Phytanic acid activation in rat liver peroxisomes is catalyzed by long-chain acyl-CoA synthetase

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Abstract In Refsum disease, disorders of peroxisome biogenesis, and rhizomelic chondrodysplasia punctata, pathological accumulation of phytanic acid results from impaired α-oxidation of this branched-chain fatty acid. Previous studies from this laboratory indicated that activation of phytanic acid to its CoA derivative precedes its α-oxidation in peroxisomes. It was reported that this reaction is catalyzed by a unique phytanoyl-CoA synthetase in human peroxisomes. We wanted to determine whether phytanic acid activation in rats required long-chain acyl-CoA synthetase (LCS), very long-chain acyl-CoA synthetase (VLCS), or a different enzyme. To test directly whether LCS could activate phytanic acid, rat liver cDNA encoding this enzyme was transcribed and translated in vitro. The expressed enzyme had both LCS activity (assayed with palmitic acid, C16:0) and phytanoyl-CoA synthetase activity; VLCS activity (assayed with lignoceric acid, C24:0) was not detectable. The ratio of phytanoyl-CoA synthetase activity to palmitoyl-CoA synthetase activity for LCS synthetized in vitro (~20%) was higher than that observed in peroxisomes isolated from rat liver (5–10%), suggesting that the expressed enzyme contained sufficient phytanoyl-CoA synthetase activity to account for all activity observed in intact peroxisomes. Further experiments were carried out to verify that phytanic acid was activated by LCS in rat liver peroxisomes. Attempts to separate LCS from phytanoyl-CoA synthetase by chromatography on several matrices were unsuccessful. Preparative isoelectric focusing revealed that phytanoyl-CoA synthetase and LCS had indistinguishable isoelectric points. Phytanoyl-CoA synthetase activity was inhibited by unlabeled palmitic acid but not by lignoceric acid. Heat treatment inactivated both phytanoyl-CoA and palmitoyl-CoA synthetase activities at similar rates. 5,8,11,14-Eicosatetraynoic acid inhibited activation of phytanic acid and palmitic acid in a parallel dose-dependent manner, whereas activation of lignoceric acid was not affected. These data support our conclusion that rat liver LCS, an enzyme known to be present in peroxisomal membranes, has phytanoyl-CoA synthetase activity.—**Watkins, P. A., A. E. Howard, S. J. Gould, J. Avigan, and S. J. Mihalik.** Phytanic acid activation in rat liver peroxisomes is catalyzed by long-chain acyl-CoA synthetase. *J. Lipid Res.* 1996. **37:** 2288–2295.

Supplementary key words fatty acid activation • α-oxidation • Refsum disease • rhizomelic chondrodysplasia punctata • peroxisome biogenesis disorders • Zellweger syndrome

The catabolism of β-methyl branched-chain fatty acids such as phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) is impaired in patients with the genetic disorders Refsum disease (reviewed in ref. 1), peroxisome biogenesis disorders (PBD) (e.g., Zellweger syndrome; reviewed in ref. 2), and in rhizomelic chondrodysplasia punctata (RCDP) (reviewed in ref. 2). PBD and RCDP are well-characterized peroxisomal disorders in which several peroxisomal metabolic processes in addition to phytanic acid oxidation are impaired. In order to gain a better understanding of normal and defective phytanic acid oxidation, we are investigating the individual steps of the pathway in rat liver.

The methyl branch on the β-carbon of phytanic acid prevents its catabolism by the β-oxidation pathway. Oxidative decarboxylation, or α-oxidation, of phytanic acid to pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) shifts the positions of the methyl branches so that further degradation can proceed by β-oxidation (3, 4). Avigan et al. (3) and Tsai et al. (5) originally proposed that the α-oxidation pathway involved first the α-hydroxylation of phytanic acid, followed by decarboxylation. To investigate the α-hydroxylation process, Zenger-Hain, Craft, and Rizzo (6) and this laboratory (7) studied deprotonation of [2,3-^{3}H]phytanic acid and found that this process was defective in fibroblasts from patients with Refsum disease, PBD, and RCDP. Furthermore, we found that deprotonation of phytanic acid was associated primarily with peroxisome-enriched fractions from rat liver (7, 8). Deprotonation in peroxi-

Abbreviations: RCDP, rhizomelic chondrodysplasia punctata; PBD, peroxisome biogenesis disorders; LCS, long-chain acyl-CoA synthetase; VLCS, very long-chain acyl-CoA synthetase; ETYA, 5,8,11,14-eicosatetraynoic acid; PCR, polymerase chain reaction; phytanic acid, 3,7,11,15-tetramethylhexadecanoic acid; pristanic acid, 2,6,10,14-tetramethylpentadecanoic acid; palmitic acid, C16:0 or hexadecanoic acid; lignoceric acid, C24:0 or tetracosanoic acid.

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somes occurred only when phytanic acid was first activated to its coenzyme A (CoA) derivative (8). Subsequent investigations revealed that in the presence of 2-oxoglutarate and Fe^{2+}, peroxisomal deprotonation of phytanic acid was associated with the formation of the product α-hydroxyphytanoyl-CoA (9). These results suggested that the initial step in the peroxisomal α-oxidation is activation of phytanic acid by an acyl-CoA synthetase.

Pahan and coworkers (10) reported that activation of phytanic acid in human skin fibroblast peroxisomes was catalyzed by an enzyme distinct from the two synthetases with known peroxisomal activities, long- and very long-chain acyl-CoA synthetases (LCS and VLCS, respectively). We wanted to determine whether phytanic acid activation in rat liver peroxisomes was also catalyzed by a unique ensyme. Both Muralidharan and Muralidharan (11) and Vanhooren et al. (12) presented indirect evidence suggesting that this might be the case. However, because of the difficulties in accurately assessing the effects of inhibitors and detergents on synthetase activity, we decided to investigate phytanoyl-CoA synthetase activity by expressing rat liver LCS cDNA in an in vitro transcription/translation system. LCS is a well-characterized enzyme that has been purified and cloned (13, 14). We report here that rat liver LCS expressed in vitro activates phytanic acid. Like Vanhooren et al. (12), we were unable to separate physically rat liver peroxisomal LCS and phytanoyl-CoA synthetase activities. Thus, we conclude that LCS, an enzyme known to be associated with peroxisomes, is capable of activating phytanic acid.

METHODS

Materials

[1-^{14}C]palmitic acid (C16:0) was purchased from DuPont/New England Nuclear (Boston, MA), [1-^{14}C]lignoceric acid (C24:0) was from Research Products International (Mount Prospect, IL), and [^{35}S]methionine was from Amersham (Arlington Heights, IL). [2,3-^{3}H]dihydrophytol was kindly provided by Dr. William Rizzo, Medical College of Virginia, Richmond, VA. [2,3-^{3}H]phytanic acid was prepared from [2,3-^{3}H]dihydrophytol was described by Zenger-Hain et al. (6). Nycodenz and Maxidens were obtained from Accurate Chemical and Scientific (Westbury, NY). CoA was obtained from Pharmacia Biotech Inc. (Piscataway, NJ). Cyclodextrins, CHAPS, unlabeled fatty acids, and assay reagents and cofactors were from Sigma Chemical Co. (St. Louis, MO). Restriction enzymes and buffers were from Boehringer Mannheim (Indianapolis, IN). TnT Coupled Transcription/Translation System was from Promega (Madison, WI). 5,8,11,14-Eicosatetraynoic acid (ETYA) was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). A cDNA clone encoding the rat long chain fatty acyl-CoA synthetase (14) was a kind gift from Dr. T. Yamamoto, Tohoku Univ. Gene Research Center, Sendai, Japan.

Fatty acyl-CoA synthetase assay

Acyl-CoA synthetase activity was measured exactly as described (8). Radiolabeled fatty acids (final concentration, 20 μM; 30–40,000 dpm/nmol) were solubilized with β-cyclodextrin (phytanic and C16:0) or α-cyclodextrin (C24:0); the final concentration of cyclodextrin in assays was 2 mg/ml. When present, unlabeled fatty acids, including ETYA, were solubilized with cyclodextrin along with the labeled fatty acid. Because lignoceric acid is not solubilized with β-cyclodextrin and phytanic acid is poorly solubilized by α-cyclodextrin, a combination of α- and β-cyclodextrins (final concentrations, 2 and 1 mg/ml, respectively) was used in experiments in which both fatty acids were present. Acyl-CoA synthetase assays contained 2–10 μg, 15–40 μg, and 50–100 μg of peroxisomal protein for palmitic, phytanic, and lignoceric acid substrates, respectively.

Isolation of peroxisomes from rat liver

Peroxisomes were isolated from livers of female CD rats (Charles River) by centrifugation through 30% (w/v) Nycodenz exactly as described (8). Peroxisomal purity was estimated by assaying the marker enzymes catalase (peroxisomes) (15), succinate dehydrogenase (mitochondria) (16), and NADPH cytochrome c reductase (microsomes) (17) in a post-nuclear supernatant and in purified peroxisomes. Recovery of the mitochondrial and microsomal enzymes in the peroxisomal fraction was less than 10% of the recovery of catalase, indicating the peroxisomes were >90% pure.

For isoelectric focusing and chromatographic separations only, peroxisomal synthetase activity was solubilized by stirring purified organelles for 30 min at room temperature in 20 mM potassium phosphate, pH 7.4, containing 0.1% CHAPS and 100 mM KCl. The supernatant obtained by centrifugation at 27,000 *g* for 10 min contained synthetase activity. Protein was determined by the method of Lowry et al. (18).

Cloning of full-length long chain acyl-CoA synthetase cDNA into pET11d

Plasmid manipulations were performed essentially as described by Sambrook, Fritsch, and Maniatis (19). The full-length cDNA encoding the rat LCS (pRACS15) (14) was cloned into pET11d (Novagen, Madison, WI). Oligonucleotide primers (Operon Technologies, Inc.,

Alameda, CA) were designed to synthesize, by the polymerase chain reaction (PCR), a DNA fragment encoding the first 120 codons of rat LCS with an *Nco*I restriction site at the initiator methionine codon and *Nsi*I and *Bam*HI sites downstream of the E120 codon. The oligonucleotides 5′-GGGGAATCCATGGAAGTCCACGAAT TGTTCCGGTATTTTCG and 5′-GGGGGATCCATGCA TCTCATATGGCTGGTTTGGCTTTC were used with pRACS15 in the PCR reaction. The purified PCR product was cleaved with *Nco*I and *Bam*HI and ligated between the *Nco*I and *Bam*HI sites of pET11d, creating pPW931. Next, a 3180 bp *Sfu*I–*Nsi*I fragment containing the C-terminal 689 codons of LCS and part of the 3′ untranslated region was cleaved from pRACS15 and cloned between the *Sfu*I and *Nsi*I sites of pPW931. This procedure yielded plasmids containing the entire LCS coding region. Two clones (pPW932 and pPW933) were independently constructed.

In vitro transcription/translation

The rat LCS was transcribed from pPW932 and pPW933 by T7 polymerase and translated in vitro in a rabbit reticulocyte lysate (Promega, Madison, WI). Control incubations contained the pET11d vector without the LCS cDNA insert. Incubations were carried out for 90 min at 30°C. In one set of experiments, [^{35}S]methionine was present; after incubation, an aliquot of the reaction mixture was subjected to SDS-PAGE (20) and fluorography. In other experiments, unlabeled methionine was present and aliquots of reaction mixture were assayed for fatty acyl-CoA synthetase activities.

Preparative isoelectric focusing

Focusing was carried out in a 110 ml LKB 8100 Ampholine Column. Carrier ampholytes (Pharmalyte, pH 3–10; Sigma Chemical Co., St. Louis, MO) were used at a final concentration of 1%. The column was filled by constructing a linear gradient from 55 ml of a ''dense solution'' (three-fourths of the total carrier ampholytes in 50% sucrose) and 55 ml of a ''light solution'' (one-fourth of the ampholytes plus CHAPS-solubilized peroxisomes in deionized water). The anode and cathode solutions contained 1% phosphoric acid and 2% ethanolamine, respectively. The column was operated at 4°C for 17 h at 500 v. Fractions of 4 ml were collected and the pH was measured. After dialysis against 20 mM potassium phosphate, pH 7.4, fractions were assayed for synthetase activities.

RESULTS

Rat LCS, expressed in vitro, activates phytanic acid

On the basis of antibody, detergent, and protease treatment experiments, Pahan and coworkers (10) reported that phytanic acid was activated to its CoA derivative in human skin fibroblast peroxisomes by an enzyme that was distinct from both the well-characterized LCS (13, 21) and VLCS (22–27). As part of our effort to characterize the individual steps of the rat phytanic acid α-oxidation pathway, we sought to determine whether a unique enzyme was responsible for phytanic acid activation in rat liver peroxisomes. Previously reported attempts to show that rat liver microsomal or peroxisomal phytanoyl-CoA synthetase and LCS are separate enzymes were based primarily on inhibitor studies and physical properties of the enzyme(s) in organelle preparations and yielded inconclusive results (11, 12). To avoid some of the problems encountered by others, we took an alternative approach and first investigated the ability of the isolated rat LCS, obtained by in vitro expression, to activate phytanic acid.

Full-length cDNA encoding rat liver LCS was cloned into pET11d and transcribed and translated in vitro. When translated in the presence of [^{35}S]methionine, a labeled 76 kDa protein was detected (**Fig. 1**). This finding was consistent with the previously reported molecular weight (M_r = 76,000) of purified LCS (13) and the molecular mass (78,177 daltons) predicted by its deduced amino acid sequence (14).

To assay this enzyme for fatty acyl-CoA synthetase activities, the protein was translated in the absence of [^{35}S]methionine but with excess unlabeled methionine. Rabbit reticulocyte lysate contained endogenous acyl-CoA synthetase activity (data not shown) which was not affected by incubation with the pET11d vector alone (**Table 1**). However, in vitro transcription/translation

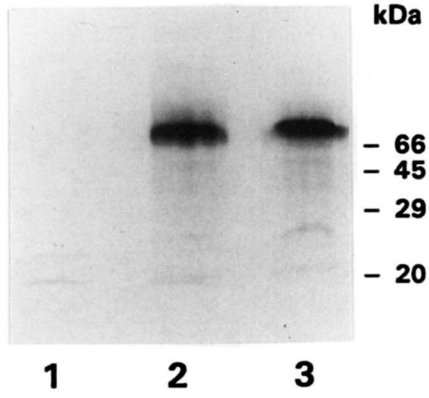

Fig. 1. In vitro transcription/translation of rat long-chain acyl-CoA synthetase. Expression vectors (pPW932 and pPW933) containing full-length cDNA encoding rat LCS were constructed as described in Methods. These constructs, or the vector (pET11d) alone, were incubated with [^{35}S]methionine in a rat reticulocyte lysate expression system for 90 min at 30°C. Labeled proteins were precipitated with ice-cold 10% trichloroacetic acid, separated by SDS-PAGE on a 10% gel, and fluorographed. Lane 1, pET11d; lane 2, pPW932; lane 3, pPW933. Positions of molecular weight markers are indicated on the right.

TABLE 1. In vitro transcription/translation of rat long-chain fatty acyl-CoA synthetase activity

	pET11d	pPW932	pPW933
		nmol/20 min/10 μl	
Expt. 1			
[1-^{14}C]palmitic	0.18	1.87	2.22
[2,3-^{3}H]phytanic	0.07	0.41	0.40
Expt. 2			
[1-^{14}C]palmitic	0.16	0.95	N.D.
[1-^{14}C]lignoceric	0.01	0.01	N.D.
[2,3-^{3}H]phytanic	0.06	0.20	N.D.

Full-length cDNA encoding rat long-chain acyl-CoA synthetase was cloned into the expression vector, pET11d, as described in Methods. In vitro transcription/translation of the vector alone (pET11d) or the synthetase cDNA (pPW932 or pPW933) was carried out in a rabbit reticulocyte lysate system. After incubation for 90 min at 37°C, aliquots were assayed for their ability to activate the indicated fatty acid substrate. N.D., not determined.

of full-length LCS cDNA from two independently constructed plasmids, pPW932 or pPW933, resulted in a 6- to 12-fold increase in activation of a long-chain fatty acid (palmitic acid, C16:0) (Table 1). No detectable increase in very long-chain fatty acid (lignoceric acid, C24:0) activation was observed under these conditions (Table 1). However, expression of the LCS cDNA resulted in a 3- to 6-fold increase in activation of phytanic acid (Table 1). The rate of phytanic acid activation by in vitro expressed LCS was approximately 20% of the rate of C16:0 activation.

Peroxisomal acyl-CoA synthetase activities

We next determined whether the phytanoyl-CoA synthetase activity of LCS expressed in vitro was sufficient to account for that observed in intact peroxisomes. Peroxisomes were isolated from rat liver and assayed for their ability to activate long-chain fatty acids, very long-chain fatty acids, or phytanic acid. As expected, peroxisomes had considerable capacity to activate palmitic acid (4444 ± 563 nmol/h per mg protein; n = 5). VLCS activity, determined using lignoceric acid as substrate, was also present; however, the specific activity of VLCS (23.3 ± 7.5 nmol/h per mg protein; n = 5) was considerably lower than that of either LCS or phytanoyl-CoA synthetase activity. Phytanoyl-CoA synthetase activity was present at 5–10% of the activity measured with palmitate (284 ± 130 nmol/h per mg protein; n = 15). This finding that the ratio of phytanoyl-CoA synthetase to palmitoyl-CoA synthetase activities is higher for the in vitro expressed LCS than that for intact peroxisomes reduces the probability that a separate, unique enzyme is responsible for phytanic acid activation.

Peroxisomal phytanoyl-CoA synthetase and LCS activities co-localized after chromatography or isoelectric focusing

In another set of experiments, we tried to find evidence for a distinct phytanoyl-CoA synthetase activity in rat liver peroxisomes by physically separating the synthetase activities. For chromatography, peroxisomal synthetase activities, found within the membrane-associated protein fraction, were solubilized with 0.1% CHAPS in the presence of 0.1 M KCl. Soluble proteins were chromatographed on several matrices, including hydroxylapatite, DEAE cellulose, cellulose phosphate, and blue-Sepharose. Under all conditions tested, phytanoyl-CoA synthetase activity co-eluted with palmitoyl-CoA synthetase activity (data not shown). As proteins that are otherwise similar often have different isoelectric points, solubilized peroxisomes were subjected to preparative isoelectric focusing on an LKB Ampholine column. As shown in **Fig. 2,** identical elution profiles for synthetase activity were observed with either C16:0 or phytanic acid as substrate. The measured pI of ~5.2 for both synthetase activities was similar to the pI of 5.5 reported for LCS purified from rat liver microsomes (28). The results of the above experiments are consis-

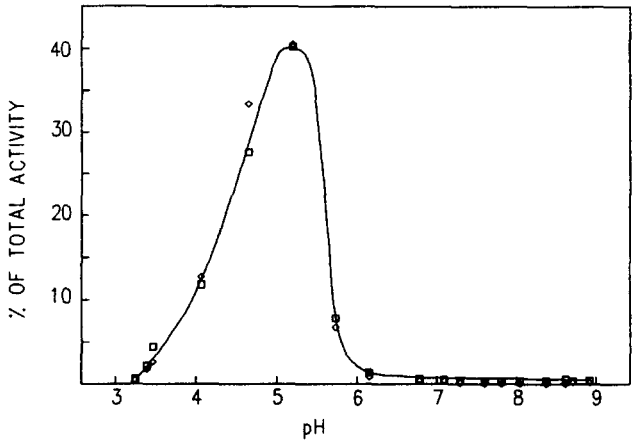

Fig. 2. Distribution of palmotoyl- and phytanoyl-CoA synthetase activities after preparative isoelectric focusing. CHAPS-solubilized rat liver peroxisomes were mixed with carrier ampholytes (pH 3–10) and subjected to preparative isoelectric focusing as described in Methods. Activity of phytanoyl-CoA synthetase (◇) and palmitoyl-CoA synthetase (□) were measured in fractions eluted from the isofocusing column. Results are expressed as percent of total recovered activity.

tent with the interpretation that in rat liver peroxisomes, phytanic acid is activated by LCS.

Competition experiments

We next carried out competition experiments to determine whether phytanoyl-CoA synthetase activity was affected by long- or very long-chain fatty acids. Phytanoyl-CoA synthetase activity of intact peroxisomes was determined in the absence or presence of either palmitic acid or lignoceric acid. While increasing concentrations of unlabeled palmitic acid significantly decreased peroxisomal phytanoyl-CoA synthetase activity, unlabeled lignoceric acid had little effect (**Fig. 3**). These data suggested that activation of phytanic acid by VLCS was unlikely. Although inhibition of phytanoyl-CoA synthetase by the long-chain fatty acid suggested that both fatty acids were activated by LCS, the kinetics of inhibition were complex (data not shown), similar to the observation of Wanders and coworkers (29) for rat liver peroxisomal pristanoyl-CoA synthetase. Thus, it was not possible to determine with certainty the type of inhibition.

Heat inactivation of peroxisomal synthetase activities

Because of the observation of Muralidharan and Muralidharan (11) that rat liver microsomal phytanic acid activation was more heat labile than was palmitate activation, we performed similar experiments using purified rat liver peroxisomes. When peroxisomes were incubated at 42°C in the presence of 1 mM AMP, a reaction product, activity with either C16:0 or phytanic acid as substrate was lost in parallel (**Fig. 4**). In the absence of AMP, there was a more rapid but equal loss of both activities at 42°C. The similarities in the inactivation curves of phytanoyl-CoA and palmitoyl-CoA synthetases did not suggest the presence of more than one enzyme.

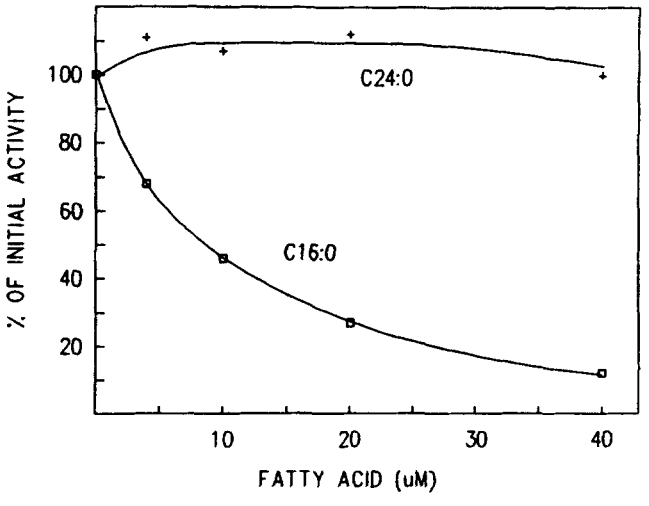

Fig. 3. Effect of long-chain and very long-chain fatty acids on phytanoyl-CoA synthetase activity in rat liver peroxisomes. Rat liver peroxisomes were assayed for phytanoyl-CoA synthetase activity, as described in Methods, in the presence of increasing concentrations (4–40 μM) of unlabeled palmitic acid (□) or lignoceric acid (+). Initial phytanoyl-CoA synthetase activity was 320 nmol/h per mg protein. Results presented are the average of two experiments.

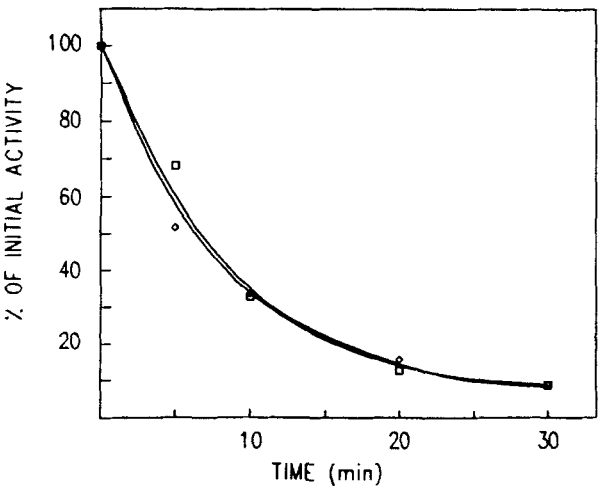

Fig. 4. Inactivation of peroxisomal phytanoyl- and palmitoyl-CoA synthetases by heat treatment. Peroxisomes (~200 μg protein) were incubated in a water bath maintained at 42.0 ± 0.5°C. Aliquots were removed at the indicated times and immediately assayed for acyl-CoA synthetase activities. (◇), Phytanoyl-CoA synthetase; (□), palmitoyl-CoA synthetase.

Inhibition of acyl-CoA synthetases by ETYA

5,8,11,14-Eicosatetraynoic acid (ETYA), an inhibitor of arachidonic acid metabolism (30), was also reported to be an inhibitor of LCS (31). Acyl-CoA synthetase activity of intact peroxisomes was measured in the presence of increasing concentrations of ETYA, using either phytanic acid, C16:0 or C24:0 as substrate (**Fig. 5**). Inhibition of C16:0 activation paralleled the decrease in phytanic acid activation, whereas the effect on C24:0 activation was negligible (Fig. 5). These results support the conclusion that phytanic acid activation by rat liver peroxisomes is most likely catalyzed by LCS.

DISCUSSION

We report here that the in vitro translation product of rat liver LCS cDNA catalyzes the activation of phytanic acid as well as the long-chain fatty acid, palmitic acid. LCS activity has a trimodal distribution in rat liver; it is present in peroxisomes, microsomes, and mitochondria (21). All evidence to date indicates that the

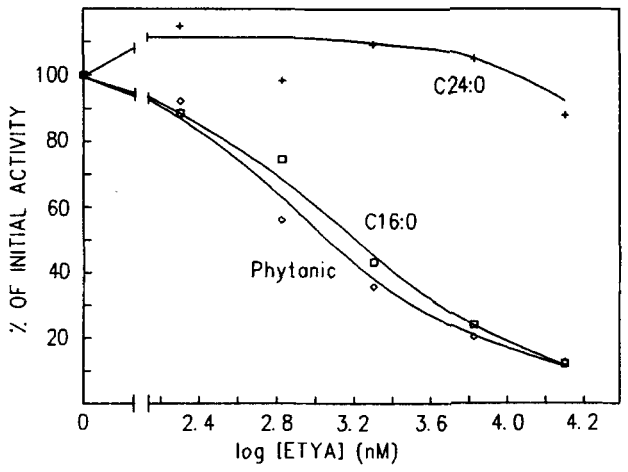

Fig. 5. Inhibition of acyl-CoA synthetases by ETYA. Rat liver peroxisomes were assayed for acyl-CoA synthetase activity in the presence of increasing concentrations of ETYA (0.2–20 μM) as described in Methods. Fatty acid substrates were [1-^{14}C]palmitic acid (C16:0) (□); [2,3-^{3}H]phytanic acid (◇); [1-^{14}C]lignoceric acid (C24:0) (+). Initial synthetase activities were 4549, 317, and 33 nmol/hr per mg protein for C16:0, phytanic acid, and C24:0 substrates, respectively.

LCS enzyme in all three organelles is identical (21). The ratio of phytanate to palmitate activation catalyzed by the in vitro expressed LCS was higher than that observed in intact peroxisomes, suggesting that the existence of a separate enzyme for branched-chain fatty acid activation is both unlikely and unnecessary. Furthermore, additional experiments designed to demonstrate the existence of separate LCS and phytanoyl-CoA synthetases of rat liver peroxisomes were unsuccessful.

The role of coenzyme A in phytanic acid α-oxidation has been controversial, with some workers suggesting that it is a required cofactor and others claiming that it is unnecessary (11, 32–36). Pahan and Singh (37) postulated that in human fibroblasts formation of the coenzyme A derivative of phytanic acid was necessary only to facilitate its entry into peroxisomes. We demonstrated that ^{3}H-release from [2,3-^{3}H]phytanic acid, a process defective in fibroblasts from patients with Refsum disease, PBD, and RCDP, required that the fatty acid first be converted to the CoA derivative (8). Subsequently, we reported that in rat liver peroxisomes, the substrate for α-hydroxylation is also phytanoyl-CoA, yielding the product α-hydroxyphytanoyl-CoA and not α-hydroxyphytanic acid (9). These results indicate that while formation of phytanoyl-CoA might be necessary for transport into peroxisomes, it is also required for subsequent steps in α-oxidation.

The identity of the acyl-CoA synthetase that activates phytanic acid and other branched-chain fatty acids has been the subject of much debate. Muralidharan and Muralidharan (11), who first studied the problem, suggested that activation of phytanic acid and palmitic acid by rat liver microsomes might involve separate enzymes. They reported that microsomal phytanoyl-CoA synthetase activity was more sensitive to heating at 45°C than was palmitate activation; however, the two assays were not carried out under identical conditions (11). The peroxisome heat inactivation experiments reported here were done at 42°C because activities of both synthetases were rapidly lost at the higher temperature (P. A. Watkins and S. J. Mihalik, unpublished results). Similar to our findings with peroxisomes, Muralidharan and Muralidharan (11) found that microsomal phytanoyl-CoA synthetase activity was inhibited by palmitic acid but not by lignoceric acid.

Wanders and coworkers (29) subsequently reported that activation of pristanic acid, the α-methyl branched-chain product of phytanic acid α-oxidation, is catalyzed by LCS in rat liver peroxisomes. They found that pristanoyl-CoA synthetase had the same subcellular distribution as LCS. Despite data demonstrating that the effects of detergent and pH on activation of pristanate and palmitate were not identical, they concluded that pristanic acid was activated by LCS on the basis of competition experiments and immunoprecipitation studies (29). These later investigators (29) and Muralidharan and Muralidharan (11) both performed competition studies and, in agreement with our findings, reported that the kinetics of inhibition of branched-chain acyl-CoA synthetase activity by other fatty acids were complex and thus difficult to interpret.

Pahan and coworkers (10) then reported that an enzyme distinct from LCS and VLCS was responsible for phytanic acid activation in human fibroblast peroxisomes. While this conclusion may be true for humans, a single enzyme may share both palmitoyl-CoA synthetase and phytanoyl-CoA synthetase activities in rats. Other enzymes of peroxisomal lipid metabolism have been found to diverge between rats and humans. For example, Vanhove et al. (38) showed that while rats contain separate acyl-CoA oxidases for bile acids and 2-methyl-branched-chain fatty acids, humans contain one enzyme catalyzing both activities.

Most recently, Vanhooren et al. (12) investigated the activation of a phytanic acid analog (3-methylmargaric acid), a pristanic acid analog (2-methylpalmitic acid), palmitic acid, and lignoceric acid in rat liver peroxisomes. These authors reported differential effects of pyrophosphate and detergents on the activation of these different substrates and concluded that different enzymes were involved. The reason for the discrepancy between their interpretation and our results with the in vitro expressed LCS is unknown at this time. Their conclusion that 2-methylpalmitate and palmitate are activated by separate enzymes also differs from that reached

by Wanders et al. (29) for pristanic acid activation by LCS. Furthermore, Vanhooren et al. (12) were also unable to separate rat liver peroxisomal LCS, 2-methylpalmitoyl-CoA synthetase, and 3-methylmargaryl-CoA synthetase activities by chromatography on hydroxylapatite, phenyl-Sepharose, or blue-Sepharose, whereas they easily separated these activities from lignoceroyl-CoA synthetase (VLCS) activity.

Suzuki and coworkers (14) cloned and sequenced the rat liver LCS cDNA. Results presented here show that in vitro transcription/translation of this cDNA yields a synthetase capable of activating palmitic acid and phytanic acid, but not lignoceric acid. These findings indicate that rat LCS is incapable of activating very long-chain fatty acids and they strengthen the argument for the existence of a separate VLCS (22–27). However, these data also provide compelling evidence that phytanic acid is activated by LCS and that this enzyme is sufficient to activate phytanic acid in vivo.

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