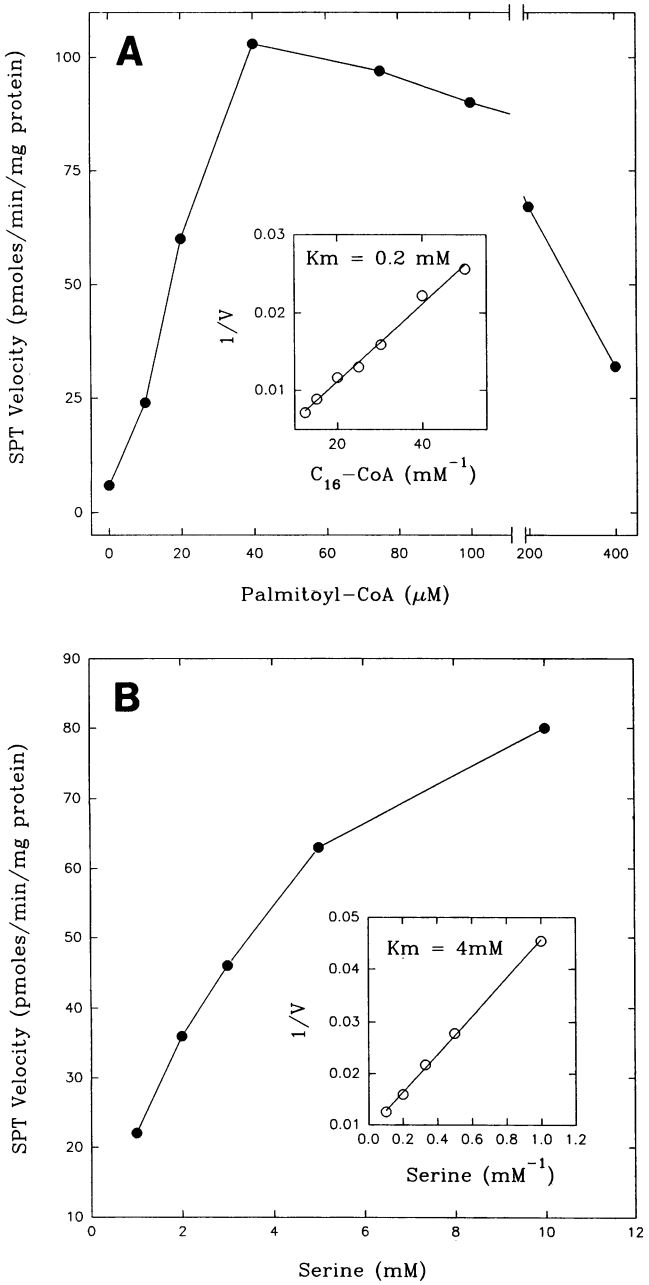

FIG. 3. Kinetics of SPT in membranes of *S. cerevisiae*. (A) Palmitoyl CoA concentration and SPT activity. Activity was assayed (method I) with strain MC6A membranes at 1 mg/ml. (B) L-Serine concentration and SPT activity. Activity was assayed (method II) with strain MC6A membranes at 1 mg/ml.

partially dependent on pyridoxal-5-phosphate (Table 2), and product formation was linear with time for at least 20 min and proportional to membrane concentrations from 0.5 to 1.5 mg of protein per ml (data not shown). Small amounts of carrier LCB added after the enzymatic reaction significantly improved recovery of the product in the $CHCl_3$ extract (Table 2). Enzyme activity increased with the palmitoyl CoA concentration up to about 40 μM, approximately its critical micelle concentration (3), with inhibition at concentrations of >100 μM (Fig. 3A); an apparent K_m of about 0.2 mM was calculated (Fig. 3A). An apparent K_m of 4 mM for serine was obtained from a Lineweaver-Burk plot (Fig. 3B). D-cycloserine and L-cycloserine were tested for inhibitory activity because of their effectiveness against bacterial and mouse brain SPTs (26). L-Cycloserine was more potent than D-cycloserine as an inhibitor of *S. cerevisiae* SPT. At a concentration of 50 mM, D-cycloserine produced 90% inhibition whereas L-cycloserine caused a 60% drop in enzyme activity at 1 mM (data not shown).

SPT activity was optimum at pH 8.0, with HEPES, phosphate, or Tris buffer, giving equivalent activity in the range of pH 7 to 9 (data not shown). SPT activity was unaffected by Tween 20 at concentrations of 0.2 to 1%; however, Brij 56, octyl glucoside, and α-cyclodextrin at a concentration of 0.2% gave 40 to 50% inhibition and 0.2% Triton X-100 and deoxycholate gave 75 and 90% decreases in enzyme activity, respectively (data not shown).

Effects of LCBs on SPT activity. Since little is known about the regulation of SPT activity, we tested the direct effects of LCBs on SPT activity in vitro. Little inhibition was observed with 100 to 1,000 μM 3-KDS, erythro-DHS, and PHS (Table 2, experiment 2). The 30% inhibition with 1,000 μM erythro-DHS is of dubious significance, since the concentration probably far exceeds physiological levels.

SPT activity in Lcb^+ and Lcb^- strains. To examine the biochemical basis for the LCB requirement of strains of *S. cerevisiae* (18, 27), we measured the SPT activity in membranes from mutant strains and the parental wild-type strains. First, the effects of sphingolipid LCBs in the growth medium on expression of SPT activity in wild-type cells were examined. The three Lcb^+ strains tested exhibited similar levels of SPT activity whether grown in synthetic or complex medium, either with or without 100 μM LCBs (Table 4); thus, there was no significant repression of SPT activity with a level of LCB adequate to support growth of Lcb^- strains (18, 27).

Very low SPT activity, at the limit of detectability, was observed in all of the Lcb^- strains tested, representing the two genetic complementation groups *LCB1* and *LCB2* (18) (Table 4). The tetrad progeny from crosses of $Lcb^+ \times Lcb^-$ strains gave 2 Lcb^+:2 Lcb^- (18); analysis of such a cross (Table 4) showed that the two Lcb^- progeny had low levels of SPT activity whereas the two Lcb^+ progeny had nearly wild-type levels of activity. The low level of SPT activity observed in Lcb^- strains is not likely to be due to repression of SPT activity by the LCB required for growth, since LCB in the culture media of LCB^+ strains did not repress SPT activity (Table 4). We conclude that lack of SPT activity accounts for the Lcb^- phenotype in the mutant strains investigated and that both *LCB1* and *LCB2* genes control SPT activity in *S. cerevisiae*.

3-KDS reductase activity in Lcb^+ and Lcb^- *S. cerevisiae*. 3-KDS reductase (Fig. 1) was measured by assaying the reduction of unlabeled 3-KDS, forming $CHCl_3$-soluble erythro-DHS (25), with [^{3}H]NADPH generated in situ from [^{3}H]glucose. With crude membranes as the enzyme source, generation of $CHCl_3$-soluble radioactivity was observed and was absolutely dependent on addition of 3-KDS and the NADPH-generating system (Table 5). Substantial activity was observed with membranes from both strains MC6A (Lcb^+) and 31A (Lcb^-) (Table 5 and Fig. 4) under assay conditions in which the enzymatic synthesis of the product was fairly proportional to incubation time and amount of enzyme (Fig. 4).

The radiolabeled $CHCl_3$-soluble product migrated with unlabeled DHS (data not shown) when chromatographed on

TABLE 4. SPT activity in mutant and wild-type strains[a]

Strain	Culture medium, LCB concn	LCB growth requirement	Mean SPT activity (pmol/min/mg of protein) ± SD (no. of expts)
Wild type			
MC6A	SYN, no LCB	No	95.0 ± 7.6 (3)
MC6A	SYN, 100 μM PHS	No	104.3 ± 2.1 (3)
MC6A	SYN, 100 μM DHS	No	94.5 ± 14.9 (2)
MC6A	PYE, no LCB	No	97.0 ± 1.4 (2)
MC6A	PYE, 100 μM DHS	No	132 ± 7.1 (2)
W303-1B	SYN, no LCB	No	82.0 ± 12.5 (3)
W303-1B	SYN, 100 μM PHS	No	84.7 ± 6.0 (3)
W303-1B	SYN, 100 μM DHS	No	70.5 ± 0.7 (2)
SJ21R	PYE, 100 μM DHS	No	79.0
Mutant			
31A (*lcb1*)	PYE, 100 μM DHS	Yes	4.3 ± 3.1 (3)
1D (*lcb2*)	SYN, 100 μM DHS	Yes	7
X23D3 (*lcb1*)	PYE, 100 μM DHS	Yes	0
X19D3 (*lcb2*)	PYE, 100 μM DHS	Yes	2.1
Tetrad progeny from W303-1B × 1B2D			
X45A	SYN, 25 μM PHS	Yes	3.5 ± 0.7 (2)
X45B	SYN, 25 μM PHS	No	84.5 ± 9.2 (2)
X45C	SYN, 25 μM PHS	No	72.0 ± 2.8 (2)
X45D	SYN, 25 μM PHS	Yes	3.5 ± 2.1 (2)

[a] Cells were cultured on complex (PYE) or synthetic (SYN) medium containing the LCBs indicated and harvested in the exponential phase. SPT activity was measured in isolated membranes by method I.

silica gel-impregnated paper (see Materials and Methods). Reduction of 3-KDS could yield two possible isomers: erythro-DHS and threo-DHS. To address this question, we carried out reverse-phase liquid chromatography with the ^{3}H-labeled *N*-biphenylcarbonyl derivative of the $CHCl_3$-soluble 3-KDS reductase products under conditions that resolve these isomers; the results showed that the product from both the Lcb$^+$ and Lcb$^-$ strains migrated with the internal erythro-DHS standard (Fig. 5). We conclude that

TABLE 5. 3-KDS reductase activities in wild-type and LCB auxotroph membranes[a]

Strain	Assay mixture	3-KDS reductase activity (pmol/min/mg of protein)
Expt 1		
MC6A	Complete	262
MC6A	Minus 3-KDS	1
MC6A	Minus membranes	1
MC6A	Minus NADPH-generating system	0
Expt 2		
MC6A	Complete	214
31A	Complete	108

[a] 3-KDS reductase was assayed as described in Materials and Methods, with the following modifications in experiment 1: 60-min incubation time, NADPH generation system components omitted: hexokinase, NADP, and glucose 6-phosphate dehydrogenase.

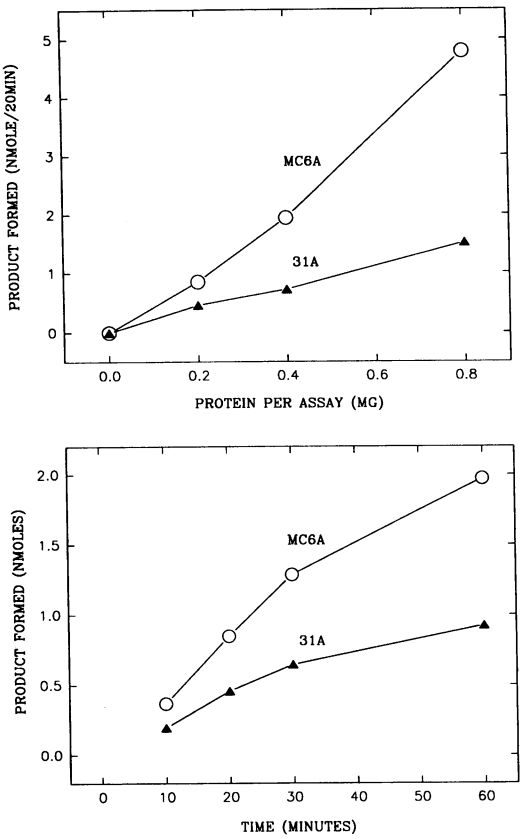

FIG. 4. 3-KDS reductase activities in membranes from Lcb$^+$ and Lcb$^-$ *S. cerevisiae*. 3-KDS reductase assays were carried out, as described in Materials and Methods, for 20 min with various amount of membranes (A) and with 0.2 mg of membrane protein for various times (B). Membranes were from Lcb$^+$ (strain MC6A) and Lcb$^-$ (strain 31A) cells.

there is NADPH–3-KDS reductase activity in *S. cerevisiae* that specifically formed erythro-DHS in Lcb$^+$ membranes, as well as in membranes from an Lcb$^-$ strain bearing a mutant allele of the *lcb1* gene.

DISCUSSION

In this study, membrane preparations of *S. cerevisiae* were shown to catalyze the first two steps in LCB synthesis (Fig. 1) as judged by product identification and the requirements of the two assay systems. SPT activity (Fig. 1, reaction 1) in crude membranes was absolutely dependent on a fatty acyl CoA, with a chain length specificity consonant with the occurrence of both C_{18} and C_{20} LCBs in *S. cerevisiae* (21); the 3-keto LCBs of various chain lengths were identified as products (Fig. 2). We found no evidence for direct inhibition of SPT activity by addition of high levels of various LCBs in vitro; likewise, no evidence for repression of active enzyme formation was observed by including LCBs in the culture medium. With cultured cerebellar cells, there is evidence that SPT and sphingosine synthesis is inhibited by addition of exogenous LCB (15).

The stereospecific NADPH-dependent reduction of 3-KDS to the erythro isomer of DHS (Fig. 1, reaction 2) was also observed in extracts of *S. cerevisiae*, both in a wild-type strain and in an Lcb$^-$ strain. It was reasonable to find 3-KDS reductase activity in an Lcb$^-$ strain for which exogenously

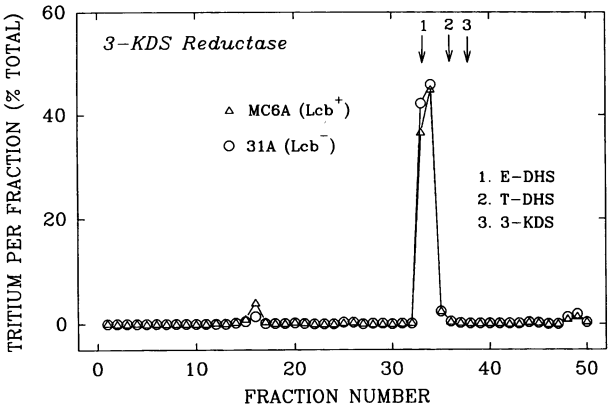

FIG. 5. HPLC analysis of products formed in the 3-KDS reductase assay. The *N*-biphenylcarbonyl derivatives of the labeled $CHCl_3$-soluble products obtained in the 3-KDS reductase assay with membranes from strain MC6As (Lcb^+) and 31A (Lcb^-) were subjected to reverse-phase HPLC (see Materials and Methods), each fraction was evaluated for ^3H. Unlabeled erythro-DHS (E-DHS), threo-DHS (T-DHS), and 3-KDS were added to the chloroform extract before derivatization, and their peaks (arrows) were located by monitoring the effluent continuously at 280 nm.

added 3-KDS satisfied the LCB growth requirement (18). PHS is the major LCB in *S. cerevisiae* lipid; however, its formation (Fig. 1, reaction 3) has yet to be observed in vitro; in vivo experiments suggest that molecular oxygen is the precursor of the 4 oxygen (13).

The first two steps of the generally accepted pathway of sphingolipid LCB biosynthesis (Fig. 1) were demonstrated over 20 years ago in extracts of the fungus *Hansenula ciferri* (22) and rat tissues (24). Subsequent work has indicated that SPT may be rate limiting in LCB synthesis in various animal tissues (10, 16). SPT has not been obtained in highly pure form from any source, although partial purification from the obligate anaerobe *Bacteroides melaninogenicus* has been achieved (14). Little is known about the regulation of LCB synthesis in any tissue, a question that will receive increased attention since exogenous sphingolipid LCBs exhibit a variety of profound effects on animal cells (9, 11). We are beginning to address some of these unanswered questions concerning sphingolipid biosynthesis in *S. cerevisiae*, an organism that permits full exploitation of molecular genetic-biochemical techniques.

Work from this laboratory has established SPT as a critical enzyme in the synthesis of sphingolipids in *S. cerevisiae*. Lcb^- strains which had an absolute growth requirement for a sphingolipid LCB and fell into two genetic complementation groups, *LCB1* and *LCB2*, were isolated (18, 27). When cultured without LCB, these strains exhibited severe loss of viability (18) and showed a marked and specific reduction in the ability to incorporate labeled precursors into the inositol-containing sphingolipids (18, 27). The experiments described here demonstrate that extracts of both *lcb1* and *lcb2* mutant strains exhibit very low SPT activity and thus these genes must somehow regulate the expression of this activity. Recent work has suggested that the *LCB1* gene may code for SPT or a portion thereof: the amino acid sequence predicted from the cloned *LCB1* DNA showed a striking similarity to the amino acid sequences of enzymes catalyzing reactions similar to that of SPT (2). The role of the *LCB2* gene is currently being explored to examine whether it codes for a subunit of SPT or somehow regulates its expression.

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