Phytanic acid oxidation: topographical localization of phytanoyl-CoA ligase and transport of phytanic acid into human peroxisomes

Kalipada Pahan and Inderjit Singh[1]

Department of Pediatrics, Medical University of South Carolina, Charleston, SC 29425

Abstract To understand the possible role of phytanoyl-CoA ligase, present in the membrane, in the oxidation of phytanic acid in the matrix of peroxisomes (Pahan, K. and I. Singh. 1993. *FEBS Lett.* **333:** 154–158) we examined the transport of phytanic acid/phytanoyl-CoA into peroxisomes and the topology of the active site of phytanoyl-CoA ligase in the peroxisomal membrane. The increase in lignoceroyl-CoA ligase as compared to no change in the activities of palmitoyl-CoA and phytanoyl-CoA ligases when peroxisomes were disrupted with detergent or sonication and inhibition of the activities of both palmitoyl-CoA and phytanoyl-CoA ligase by impermeable inhibitor of acyl-CoA ligases (mercury-dextran) and trypsin treatment in the intact peroxisomes. On the other hand, the lignoceroyl-CoA ligase activity was inhibited by mercury-dextran and trypsin only in the disrupted peroxisomes. Taken together, these studies support the conclusion that the enzymatic site of phytanoyl-CoA ligase is on the cytoplasmic surface of peroxisomal membrane. This implies that phytanoyl-CoA is synthesized on the cytoplasmic surface of peroxisomal membrane and is translocated through the membrane for its α-oxidation to pristanic acid in the matrix of peroxisomes. To delineate the transport for phytanic acid through the peroxisomal membrane, we examined cofactors and energy requirements for its transport into peroxisomes. The similar rates of transport of phytanoyl-CoA and phytanic acid under conditions favorable for fatty acid activation (presence of ATP, CoASH, and $MgCl_2$) and the lack of transport of phytanic acid when ATP and/or CoASH were removed or replaced with their inactive analogues (ATP and/or CoASH) from assay medium clearly demonstrates that the transport of phytanic acid requires prior synthesis of phytanoyl-CoA by phytanoyl-CoA ligase. The prerequisite activation of phytanic acid to phytanoyl-CoA for its α-oxidation only in intact peroxisomes, and oxidation of free phytanic acid in digitonin-permealized peroxisomes or isolated matrix, suggests that phytanoyl-CoA ligase (in peroxisomal membrane) regulates the oxidation of phytanic acid in peroxisomes by providing phytanoyl-CoA for its transport into peroxisomes.—**Pahan, K., and I. Singh.** Phytanic acid oxidation: topographical localization of phytanoyl-CoA ligase and transport of phytanic acid into human peroxisomes. *J. Lipid Res.* 1995. **36:** 986–997.

Supplementary key words acyl-CoA ligase • topology • α-oxidation • pristanic acid

Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid), a highly branched isoprenoid-derived fatty acid, accumulates in excessive amounts in the tissues and body fluids of patients with classical Refsum disease (1) and in patients with defects in the biogenesis of peroxisomes (2, 3). Phytanic acid is not synthesized de novo in humans but is taken exogenously. The ordinary human diet contains 50–100 mg of phytanic acid per day mainly as a constituent of animal, dairy, and plant products. Due to the β-methyl group, phytanic acid cannot be β-oxidized; therefore, α-oxidation is the only feasible route for its catabolism (1–4). Studies from our laboratory have demonstrated that in humans phytanic acid is α-oxidized to pristanic acid in peroxisomes, but in rodents this process occurs in mitochondria (5, 6). In human skin fibroblasts the rate of phytanic acid oxidation in peroxisomes was 26 and 130 times greater than that found in mitochondria and microsomes. Moreover, the excessive accumulation of phytanic acid and defective α-oxidation of phytanic acid in peroxisomes, but not mitochondria or microsomes, from cultured skin fibroblasts of patients with Refsum disease (7) and rhizomelic chondrodysplasia punctata (RCDP) (6) also support the conclusion that in humans the major site of α-oxidation of phytanic acid to pristanic acid is the peroxisome.

The oxidation of phytanic acid to pristanic acid involves at least four steps: activation of phytanic acid to phytanoyl-CoA, α-hydroxylation to α-hydroxyphytanic acid, conversion to 2-ketophytanic acid, and finally decarboxylation to a 19-carbon homologue, pristanic acid. The enzyme for activation of phytanic acid (phytanoyl-CoA li-

[1]To whom correspondence should be addressed at: Medical University of South Carolina, Department of Pediatrics, 171 Ashley Avenue, Charleston, SC 29425.

gase) in peroxisomes is localized in the peroxisomal membrane, and it is an enzyme distinct from the other two acyl-CoA ligases (palmitoyl-CoA ligase and lignoceroyl-CoA ligase) which are known to be present in peroxisomal membranes (8). The enzyme system involved in the next three steps (i.e., α-hydroxylation, dehydrogenation, and α-decarboxylation) was found to be present in the peroxisomal matrix (9). Surprisingly, the activation of phytanic acid to phytanoyl-CoA by phytanoyl-CoA ligase, present in peroxisomal membrane, was required for the oxidation of phytanic acid only in intact peroxisomes (9) but not in isolated peroxisomal matrix or permealized peroxisomes (9). Unlike the β-oxidation system for saturated and unsaturated unbranched fatty acids, the substrate for α-oxidation of phytanic acid, a branched chain fatty acid, in isolated matrix and permealized peroxisomes was free phytanic acid (9). This implies that phytanic acid is converted to phytanoyl-CoA in the peroxisomal membrane and after its translocation into peroxisomes the free phytanic acid is the substrate for α-oxidase enzyme system in the matrix of peroxisomes.

In an effort to understand the function of phytanoyl-CoA ligase in peroxisomes, the present study was undertaken to determine the transverse topology of the active site of this enzyme in the (plane) peroxisomal membranes and transport of phytanic acid/phytanoyl-CoA into peroxisomal matrix. The results described in this report demonstrate that the active site of phytanoyl-CoA ligase is on the cytoplasmic surface of the peroxisomal membrane, activation of phytanic acid to phytanoyl-CoA is a prerequisite for its transport into peroxisomes, and the translocation of phytanoyl-CoA through the peroxisomal membrane does not require energy. A clear understanding of the activation, transport, and α-oxidation of phytanic acid will be important for elucidation of the metabolic pathophysiology in peroxisomal diseases (2, 3) in which the precise molecular cause of inborn error in phytanic acid oxidation has not yet been identified.

EXPERIMENTAL PROCEDURES

Materials

Nycodenz was obtained from Accurate Chemical and Scientific Corp., Westbury, NY. ATP and CoASH were purchased from P-L Biochemicals, Milwaukee, WI. Acetylated trypsin, soybean trypsin inhibitor, digitonin, α-cyclodextrin, NADPH, FAD, cytochrome c, adenosine 5' (β, δ-methylene) triphosphate (AMP-PCP), desulfocoenzyme A-agarose (desulfoCoA-agarose), and α, β-methyleneadenosine 5' triphosphate (AMPCPOP) were purchased from Sigma Chemical Co., St. Louis, MO. Triton X-100 and octyl-β-D-glucopyranoside were purchased from Bio-Rad, Richmond, VA. Protein A Sepharose was purchased from Pharmacia LKB, Sweden. [1-^{14}C]Phytanic acid (55 mCi/mmol) was purchased from Amersham International, Arlington Heights, IL. [1-^{14}C]Palmitic acid (58.7 mCi/mmol), and K^{14}CN (52.0 mCi/mmol) were purchased from DuPont-New England Nuclear. [1-^{14}C]Lignoceric acid was synthesized by treatment of n-tricosanoyl bromide with K^{14}CN as described previously (10). [1-^{14}C]Phytanoyl-CoA was synthesized as described (11). Mercury-dextran was prepared from dextran ($M_{r,av}$ 10500) and mercuric acetate by the method of Pitha (12).

Isolation of peroxisomes from human liver

Peroxisomes from human liver were isolated according to the procedures described previously (6). Briefly, the liver homogenate was first fractionated by differential centrifugation to prepare the light mitochondrial fraction, the "lambda" fraction (13), and peroxisomes from the lambda fraction were prepared by isopycnic equilibrium centrifugation in a gradient consisting of 28 ml of 0–50% (w/v) Nycodenz with 4 ml of 55% (w/v) Nycodenz as a cushion in 39-ml tubes as described previously (6). The gradient fractions were analyzed for marker enzymes for different subcellular organelles: cytochrome c oxidase for mitochondria (14), NADPH-cytochrome c reductase for microsomes (15), and catalase for peroxisomes (16). Protein concentration was determined by the procedures of Bradford (17). The fractions containing the catalase peak were pooled and dialyzed against the homogenization buffer (6, 18) for 1 h with two changes to lower the concentration of Nycodenz. This peroxisomal preparation was used for further studies.

Peroxisomal integrity was measured by the latency of catalase activity, a matrix enzyme. Total catalase activity was measured by diluting peroxisomes 1:1 with 2% of Triton X-100, and free catalase activity was measured by suspending peroxisomes in isotonic solution (homogenization buffer) in the absence of Triton X-100. The percent of total catalase activity minus percent of free catalase is an index of peroxisomal integrity.

Activation and oxidation of 1-^{14}C-labeled fatty acids

The activities for palmitoyl-CoA, lignoceroyl-CoA, and phytanoyl-CoA ligases were measured as described previously (8, 19) except that fatty acids were first solubilized with α-cyclodextrin. Briefly, the reaction mixture in 0.5 ml contained 12 μM 1-^{14}C-labeled fatty acid, 50 mM KCl, 5 mM $MgCl_2$, 50 μM CoASH, 30 mM MOPS-HCl buffer, pH 7.8 (8, 19). The α-oxidation of [1-^{14}C]phytanic acid to pristanic acid was measured as liberated $^{14}CO_2$ as described previously (5, 6). Briefly, the reaction volume of 0.25 ml contained 12 μM [1-^{14}C]phytanic acid, 30 mM KCl, 5 mM $MgCl_2$, 50 μM CoASH, 10 mM ATP, 0.25 mM NADPH, 0.17 mM FAD, and 20 mM MOPS-HCl buffer, pH 7.8, in 0.25 M sucrose (5, 6). In both reactions, fatty acids were solubilized with α-cyclodextrin as follows. Fatty acids (20 × 10^6 dpm) were first dried in a

tube under nitrogen and then resuspended in 3.5 ml of 0.25 M sucrose buffer (20 mg α-cyclodextrin/ml) by sonication for 30 min.

Treatment of peroxisomes with impermeable probes and trypsin

Purified intact peroxisomes suspended in 0.25 M sucrose were incubated with different concentrations of Hg-dextran for 30 min at 4°C. To remove excess Hg-dextran, peroxisomes, after treatment, were diluted with 0.25 M sucrose and sedimented by centrifuging at 9130 *g* for 10 min at low acceleration and deceleration in a Beckman TL-100 tabletop ultracentrifuge. For trypsin treatment, peroxisomes suspended in 0.25 M sucrose were treated with trypsin (peroxisomes to trypsin ratio 20:1 by protein) for 20 min at 37°C in the presence or absence of detergent mixture (0.03% Triton X-100 and 0.02% octyl β, D-glucopyranoside). Trypsin was inhibited by adding soybean trypsin inhibitor (trypsin to soybean trypsin inhibitor ratio 1:2 by protein).

Transport of phytanic acid into peroxisomes

Peroxisomal fractions were incubated under isotonic conditions (homogenization buffer) with [1-^{14}C]phytanic acid or [1-^{14}C]phytanoyl-CoA solubilized with α-cyclodextrin in the presence or absence of CoASH and ATP or their analogs as described earlier (18). Briefly, peroxisomes, after incubation, were separated from the incubation medium by centrifugation through an organic layer of brominated hydrocarbons (20). This was done using microtubes (1.5 ml) containing 50 μl of 0.396 M sucrose, 1 mM EDTA in 3 mM imidazole buffer, pH 7.4 (as cushion), an organic layer (400 μl) consisting of a mixture of bromododecane and bromodecane (7:4, v/v), and an upper layer (500 μl) consisting of 0.25 M sucrose, 30 mM MOPS buffer, pH 7.8. The substrate concentration used was 12 μM for [1-^{14}C]phytanic acid, [1-^{14}C]phytanoyl-CoA, and [1-^{14}C]palmitic acid. Incubations were started with the addition of 10–80 μg of peroxisomal protein as indicated in the captions for each experiment. After 5 min incubation at 25°C, the tubes were centrifuged in a Beckman TL-100 ultracentrifuge (TLA-100.2 rotor) at 9130 *g* for 7 min at low acceleration and deceleration. The upper aqueous and organic layers were removed. The radioactivity in the bottom layer containing the sedimented peroxisomes was measured. Catalase activity and latency of catalase were measured in the upper and lower aqueous layers after centrifugation. Appropriate blanks with disrupted peroxisomes were also included in these studies. The rates of transport for phytanic acid, phytanoyl-CoA, and palmitic acid using disrupted (by sonication) peroxisomes (blanks) were 3.8, 3.65, and 2.82%, respectively, of the rates of these fatty acids transported into intact peroxisomes.

Effect of antibodies

Intact and disrupted (in the presence of detergent mixture) peroxisomes were treated with antisera raised against purified palmitoyl-CoA ligase (21). The ratio of peroxisomes to antibody protein was 1:4 (by protein) and controls contained the same ratio with preimmune sera. Peroxisomes were incubated with antibodies for 2 h at 4°C. Eleven percent protein-A-Sepharose was added to a final concentration of 1% with only disrupted peroxisomes. The resulting mixture was kept for another 30 min at 4°C and then centrifuged at 2,000 rpm for 15 min to remove sediment. Supernatant was used for acyl-CoA ligase assays. Intact peroxisomes were reisolated by centrifuging the incubation mixture over 0.25 M sucrose at 9130 *g* for 10 min at 4°C at low acceleration and deceleration in a Beckman TL-100 tabletop ultracentrifuge. The pellet was gently suspended in 0.25 M sucrose and used for activation, transport, and oxidation transport assays.

RESULTS

Transverse topographical localization of the active site of phytanoyl-CoA ligase in the plane of peroxisomal membranes

The peroxisomes prepared by the procedure described above were approximately 98% pure with minor contamination of mitochondria (0.08 ± 0.01%) and microsomes (1.7 ± 0.2%) as protein. Percent purity of peroxisomal fractions was calculated according to the method given by Fujiki et al. (22). As judged by the latency of catalase activity, the integrity of peroxisomes was approximately 83 ± 4%. The topographical localization of the activity of phytanoyl-CoA ligase in the plane of peroxisomal membrane was examined by treatment of peroxisomes with detergents, proteolytic enzymes, impermeable inhibitors, and antibodies against palmitoyl-CoA ligase. The results for phytanoyl-CoA ligase were compared with results obtained for palmitoyl-CoA ligase, an enzyme with active site on the cytoplasmic surface, and that of lignoceroyl-CoA ligase with active site on the luminal surface of peroxisomes.

Effect of disruption of peroxisomes on the activities of acyl-CoA ligases

The free catalase activity increased with disruption of peroxisomes by sonication (**Fig. 1A**) and exposure to digitonin (Fig. 1B). The disruption of peroxisomes either with sonication or permealization with digitonin had no effect on the activity of palmitoyl-CoA ligase; whereas activity of lignoceroyl-CoA increased by 74% and 59% in sonicated and digitonin treated peroxisomes, respectively.

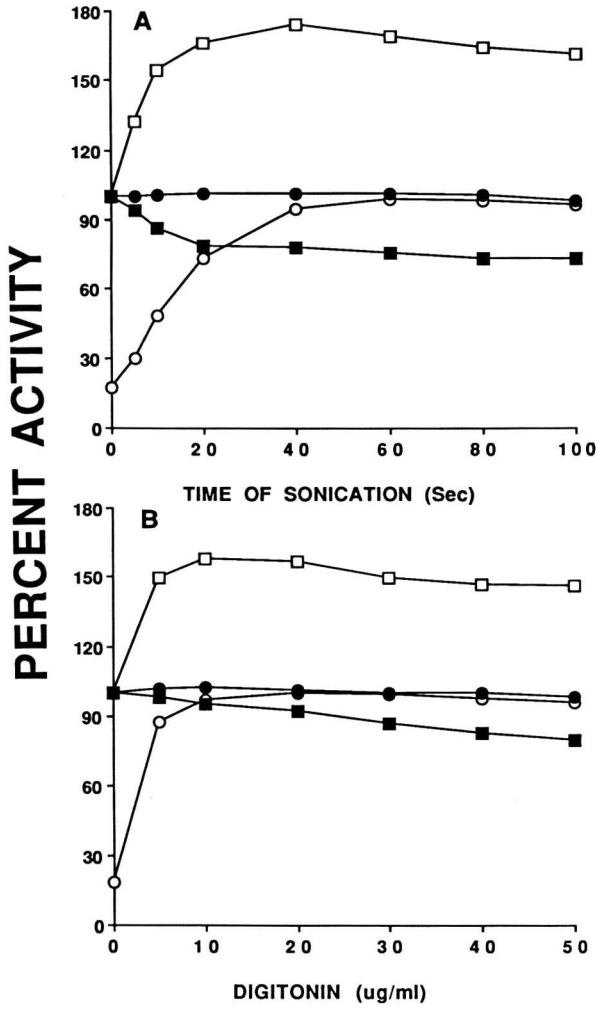

Fig. 1. Effect of sonication (A) and digitonin (B) on peroxisomal integrity and fatty acyl-CoA ligase activities. Purified peroxisomes suspended in 0.25 M sucrose were sonicated for different periods of time and activities for catalase and fatty acyl-CoA ligases were measured. In different sets of experiments, peroxisomes suspended in 0.25 M sucrose were incubated with different amounts of digitonin for 1 h at 4°C. The activities of palmitoyl-CoA ligase (●), lignoceroyl-CoA ligase (□), and phytanoyl-CoA ligase (■) in intact peroxisomes (without sonication and digitonin treatment) were considered as 100%, while free catalase activity (○) was considered to be 100% in disrupted peroxisomes. Specific activities of palmitoyl-CoA ligase, lignoceroyl-CoA ligase, and phytanoyl-CoA ligase were 14.2 ± 2.3, 0.56 ± 0.13, and 6.7 ± 1.6 nmol/min/mg protein, respectively. These results are the average of two independent determinations done in triplicate.

The disruption of peroxisomal integrity either with digitonin or sonication did not result in appearance of any "latent" pool of phytanoyl-CoA ligase. This suggests that the active site of phytanoyl-CoA ligase, like that of palmitoyl-CoA ligase, is located on the cytoplasmic surface of peroxisomes.

Effect of mercury-dextran (Hg-dextran) on acyl-CoA ligase activities in intact peroxisomes

The effect of mercury as a sulfhydryl inhibitor was examined on the activities of acyl-CoA ligases in intact peroxisomes. Although most of the inorganic and small organic derivatives of mercury are cell-permeable, Hg-dextran cannot penetrate through organellar membranes (12, 19, 23). Treatment of intact peroxisomes with Hg-dextran decreased the activity of both palmitoyl-CoA and phytanoyl-CoA ligases by about 80% and 90% at the concentrations of 50 μM and 100 μM, respectively (**Fig. 2**). In contrast, Hg-dextran had no significant effect on lignoceroyl-CoA ligase in intact peroxisomes; whereas in disrupted peroxisomes lignoceroyl-CoA ligase activity was inhibited by 83.5%. The loss of 15–20% of lignoceroyl-CoA ligase activity in intact peroxisomes reflects the percent of the disrupted peroxisomes under these experimen-

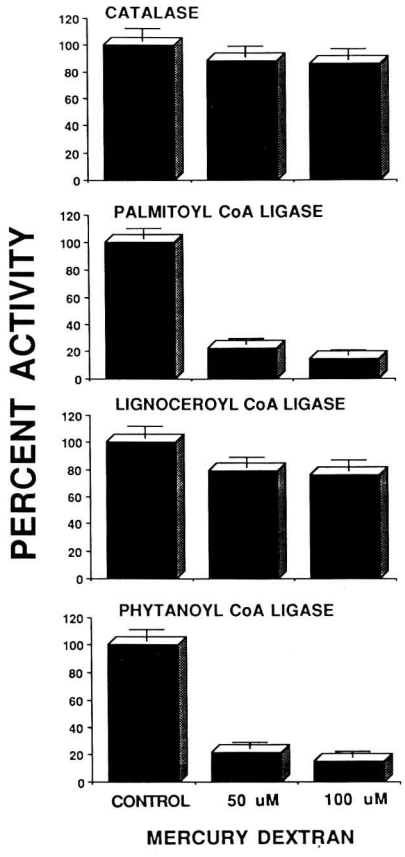

Fig. 2. Effect of mercury-dextran on peroxisomal fatty acyl-CoA ligase activities. Purified intact peroxisomes suspended in 0.25 M sucrose were incubated with different concentrations of Hg-dextran for 30 min at 4°C. To remove excess of Hg-dextran, the peroxisomes after treatment were gently diluted with 0.25 M sucrose and reisolated by centrifuging at 9130 *g* for 10 min at low acceleration and deceleration in a Beckman TL-100 tabletop ultracentrifuge. Catalase and fatty acyl-CoA ligase activities were measured by usual procedures in the pellet after dilution with 0.25 M sucrose. Integrity of this peroxisomal suspension was 83–85% as measured by the latency of catalase activities. One hundred percent activity for total catalase was 3.9 ± 0.7 U/mg protein and 100% activities for palmitoyl-CoA ligase, lignoceroyl-CoA ligase, and phytanoyl-CoA ligase were 14.2 ± 2.3, 0.56 ± 0.13, and 6.7 ± 1.6 nmol/min/mg protein, respectively. The results are the mean ± SD of six values.

tal conditions. The sensitivity of palmitoyl-CoA ligase and phytanoyl-CoA ligase to Hg-dextran in intact peroxisomes implies that like palmitoyl-CoA ligase, the active site of phytanoyl-CoA ligase is located on the cytoplasmic surface of peroxisomes. The absence of similar Hg-dextran sensitivity for lignoceroyl-CoA ligase in intact peroxisomes is in agreement with our previous conclusion (24) that the active site of lignoceroyl-CoA ligase is located on the luminal surface of peroxisomal membranes.

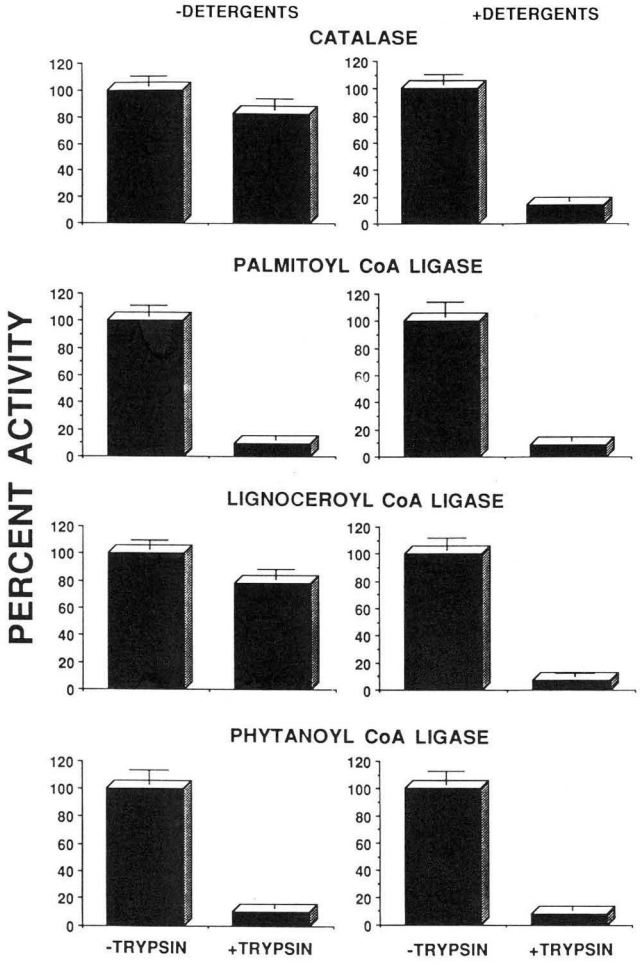

Fig. 3. Effect of trypsin treatment on peroxisomal integrity and fatty acyl-CoA ligase activities. Purified peroxisomes suspended in 0.25 M sucrose were treated with trypsin in the absence and in the presence of a detergent mixture containing 0.03% Triton X-100 and 0.02% octy β, D-glucopyranoside for 20 min at 30°C. Peroxisome to trypsin ratio was 20:1 by protein. Trypsin was inhibited by adding soybean trypsin inhibitor (trypsin and trypsin inhibitor ratio was 1:2 by protein). After proper incubations, catalase and fatty acyl-CoA ligase activities in peroxisomal samples were measured by standard procedures. The integrity of peroxisomes in these samples incubated in the absence of detergent mixture was 79–80% as judged by the latency of catalase. Total catalase activity in peroxisomes incubated with and without digitonin was 3.9 ± 0.7 U/mg protein. One hundred percent activities for palmitoyl-CoA ligase, lignoceroyl-CoA ligase, and phytanoyl-CoA ligase in peroxisomes incubated without detergent mixture were 14.2 ± 2.3, 0.56 ± 0.13, and 6.7 ± 1.6 nmol/min/mg protein, respectively, and in peroxisomes incubated with detergent mixture were 13.7 ± 1.8, 0.45 ± 0.11, and 4.02 ± 0.8 nmol/min/mg protein, respectively. Data are expressed as mean ± SD of six values.

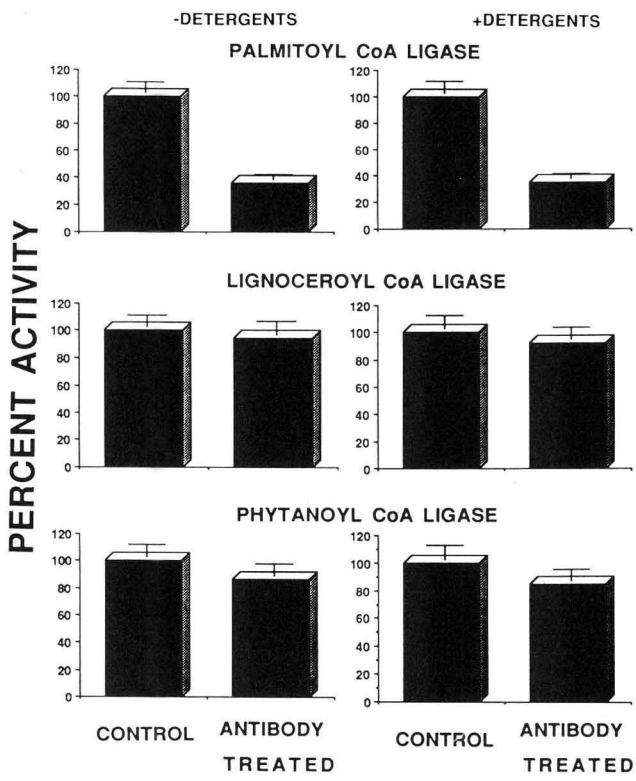

Fig. 4. Effect of antibodies against palmitoyl-CoA ligase on acyl-CoA ligase activities in peroxisomes in the absence and in the presence of detergent mixture containing 0.03% Triton X-100 and 0.02% octyl β,D-glucopyranoside. The ratio of peroxisomes to antibodies was 1:4 (by protein) and controls contained the same amount of preimmune sera. This mixture was incubated for 2 h at 4°C. With disrupted peroxisomes, 11% protein A-Sepharose was added to a final concentration of 1%. The resulting mixture was kept for 30 min at 4°C and then centrifuged at 2,000 rpm for 15 min to remove sediment. Supernatant was used for acyl-CoA ligase assays. After 2 h incubation at 4°C, intact peroxisomes were separated from unbound antibodies by centrifuging at 9130 *g* for 10 min at 4°C at low concentration and deceleration in a Beckman TL-100 tabletop centrifuge. The pellet was gently suspended in sucrose buffer. The integrity of peroxisomes in this suspension was 80–81% as measured by latency of catalase activities. This peroxisomal suspension was used for acyl-CoA ligase assays. One hundred percent activities for palmitoyl-CoA, phytanoyl-CoA, and lignoceroyl-CoA ligases in peroxisomes without detergent were 13.9 ± 2.1, 6.15 ± 0.9, and 0.51 ± 0.2 nmol/min/mg protein, respectively. When detergent was added the total activities were 12.8 ± 1.7, 3.23 ± 0.45, and 0.38 ± 0.12 nmol/min/mg protein, respectively. Values are mean ± SD of three separate experiments.

Effect of trypsin on acyl-CoA ligase activities in intact and disrupted peroxisomes

The activities of acyl-CoA ligases were determined after trypsin treatment of intact peroxisomes and peroxisomes were disrupted with detergent mixture containing 0.03% Triton X-100 and 0.02% octyl β, D-glucopyranoside. Treatment with trypsin of both intact (in the absence of detergent mixture) and disrupted (in the presence of detergent mixture) peroxisomes decreased both palmitoyl-CoA ligase and phytanoyl-CoA ligase activities by about 90% (**Fig. 3**). In contrast, lignoceroyl-CoA ligase activity

in intact peroxisomes was found to be inhibited by only 21%. This corresponds to the percentage of disrupted peroxisomes in these preparations as judged by latency of catalase. In contrast, trypsin decreased the lignoceroyl-CoA ligase activity by 88% in peroxisomal preparation disrupted by detergent mixture.

Effect of antibodies against palmitoyl-CoA ligase on the activity of acyl-CoA ligase in intact and disrupted peroxisomes

In these studies we examined the effect of antibodies against palmitoyl-CoA ligase on the acyl CoA-ligase activities for palmitic, lignoceric, and phytanic acids. Antibodies against palmitoyl-CoA ligase inhibited the activity of palmitoyl-CoA ligase but had no effect on the activities of phytanoyl-CoA ligase and lignoceroyl-CoA ligase both in intact and disrupted peroxisomes (**Fig. 4**).

Transport of phytanic acid and phytanoyl-CoA into peroxisomes isolated from human liver

Table 1 shows the integrity of peroxisomes under conditions used for the study of transport of phytanic acid or phytanoyl-CoA. Neither exposure of peroxisomes to phytanic acid or phytanoyl-CoA and cofactors for 5 min at 25°C nor the centrifugal force (9130 *g*) used in the fatty acid transport experiments had any effect on the integrity of peroxisomes. Transport of phytanic acid and phytanoyl-CoA was linear during the first 5 min (**Fig. 5A**). The transport of phytanic acid or its CoA derivative increased with increasing temperature from 4°C to 37°C (Fig. 5B); however, peroxisomes become more fragile at temperatures greater than 25°C (18, 24), therefore, all subsequent studies were performed at 25°C. The rates of transport of phytanic acid or phytanoyl-CoA at different concentrations of this fatty acid or its CoA-derivative and with different quantities of peroxisomal protein are shown in Figs. 5C and 5D, respectively. The transport of phytanic acid or its CoA-derivative was linear between 15 and 75 μg of peroxisomal protein. Therefore, for the additional studies assessing transport of fatty acids, 12 μM phytanic acid or phytanoyl-CoA was used with 15–60 μg of peroxisomal protein for 5 min.

The transport of [1-^{14}C]phytanic acid or [1-^{14}C]phytanoyl-CoA into purified peroxisomes was measured in the presence or absence of cofactors and metal ions (CoASH, ATP, and Mg^{2+}) required for the activation of fatty acids. The rate of transport of phytanoyl-CoA was much greater than the free phytanic acid in the absence of CoASH, ATP, and Mg^{2+} (Fig. 5). The specific activities for the rates of transport of phytanic acid or its CoA-derivative in the presence or absence of cofactors and metal ions are summarized in **Table 2.** The rates of transport of phytanoyl-CoA (23.39 ± 2.26 nmol/h/mg protein) and phytanic acid (22.05 ± 2.5 nmol/h/mg protein) in the presence all activating cofactors (CoASH, ATP, and Mg^{2+}) were nearly similar. In the absence of cofactors, the rate of transport for free phytanic acid was only 12–14% of that seen with phytanoyl-CoA. Results presented in Fig. 5 and Table 2 clearly show that the transport of phytanic acid was completely dependent on the presence of CoASH, ATP, and Mg^{2+}, whereas the transport of phytanoyl-CoA was independent of these cofactors. These results suggest that phytanic acid is transported into peroxisomes as phytanoyl-CoA not as free fatty acid. These conclusions are also supported by the observations that the transport of phytanic acid is inhibited by substitution of ATP and/or CoASH with their respective analogs (AMPCPOP or desulfoCoA-agarose) which do not support the activation of phytanic acid to phytanoyl-CoA (Table 2). On the other hand, AMPCPOP and desulfoCoA-agarose have no effect on the transport of phytanoyl-CoA. Similar transport of phytanoyl-CoA and phytanic acid in fatty acid-activating conditions and the absence of an effect of substitution of ATP by AMP-PCP on the transport of phytanoyl-CoA suggest that the translocation of phytanoyl-CoA across the peroxisomal membrane does not require energy (ATP) (Table 2). The transport of phytanic acid into peroxisomes seems to be similar to the transport of palmitic acid but not to the transport of lig-

TABLE 1. Effect of phytanic acid and phytanoyl-CoA on the integrity of human liver peroxisomes

	Phytanic Acid		Phytanoyl-CoA	
Peroxisomal Fraction	Catalase Activity	Free Catalase	Catalase Activity	Free Catalase
	units/mg protein	%	*units/mg protein*	%
Before dialysis	3.98 ± 0.72	17.2 ± 1.8	4.15 ± 0.57	16.9 ± 2.4
After dialysis	3.72 ± 0.43	18.5 ± 2.25	3.91 ± 0.68	17.8 ± 2.2

Peroxisomal fractions before or after dialysis were incubated with 12 μM phytanic acid or phytanoyl-CoA in the presence of 10 mM ATP, 50 μM CoASH, and 5 mM Mg^{2+}. The peroxisomal integrity, as percent of free catalase, was measured after incubation for 5 min at 25°C. The results are expressed as average ± SD of three separate experiments.

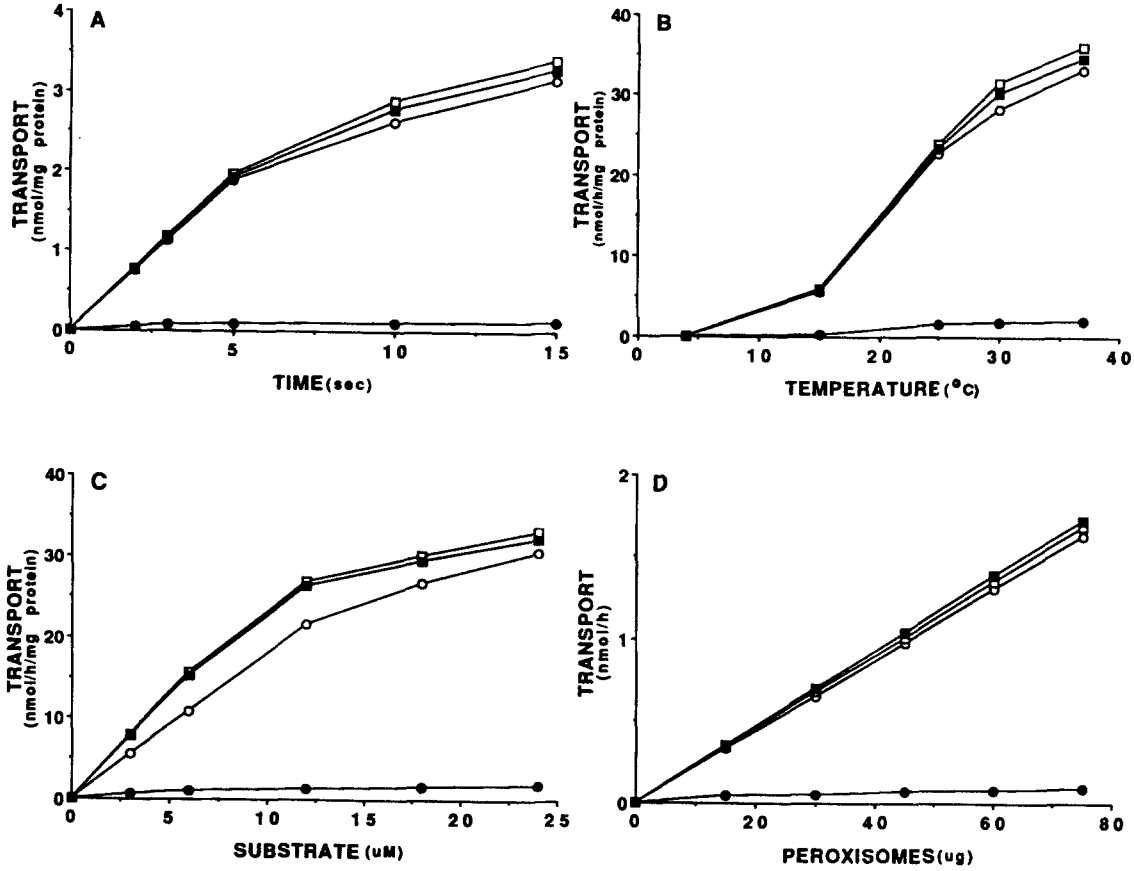

Fig. 5. Transport of phytanic acid and phytanoyl-CoA into human liver peroxisomes. The transport of phytanic acid (○, in the presence and ●, in the absence of fatty acid activating conditions) and phytanoyl CoA (□, in the presence and ■, in the absence of fatty acid-activating conditions) was studied in purified peroxisomes as described in the text. Panel A shows the time course of the transport at 25°C using 12 μM substrate and 30 μg peroxisomes. Panel B shows the temperature dependance of transport under similar conditions. The effects of varying substrate concentration and peroxisomal protein are seen in panels C and D, respectively. The results are the average of two independent determinations done in duplicate.

noceric acid because palmitic acid is also converted to palmitoyl-CoA on the cytoplasmic surface of peroxisomal membrane and then transported into peroxisomes; whereas lignoceric acid is transported into peroxisomes as such and then activated by lignoceroyl-CoA ligase on the luminal surface of the peroxisomal membrane (18).

To delineate the relationship between the transport processes of palmitic acid and phytanic acid in human liver peroxisomes, we examined the effect of unlabeled fatty acids and of antibodies against palmitoyl-CoA ligase on the activation and transport of these two fatty acids. A fourfold excess of unlabeled palmitic acid had no effect on the transport of [1-^{14}C]phytanic acid or [1-^{14}C]phytanoyl-CoA into peroxisomes. In contrast, unlabeled palmitic acid strongly inhibited the transport of [1-^{14}C]palmitic acid (**Fig. 6**). The antibodies against palmitoyl-CoA ligase inhibited the activation, transport, and oxidation of palmitic acid by peroxisomes by 60–70% (**Fig. 7**) supporting our previous results (18) that palmitic acid must be converted to palmitoyl-CoA prior to its transport and oxidation in peroxisomes. However, these antibodies had no effect on the activation, transport, and oxidation of phytanic acid suggesting that palmitoyl-CoA ligase is not associated with these steps of phytanic acid catabolism. These results suggest that although both phytanic and palmitic acid need to be converted to their CoA-derivatives on the cytoplasmic surface of peroxisomes, the

TABLE 2. Transport of phytanic acid and phytanoyl-CoA into human liver peroxisomes

Assay Conditions	Phytanic Acid	Phytanoyl-CoA
	nmol/h/mg protein	
ATP + CoASH + Mg^{2+}	22.05 ± 2.50	23.39 ± 2.26
− ATP/ + AMPCPOP	1.78 ± 0.20	24.26 ± 2.17
− CoASH/ + desulfo CoA agarose	1.62 ± 0.12	23.76 ± 3.11
− CoASH/ − ATP/ − Mg^{2+}	1.74 ± 0.22	22.57 ± 1.80
− ATP/ + AMP-PCP		24.56 ± 3.06

Transport of phytanic acid and phytanoyl-CoA into peroxisomes in the presence (+) or absence (−) of different cofactors was measured as described in the text. The results are expressed as average ± SD of three separate experiments.

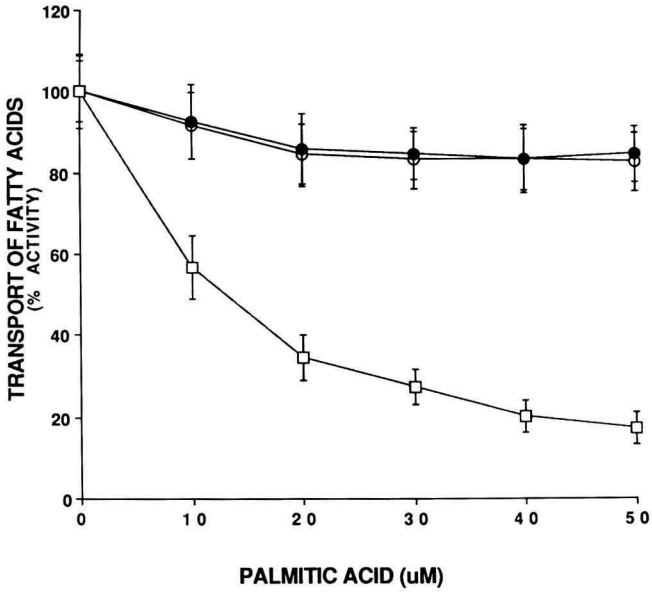

Fig. 6. The effect of unlabeled palmitic acid on the transport of fatty acids into human liver peroxisomes. The transport of [1-^{14}C]phytanic acid (○), [1-^{14}C]phytanoyl CoA (●), and [1-^{14}C]palmitic acid (□) was measured in the presence of increasing concentrations of unlabeled palmitic acid in the presence of fatty acid activation medium as described in the text. These results are expressed as mean ± SD of six values. The rates of transport of phytanic acid (22.05 ± 2.5 nmol/h/mg protein), phytanoyl-CoA (23.39 ± 2.26 nmol/h/mg protein), and palmitic acid (45.8 ± 6.3 nmol/h/mg protein) were considered as 100% activity.

acyl-CoA ligases for activation of phytanic acid and palmitic acid and the site (proteins) for the transport of palmitoyl-CoA and phytanoyl-CoA through the peroxisomal membrane seem to be different.

To investigate the fate of phytanoyl-CoA transported into peroxisomes, the transport of phytanoyl-CoA into intact peroxisomes was studied as described before, and the status of phytanoyl-CoA in peroxisomes was studied by Dole partition (25) of the radioactive fatty acid after transport assay into aqueous phase (phytanoyl-CoA) and organic phase (phytanic acid). As shown in **Fig. 8,** the signal for radioactivity in organic phase (phytanic acid) gradually increased from 0 min to 60 min; whereas in the aqueous phase (phytanoyl-CoA) it gradually decreased from 0 min to 60 min after transport assay indicating that phytanoyl-CoA, after its transport into peroxisomes, is quickly hydrolyzed to free phytanic acid.

DISCUSSION

A deficit in the catabolism of phytanic acid to pristanic acid in patients with Refsum disease was demonstrated about 25 years ago (1), but the identification of the organelle and the enzyme system involved remains elusive (5, 26–29). Recent studies have provided more definitive evidence that in humans α-oxidation of phytanic acid occurs in peroxisomes while in rodents it occurs in mitochondria (6). The activation of phytanic acid to phytanoyl-CoA by phytanoyl-CoA ligase is prerequisite for its oxidation in intact peroxisomes. The human peroxisomes contain three distinct acyl-CoA ligases for long chain (palmitic acid), very long chain (lignoceric acid), and branched chain (phytanic acid) fatty acids (8, 30–35). At the subcellular level, palmitoyl-CoA ligase (76 kDa) is present in peroxisomes, mitochondria, and endoplasmic reticulum (ER); whereas lignoceroyl-CoA ligase is present in peroxisomes and ER but not in mitochondria (32, 35). Lignoceric acid is primarily and possibly exclusively oxidized in peroxisomes (36). Mitochondria cannot oxidize lignoceric acid because they lack lignoceroyl-CoA ligase (32).

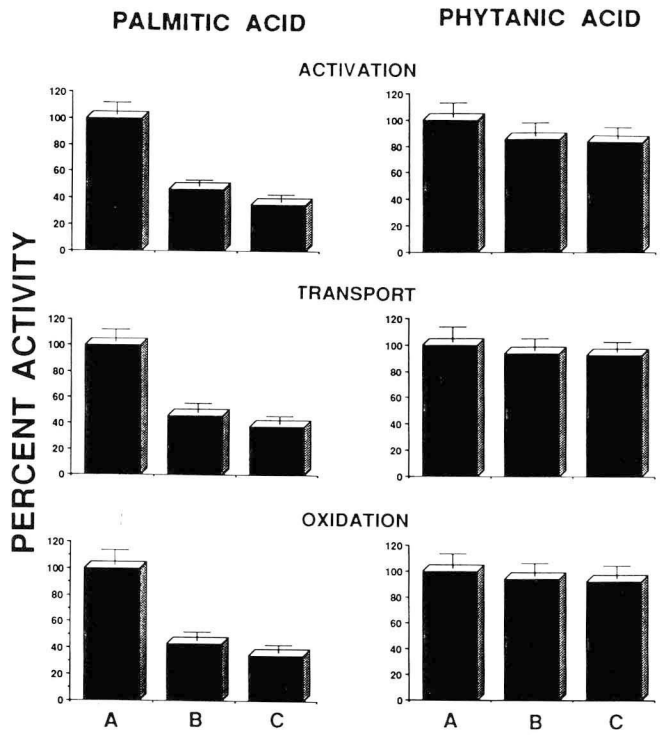

Fig. 7. Effect of antisera raised against palmitoyl-CoA ligase on the activation, transport, and oxidation of palmitic acid and phytanic acid. Purified peroxisomes suspended in 0.25 M sucrose were incubated with different concentrations of antisera against palmitoyl-CoA ligase at 4°C (A, preimmune sera; B, antibody:peroxisome ratio of 2:1 by protein; C, antibody:peroxisome ratio of 4:1). After 2 h, this preparation was centrifuged at 9130 *g* for 10 min at 4°C at low acceleration and deceleration in a Beckman TL-100 tabletop ultracentrifuge. The pellet was gently suspended in 0.25 M sucrose and used for activation, transport, and oxidation assays. Integrity of these peroxisomal suspensions was 83–85% as judged by latency of catalase activities. One hundred percent activity for palmitoyl-CoA and phytanoyl-CoA ligases were 13.9 ± 2.1 and 6.15 ± 0.9 nmol/min/mg protein, respectively. One hundred percent activities for transport of palmitic acid and phytanic acid were 45.8 ± 6.3 and 22.05 ± 2.5 nmol/h/mg protein. One hundred percent of activities for oxidation of palmitic acid and phytanic acid were 24.7 ± 3.6 nmol/h/mg protein and 33.5 ± 4.1 pmol/h/mg protein, respectively. The results are mean ± SD of six values.

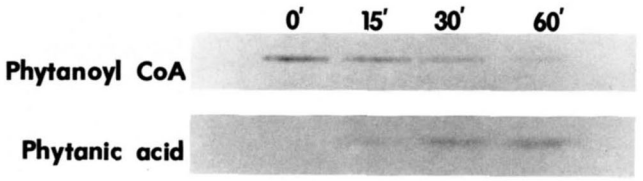

Fig. 8. The status of phytanoyl-CoA after transport into peroxisomes. Purified peroxisomes were incubated for 5 min with [1-^{14}C]phytanoyl-CoA at 25°C under isotonic conditions, and transport of phytanoyl-CoA was measured as described in the text. The status of phytanoyl-CoA, transported into peroxisomes, was measured by Dole partition (25) of radioactivity in peroxisomes into aqueous (phytanoyl-CoA) and organic (free phytanic acid) phases for different time intervals after transport assay. The organic and aqueous phases were removed, dried to small volumes, and resolved by thin-layer chromatography (TLC) using hexane–diethyl ether–acetic acid 60:40:1 (v/v) as the solvent system for the organic phase, and diethyl ether –benzene 60:40 (v/v) as the solvent system for the aqueous phase. The phytanic acid and phytanoyl-CoA bands were identified by unlabeled standards. TLC plates were exposed for 24 h at −70°C to X-ray films. After transport assay the lower aqueous layer at zero time contained 6640 dpm of [1-^{14}C]phytanoyl-CoA.

Phytanoyl-CoA ligase activity is found in microsomes, mitochondria, and peroxisomes (8). Consistent with the conclusion that the peroxisome is the primary site of α-oxidation of phytanic acid in humans, the specific activity of phytanoyl-CoA ligase in peroxisomes is 8 to 12 times higher than in mitochondria. In rat tissue, however, the mitochondrion is the primary site of phytanic acid oxidation (6), with the specific activities of phytanoyl-CoA ligase in mitochondria being 7 to 8 times higher than that found in peroxisomes (8). These acyl-CoA ligases control the metabolism of fatty acids in these organelles by providing acyl-CoA derivatives for complex lipid biogenesis in ER and for their catabolism in peroxisomes and mitochondria (32, 34, 35).

The intraorganellar distribution of enzymes in the system for α-oxidation of phytanic acid in peroxisomes is similar to the peroxisomal β-oxidation enzyme system. Similar to palmitoyl-CoA and lignoceroyl-CoA ligases, the phytanoyl-CoA ligase is present in peroxisomal membrane and the enzyme system for subsequent steps in the α-oxidation of phytanic acid is present in the peroxisomal matrix (9). Surprisingly, we found that in contrast to the β-oxidation system for fatty acids, the substrate for α-oxidation is free phytanic acid (9). The activation of phytanic acid to phytanoyl-CoA was required only in intact peroxisomes; whereas in isolated matrix or digitonin-permealized peroxisomes the free phytanic acid underwent α-oxidation as efficiently as phytanoyl-CoA (9). This implies that phytanoyl-CoA is synthesized in the peroxisomal membrane and then hydrolyzed to free fatty acid prior to its α-oxidation to pristanic acid in the matrix. Consistent with this hypothesis, phytanoyl-CoA is quickly hydrolyzed to free phytanic acid in the matrix of peroxisomes (9). The inability of Naproxen, an inhibitor of acyl-CoA ligases, to inhibit the α-oxidation of phytanic acid in isolated matrix or permealized peroxisomes suggests that free phytanic acid is the true substrate for the α-oxidation enzyme system of phytanic acid. This implies that the active site of phytanoyl-CoA ligase should be on the cytoplasmic surface of peroxisomal membrane where it controls the metabolism of phytanic acid by providing phytanoyl-CoA for transport into peroxisomes.

Studies from our laboratory have previously demonstrated that the active site of palmitoyl-CoA ligase is on the cytoplasmic surface of peroxisomes; whereas the active site of lignoceroyl-CoA ligase is on the luminal surface of the peroxisomal membrane (24). Several lines of evidence reported in this study demonstrate that the enzymatic site of phytanoyl-CoA ligase is on the cytoplasmic surface of peroxisomes. First, disruption of peroxisomes by sonication or permealization with digitonin results in release of a "latent" pool of catalase (a matrix enzyme) and lignoceroyl-CoA ligase activity; whereas the activities of palmitoyl-CoA and phytanoyl-CoA ligases were relatively unaffected. Second, proteolytic enzyme (trypsin) treatment inhibits the activities of phytanoyl-CoA and palmitoyl-CoA ligases in intact peroxisomes, while lignoceroyl-CoA ligase is only inhibited when peroxisomal integrity was disrupted before treatment with trypsin. And finally, the impermeable sulfhydryl inhibitor (Hg-dextran) inhibits the activity of phytanoyl-CoA and palmitoyl-CoA ligases in intact peroxisomes; whereas lignoceroyl-CoA ligase activity remains relatively unaffected. These results clearly demonstrate that similar to palmitoyl-CoA ligase (24, 30, 37) the active site of phytanoyl-CoA ligase is located on the cytoplasmic surface of peroxisomes. The inhibition of palmitoyl-CoA ligase but not of phytanoyl-CoA ligase by antibodies raised against palmitoyl-CoA ligase supports the conclusion that although the active sites of both enzymes are on the

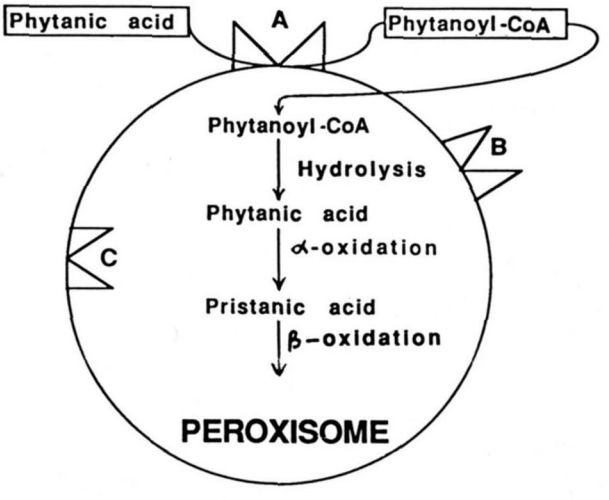

Fig. 9. Topographical model of enzyme system for activation and α-oxidation of phytanic acid in human liver peroxisomes. A, B, and C represent the enzymatic sites of phytanoyl-CoA ligase, palmitoyl-CoA ligase, and lignoceroyl-CoA ligase, respectively.

cytoplasmic surface, they are different enzymes. The results from this study also support our previous conclusion (24) that the active site of lignoceroyl-CoA ligase is on the luminal surface of the peroxisomal membrane (24). Another study using only one approach, the sensitivity of lignoceroyl-CoA ligase to trypsin, has postulated that the active site of lignoceroyl-CoA ligase is on the cytoplasmic surface of peroxisomes (30). Unfortunately, the integrity of peroxisomes was not very well controlled in that study. In fact, the marker enzymes (*d*-amino acid and *l*-hydroxy acid oxidases) used in this study are poor indicators of peroxisomal integrity as more than 50% of their activity stays with the membranes even when 98% of the catalase activity is lost. Moreover, the transport of fatty acids through the peroxisomal membrane agrees with the topology of the active site of these ligases in the peroxisomal membrane (18).

The transport of proteins and metabolites into peroxisomes is an active area of interest. The mammalian peroxisomal matrix proteins studied so far are synthesized on free polysomes and are translocated post-translationally into existing peroxisomes by an unique tripeptide sequence (SKL or SRL or SHL) at the C-terminal that targets these proteins to peroxisomes (38). The transport mechanism of free fatty acids and other metabolites into peroxisomes is not very well understood. The hydrophilic molecules smaller than 800 daltons (e.g., sucrose, CoASH, ATP, and carnitine) are known to be permeable through the nonspecific pores present in peroxisomal membranes (39). Recent studies using synthetic phospholipid vesicles have demonstrated that peroxisomal membrane proteins (22- and 28-kDa) are associated with these pores which allow nonspecific permeability of small hydrophilic molecules (39). The selective transport of phytanoyl-CoA but not of phytanic acid suggests that transport of phytanic acid may not be mediated by these nonspecific pores. Consistent with the topology of the active site of phytanoyl-CoA ligase in the peroxisomal membrane, the transport of phytanoyl-CoA was 12 to 14 times higher than that of free fatty acid in the absence of fatty acid-activating cofactors. The inability of palmitic acid to inhibit the transport of phytanic acid or phytanoyl-CoA into peroxisomes suggests that different peroxisomal membrane proteins (sites) may be associated with the transport of phytanoyl-CoA and palmitoyl-CoA into peroxisomes. The transport of proteins through the peroxisomal membrane requires energy (ATP) (38); however, the deletion of ATP from the assay medium or substitution of ATP by AMP-PCP had no effect on the transport of phytanoyl-CoA into peroxisomes.

In summary, we have demonstrated that in human liver peroxisomes: *a)* phytanoyl-CoA ligase is a unique protein distinct from palmitoyl-CoA ligase and lignoceroyl-CoA ligase; *b)* the active site of this phytanoyl-CoA ligase lies at the cytoplasmic surface of the peroxisomal membranes (similar to palmitoyl-CoA ligase); and *c)* phytanic acid is transported into the peroxisomes as phytanoyl-CoA not as free fatty acid. This enzyme provides phytanoyl-CoA which is transported into peroxisomes where it is hydrolyzed to free phytanic acid and where it then undergoes α-oxidation to pristanic acid (9). Although the subcellular site for β-oxidation of pristanic acid, a product of phytanic acid α-oxidation, has not been clearly demonstrated, the identification of pristanoyl-CoA oxidase in peroxisomes (40) and excessive accumulation and defective oxidation of pristanic acid in diseases which lack peroxisomes (2, 3) suggest that the catabolism of pristanic acid by β-oxidation in human tissue also takes place in peroxisomes. Based on these findings, we propose a possible biochemical model for catabolism of phytanic acid in human liver peroxisomes which should provide a better understanding of the physiological role of this organelle in the catabolism of phytanic acid (**Fig. 9**). A clear understanding of the metabolic biology of phytanic acid will be helpful in delineating the molecular basis for the inherent loss of phytanic acid α-oxidase activity in diseases that lack peroxisomes (e.g., Zellweger Syndrome, neonatal adrenoleukodystrophy, and infantile Refsum disease), diseases with morphologically visible peroxisomes and with multiple enzyme deficiencies (e.g., rhizomelic chondrodysplasia punctata, and pseudo infantile Refsum disease) (2, 3, 41), adult Refsum disease (1), and disease with isolated phytanic acid oxidation deficiency (42).

This study was supported by a grant from the National Institutes of Health (NS-22576). We are thankful to Mrs. Carol Frazier for typing this manuscript.

Manuscript received 7 July 1994 and in revised form 9 December 1994.

REFERENCES

1. Steinberg, D. 1989. Refsum disease. *In* The Metabolic Basis of Inherited Disease. 6th ed. C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle, editors. McGraw-Hill Book Co., New York. 1533-1550.
2. Lazarow, P. B., and H. W. Moser. 1989. Disorders of peroxisome biogenesis *In* The Metabolic Basis of Inherited Disease. 6th ed. C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle, editors. McGraw-Hill Book Co., New York. 1479-1509.
3. Brown, F. R., R. Voight, A. K. Singh, and I. Singh. 1993. Peroxisomal disorders. Neurodevelopmental and biochemical aspects. *Am. J. Dis. Child.* **147:** 617-626.
4. Herndon, J. H., Jr., D. Steinberg, B. W. Uhlendorf, and H. M. Fales. 1969. Refsum's disease: characterization of enzyme defect in cell culture. *J. Clin. Invest.* **48:** 1017-1032.
5. Singh, I., O. Lazo, K. Pahan, and A. K. Singh. 1992. Phytanic acid α-oxidation in human cultured skin fibroblasts. *Biochim. Biophys. Acta.* **1180:** 221-224.
6. Singh, I., K. Pahan, G. S. Dhaunsi, O. Lazo, and P. Ozand. 1993. Phytanic acid α-oxidation. Differential subcellular localization in rat and human tissues and its inhibition by

nycodenz. *J. Biol. Chem.* **268:** 9972–9979.
7. Singh, I., K. Pahan, A. K. Singh, and E. Barbosa. 1993. Refsum disease: a defect in the α-oxidation of phytanic acid in peroxisomes. *J. Lipid Res.* **34:** 1755–1764.
8. Pahan, K., J. Cofer, P. Baliga, and I. Singh. 1993. Identification of phytanoyl-CoA ligase as a distinct acyl-CoA ligase in peroxisomes from cultured skin fibroblasts. *FEBS Lett.* **322:** 101–104.
9. Pahan, K., and I. Singh. 1993. Intraorganellar localization of CoASH-independent phytanic acid oxidation in human liver peroxisomes. *FEBS Lett.* **333:** 154–158.
10. Hoshi, M., and Y. Kishimoto. 1973. Synthesis of cerebronic acid from lignoceric acid by rat brain preparation. Some properties and distribution of the hydroxylation system. *J. Biol. Chem.* **248:** 4123–4130.
11. Akanuma, H., and Y. Kishimoto. 1979. Synthesis of ceramides and cerebrosides containing both α-hydroxy and nonhydroxy fatty acids from lignoceroyl-CoA by rat brain microsomes. *J. Biol. Chem.* **254:** 1050–1056.
12. Pitha, J. 1978. Reagents specific for cell surface components. *Eur. J. Biochem.* **82:** 285–292.
13. Leighton, F., B. Poole, H. Beaufay, P. Baudhin, J. W. Coffey, S. Fowler, and C. de Duve. 1968. The large scale preparation of peroxisomes, mitochondria and lysosomes from the livers of rats injected with triton WR-1339. Improved isolation procedures, automated analysis, biochemical and morphological properties of fractions. *J. Cell Biol.* **37:** 482–512.
14. Cooperstein, S. J., and A. Lazarow. 1971. Microspectrophotometric method for the determination of cytochrome oxidase. *J. Biol. Chem.* **189:** 665–670.
15. Beaufay, H., A. Amar-Costesec, E. Feytmans, D. Thines-Sempoux, M. Wibo, M. Robbi, and T. Berthet. 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. I. Biochemical methods. *J. Cell. Biol.* **61:** 188–200.
16. Baudhin, P., H. Beaufay, Y. Rahman-Li, O. Z. Sellinger, R. Wattiaux, P. Jacques, and C. de Duve. 1964. Tissue fractionation studies. Intracellular distribution of monoamine oxidase, aspartate aminotransferase, alanine aminotransferase, D-amino acid oxidase and catalase in rat-liver tissue. *Biochem. J.* **92:** 179–184.
17. Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.
18. Singh, I., O. Lazo, G. S. Dhaunsi, and M. Contreras. 1992. Transport of fatty acids into human and rat peroxisomes: differential transport of fatty acids into peroxisomes. *J. Biol. Chem.* **267:** 13306–13313.
19. Singh, I., R. P. Singh, A. Bhusan, and A. K. Singh. 1985. Lignoceroyl-CoA ligase activity in rat brain microsomal fraction: topographical localization and effect of detergents and α-cyclodextrin. *Arch. Biochem. Biophys.* **236:** 418–426.
20. Cornell, N. W. 1980. Rapid fractionation of cell suspensions with the use of brominated hydrocarbons. *Anal. Biochem.* **102:** 326–331.
21. Singh, I., A. Bhusan, N. K. Relan, and T. Hashimoto. 1988. Acyl-CoA ligases from rat brain microsomes. An immunochemical study. *Biochim. Biophys. Acta.* **963:** 509–514.
22. Fujiki, Y., S. Fowler, H. Shio, A. L. Hubbard, and P. B. Lazarow. 1982. Polypeptide and phospholipid composition of the membrane of rat liver peroxisomes: comparison with endoplasmic reticulum and mitochondrial membranes. *J. Cell Biol.* **93:** 103–110.
23. Coleman, R. A., and R. M. Bell. 1983. Topography of membrane bound enzymes that metabolize complex lipids. *In* The Enzymes. P. D. Boyer, editor. Academic Press, New York. 605–619.
24. Lazo, O., M. Contreras, and I. Singh. 1990. Transverse topographical localization of peroxisomal acyl-CoA ligases: differential localization of palmitoyl-CoA and lignoceroyl-CoA ligases. *Biochemistry.* **29:** 3981–3986.
25. Dole, V. P. 1956. A relation between non-esterified fatty acids in plasma and the metabolism of glucose. *J. Clin. Invest.* **35:** 150–154.
26. Muralidharan, V. B., and Y. Kishimoto. 1984. Phytanic acid α-oxidation in rat liver. *J. Biol. Chem.* **259:** 13021–13026.
27. Watkins, P. A., S. J. Mihalik, and O. H. Skjeldal. 1990. Mitochondrial oxidation of phytanic acid in human and monkey liver: implications that Refsum's disease is not a peroxisomal disorder. *Biochem. Biophys. Res. Commun.* **167:** 580–586.
28. Wanders, R. J. A., and C. W. T. Van Roermund. 1993. Studies on phytanic acid α-oxidation in rat liver and cultured skin fibroblasts. *Biochim. Biophys. Acta.* **1167:** 345–350.
29. Huang, S., P. P. Van Veldhoven, F. Vanhoutte, G. Parmetier, H. J. Eyssen, and G. P. Mannaerts. 1992. Alpha-oxidation of 3-methyl-substituted fatty acids in rat liver. *Arch. Biochem. Biophys.* **296:** 214–223.
30. Lageweg, W., J. M. Tager, and R. J. A. Wanders. 1991. Topography of very long chain fatty acid activating activity in peroxisomes from rat liver. *Biochem. J.* **276:** 53–56.
31. Lazo, O., M. Contreras, M. Hashmi, W. Stanley, C. Irazu, and I. Singh. 1988. Peroxisomal lignoceroyl CoA ligase deficiency in childhood adrenoleukodystrophy and adrenomyeloneuropathy. *Proc. Natl. Acad. Sci. USA.* **85:** 7467–7651.
32. Lazo, O., M. Contreras, Y. Yoshida, A. K. Singh, W. Stanley, M. Weise, and I. Singh. 1990. Cellular oxidation of lignoceric acid is regulated by the subcellular localization of lignoceroyl-CoA ligases. *J. Lipid Res.* **31:** 583–595.
33. Singh, H., and A. Poulos. 1988. Distinct long chain and very long chain fatty acyl-CoA synthetases in rat liver peroxisomes and microsomes. *Arch. Biochem. Biophys.* **266:** 486–495.
34. Groot, P. H. E., H. R. Scholte, and W. C. Hulsman. 1976. Fatty acid activation: specificity, localization, and function. *Adv. Lipid Res.* **14:** 75–126.
35. Miyazawa, S., T. Hashimoto, and S. Yokoda. 1985. Identity of long-chain acyl-coenzyme A synthetase of microsomes, mitochondria and peroxisomes in rat liver. *J. Biochem.* **98:** 723–733.
36. Singh, I., A. B. Moser, S. Goldfisher, and H. W. Moser. 1984. Lignoceric acid oxidized in the peroxisome: Implications for the Zellweger cerebro-hepato-renal syndrome and adrenoleukodystrophy. *Proc. Natl. Acad. Sci. USA.* **81:** 4206–4207.
37. Mannaerts, G. P., P. Van Veldhoven, A. Van Broekhoven, G. Vandebroek, and L. J. Debeer. 1982. Evidence that peroxisomal acyl-CoA synthetase is located at the cytoplasmic side of the peroxisomal membrane. *Biochem. J.* **204:** 17–23.
38. Subramani, S. 1993. Protein import into peroxisomes and biogenesis of the organelle. *Annu. Rev. Cell Biol.* **9:** 445–478.
39. Van Veldhoven, P. P., W. W. Just, and G. P. Mannaerts. 1987. Permeability of peroxisomal membrane to cofactors of β-oxidation. Evidence for the presence of a pore-forming protein. *J. Biol. Chem.* **262:** 4310–4318.
40. Van Veldhoven, P. P., G. Vanhove, S. Asselberghs, H. J. Eyssen, and G. P. Mannaerts. 1992. Substrate specificities

of rat liver peroxisomal acyl-CoA oxidases: palmitoyl-CoA oxidase (inducible acyl-CoA oxidase), pristanoyl-CoA oxidase (non-inducible acyl-CoA oxidase) and trihydroxycoprostanoyl-CoA oxidase. *J. Biol. Chem.* **267:** 20065–20074.

41. Aubourg, P., K. Kremser, M. O. Roland, M. Hadchonel, and I. Singh. 1993. Pseudo infantile Refsum's disease: catalase-deficient peroxisomal particles with partial deficiency of plasmalogen synthesis and oxidation of fatty acids. *Pediatr. Res.* **34:** 270–276.

42. Pai, G. S., K. Kremser, K. Pahan, and I. Singh. 1993. Phytanic acid oxidation defect and normal plasmalogen synthesis in two patients with rhizomelic chondrodysplasia punctata (RCDP). *Am. J. Hum. Genet.* **53:** A937.