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# Topographical Localization of Peroxisomal Acyl-CoA Ligases: Differential Localization of Palmitoyl-CoA and Lignoceroyl-CoA Ligases†

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ABSTRACT: We found that peroxisomal lignoceroyl-CoA ligase, like palmitoyl-CoA ligase, is present in the peroxisomal membrane whereas the peroxisomal β-oxidation enzyme system is localized in the matrix. To further define the role of peroxisomal acyl-CoA ligases (membrane component) in providing acyl-CoA for peroxisomal β-oxidation, we examined the transverse topographical localization of enzymatic sites of palmitoyl-CoA and lignoceroyl-CoA ligases in the peroxisomal membranes. The disruption of peroxisomes by various techniques resulted in the release of a "latent" pool of lignoceroyl-CoA ligase activity while palmitoyl-CoA ligase activity remained the same. Proteolytic enzyme treatment inhibited palmitoyl-CoA ligase activity in intact peroxisomes but had no effect on lignoceroyl-CoA ligase activity. Lignoceroyl-CoA ligase activity was inhibited only if peroxisomes were disrupted with detergent before trypsin treatment. Antibodies to palmitoyl-CoA ligase and to peroxisomal membrane proteins (PMP) inhibited palmitoyl-CoA ligase in intact peroxisomes, and no pool of "latent" activity appeared when antibody-treated peroxisomes were disrupted with detergent. On the other hand, disruption of PMP antibody-treated peroxisomes with detergent resulted in the appearance of a "latent" pool of lignoceroyl-CoA ligase activity. These results demonstrate that the enzymatic site of palmitoyl-CoA ligase is on the cytoplasmic surface whereas that for lignoceroyl-CoA ligase is on the luminal surface of peroxisomal membranes. This implies that palmitoyl-CoA is synthesized on the cytoplasmic surface and is then transferred to the matrix through the peroxisomal membrane for β-oxidation in the matrix. Lignoceric acid, on the other hand, is first transported through the peroxisomal membrane as such and is then activated to lignoceroyl-CoA on the luminal surface of the membrane before it is oxidized by the β-oxidation system in the matrix, and implications of these findings are discussed for X-linked adrenoleukodystrophy, a disorder with deficient activity of peroxisomal lignoceroyl-CoA ligase.

Activation of fatty acids to acyl-CoA derivatives by acyl-CoA ligases is the initial and obligatory step in their metabolism (e.g., oxidation, elongation, and synthesis of complex lipids). Different acyl-CoA ligases have been shown to activate fatty acids of different chain length (Groot et al., 1976). At the subcellular level, these acyl-CoA ligases are present in mitochondria, microsomes (Groot et al., 1976), and peroxisomes (Shindo & Hashimoto, 1978). Fatty acids greater than $C_{22}$ are primarily and possibly exclusively β-oxidized in peroxisomes (Singh et al., 1984a) by the peroxisomal β-oxidation system (Lazarow & De Duve, 1976), and fatty acids shorter then $C_{10}$ are β-oxidized in mitochondria (Mannaerts & Debeer, 1982). Fatty acids of chain length $C_{12}$–$C_{22}$ are β-oxidized in both mitochondria and peroxisomes.

An understanding of the activation, transport, and β-oxidation of very long chain (VLC) fatty acids (>$C_{22}$) in peroxisomes is of particular interest because pathognomic amounts of these VLC fatty acids accumulate as a result of either defect of a single enzyme (e.g., X-linked adrenoleukodystrophy) or β-oxidation enzyme pathways (e.g., Zellweger syndrome) (Singh et al., 1981, 1984a,b). The normal β-oxidation of lignoceroyl-CoA as compared to the defective β-oxidation of lignoceric acid ($C_{24:0}$) in homogenates of cultured skin fibroblast of X-linked adrenoleukodystrophy (X-ALD) suggested a defect in peroxisomal lignoceroyl-CoA ligase activity (Hashmi et al., 1986). This was later confirmed by direct demonstration of a deficiency for lignoceroyl-CoA ligase activity in peroxisomes isolated from cultured skin fibroblasts of X-ALD (Lazo et al., 1988; Wanders et al., 1988). In contrast to the defective activation and β-oxidation of lignoceric acid (Lazo et al., 1988), the activation and β-oxidation of palmitic acid in X-ALD are normal (Singh et al., 1984a; Lazo et al., 1988). By using antibodies to palmitoyl-CoA ligase, we previously demonstrated that residual activity for activation (17% of control) and β-oxidation (12% of control) of lignoceric acid in X-ALD was derived from the activation of lignoceric acid by peroxisomal palmitoyl-CoA ligase activity (Lazo et al., 1989).

Peroxisomes are single membranous organelles surrounding matrix proteins. Peroxisomal palmitoyl-CoA ligase is a con-

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stituent of this membrane (Krisans et al., 1980), while the β-oxidation enzymes (acyl-CoA oxidase, bifunctional enzyme, and β-ketoacyl-CoA thiolase) are present in the matrix (Applekvist & Dallner, 1980; Huttinger et al., 1980). The enzymatic site of palmitoyl-CoA ligase is localized on the cytoplasmic surface of the peroxisomal membranes (Mannaerts et al., 1982). Therefore, palmitoyl-CoA synthesized on the cytoplasmic surface has to be transported through the peroxisomal membranes before it is oxidized by the β-oxidation pathway. The present study was undertaken to identify the localization of lignoceroyl-CoA ligase in peroxisomes and to gain a better understanding of the role these two acyl-CoA ligases play in the manifestation of the peroxisomal disorders.

## Experimental Procedures

Acetylated trypsin, soybean trypsin inhibitor, malate, FAD, NAD, L-carnitine, and α-cycylodextrin were purchased from Sigma (St. Louis, MO). ATP and CoASH were obtained from P-L Biochemicals (Milwaukee, WI). Octyl glucoside (D-glucopyranoside) was purchased from United States Biochemical Corp. (Cleveland, OH), and Triton X-100 was purchased from Bio-Rad (Richmond, CA). [1-$^{14}$C]Palmitic acids (58.7 mCi/mmol) and K$^{14}$CN (52.0 mCi/mmol) were purchased from New England Nuclear (Boston, MA). Nycodenz was obtained from Accurate Chemical and Scientific Corp. (Westbury, NY). [1-$^{14}$C]Lignoceric acid was synthesized by treatment of *n*-tricosanoyl bromide with K$^{14}$CN as described previously (Hoshi & Kishimoto, 1973).

*Isolation of Peroxisomes from Rat Liver.* Liver peroxisomes were prepared from Sprague-Dawley rats, fasted overnight and weighing approximately 250 g. Livers were homogenized in 10 times (w/v) of a medium containing 0.25 M sucrose, 1 mM EDTA, 1 μg/mL antipain, 1 μg/mL leupeptin, 2 μg/mL aprotinin, 0.7 μg/mL of pepstatin, 0.2 mM phenylmethanesulfonyl fluoride, 0.1% ethanol, and 3 mM imidazole buffer, pH 7.4, at 4 °C. The homogenate was first fractionated by differential centrifugation to prepare the light mitochondrial "L" fraction (de Duve et al., 1955), and peroxisomes from the "L" fraction were prepared by isopycnic equilibrium centrifugation in a continuous gradient consisting of 28 mL of a 0–50% gradient of Nycodenz with 4 mL of 55% Nycodenz as a cushion in 39-mL tubes for a JV-20 Beckman vertical rotor. The tubes were sealed and then centrifuged at 33700*g* for 60 min at 8 °C in a JV-20 Beckman centrifuge with low acceleration and deceleration. The gradient was collected from the bottom, each fraction was analyzed for marker enzyme activities, and the densities of gradient fractions were determined with a hand refractometer (Atago type 500). Gradient fractions were analyzed for the following subcellular enzyme markers: cytochrome *c* oxidase for mitochondria (Cooperstein & Lazarow, 1951), NADPH–cytochrome *c* reductase for microsomes (Beaufay et al., 1974), catalase for peroxisomes (Baudhuin et al., 1964), *N*-acetyl-β-glucosaminidase for lysosomes (Sellinger et al., 1960), and phosphoglucomutase for cytosol (Bronfman et al., 1984). The purity of peroxisomes was approximately 95% as observed previously (Lazo et al., 1989). Protein concentration was determined by the procedure of Bradford (1976).

Peroxisomal integrity was measured by the latency of catalase activity, a matrix enzyme. Catalase activity was measured as described by Baudhuin et al. (1964). Total catalase activity was measured by diluting peroxisomes 1:1 with 2% of Triton X-100, and free catalase activity was measured in peroxisomes suspended in 0.25 M sucrose in the absence of Triton X-100.

*Enzyme Assay for Activation of Palmitic and Lignoceric Acids.* The activities for palmitoyl-CoA and lignoceroyl-CoA ligases were measured as described perviously (Singh et al., 1985) except that [1-$^{14}$C]palmitic and lignoceric acids were solubilized with α-cyclodextrin (Singh & Kishimoto, 1983). The fatty acid (20 × 10$^6$ cpm) was first dried in a test tube under nitrogen and then resuspended in 3.5 mL of α-cyclodextrin (20 mg/mL) by sonication for 1 h.

*Production of Antibodies to Palmitoyl-CoA Ligase and Peroxisomal Membranes.* Antibodies against microsomal palmitoyl-CoA ligase were prepared as described previously (Singh et al., 1988). This antibody reacts with palmitoyl-CoA ligase from mitochondria, microsomes, and peroxisomes (Miyazawa et al., 1985). Peroxisomal membrane proteins (PMP) prepared by pyrophosphate treatment (Leighton et al., 1969) were emulsified with complete Freund's adjuvant and injected subcutaneously at various sites in rabbits followed by boost injections of PMP in incomplete Freund's adjuvant as essentially described for palmitoyl-CoA ligase (Singh et al., 1988).

## Results

*Transverse Topographical Localization of Enzymatic Sites of Palmitoyl-CoA and Lignoceroyl-CoA Ligases in the Peroxisomal Membrane.* The topographical localization of the enzymatic sites for palmitoyl-CoA and lignoceroyl-CoA ligases in the transverse plane of the peroxisomal membranes was examined in intact and disrupted peroxisomes by treatment with proteolytic enzymes and antibodies to PMP and palmitoyl-CoA ligase.

*Localization of Lignoceroyl-CoA Ligase Activity in Peroxisomes.* We first determined the localization of lignoceroyl-CoA ligase activity in peroxisomes. Peroxisomes were disrupted by pyrophosphate treatment for various times at 4 °C, and peroxisomal membranes were separated from matrix proteins by centrifugation. Catalase and acyl-CoA ligase activities were measured in the membrane pellet sedimented by centrifugation. Matrix proteins are released into the medium when peroxisomal membranes are disrupted. We measured membrane-bound catalase, a matrix enzyme, as an index of intact peroxisomes. After 14 h of treatment with pyrophosphate, 77% of peroxisomal catalase was released, and after 18 h of treatment, only 5% of the total catalase was present in the membranes. Lignoceroyl-CoA ligase activity was observed only in peroxisomal membranes but not in the matrix. After 18 h of pyrophosphate treatment, lignoceroyl-CoA ligase and catalase activities in the membrane were 87 and 5%, respectively. Lignoceroyl-CoA ligase activity was sensitive to long-term treatment with pyrophosphate because this activity decreased in the membrane without its appearance in the soluble fraction.

*Effect of Temperature and Fatty Acids on Integrity of Peroxisomes.* We next examined the integrity of peroxisomes in conditions (e.g., different temperatures and concentrations of fatty acids) to which peroxisomes will be exposed to measure the transverse topographical localization of acyl-CoA ligases in the subsequent studies. Because peroxisomes are fragile, it was essential to know the percent of intact peroxisomes in an experiment to interpret the data from the transverse topographical studies. The percent release of catalase from peroxisomes (free catalase) is an index of disrupted peroxisomes, and the total catalase activity measured in the presence of 0.1% Triton X-100 is an index of both intact and disrupted peroxisomes.

Figure 1A shows the percent of free catalase when peroxisomes were exposed to three different temperatures (4, 25, or 37 °C) in the absence or presence of fatty acid (lignoceric

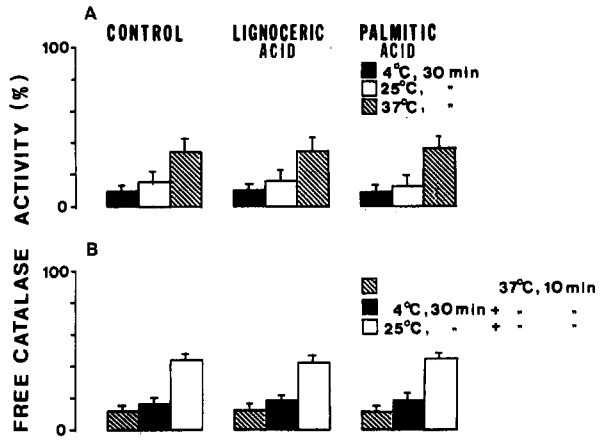


FIGURE 1: Effect of temperature and fatty acids on the integrity of peroxisomes. The integrity of peroxisomes suspended in 0.25 M sucrose was measured as described in the text after treatment of peroxisomes at different temperatures for different time periods in the absence or presence of fatty acids solubilized with α-cyclodextrin. (A) represents treatment of peroxisomes at different temperatures for a period of 30 min, and (B) represents subsequent treatment of peroxisomes to two different temperatures for different periods of time. The columns under lignoceric acid and palmitic acid represent the presence of either 4 μM lignoceric or 6 μM palmitic acids in the incubation mixture. The results are an average of four independent determinations ± the standard deviations.

acid or palmitic acid) solublized with α-cyclodextrin for 30 min. The disruption of peroxisomes increased with an increase in temperature, but the presence of fatty acid solubilized with α-cyclodextrin in the incubation mixture had no effect (Figure 1A). The percent of free catalase after 30-min incubation at 4, 25, and 37 °C was 10, 15, and 34%, respectively. Next, we examined the integrity of peroxisomes after subsequent exposure to two different temperatures for different times, conditions to which peroxisomes were subjected to in subsequent experiments (Figure 1B). The incubation of peroxisomes at 4 °C for 30 min (Figure 1A) or at 37 °C for 10 min (Figure 1B) resulted in 9 and 12% of free catalase, respectively. Subsequent exposure at 4 °C for 30 min and then at 37 °C for 10 min increased the percentage of free catalase to 16%. The greatest disruption of peroxisomes (44% free catalase) was observed when peroxisomes were first treated at 25 °C for 30 min and then at 37 °C for 10 min. The presence or absence of fatty acids solubilized with α-cyclodextrin had no effect (Figure 1B). These results demonstrate that the disruption of peroxisomal integrity increased with an increase in temperature and incubation time but that fatty acids solubilized with α-cyclodextrin had no effect.

*Effect of Disruption of Peroxisomes on the Activities of Acyl-CoA Ligases.* The disruption of peroxisomal integrity increased with an increase in the duration of sonication of purified peroxisomes as measured by an increase in the free catalase activity (Figure 2). The disruption of peroxisomes had no effect on the activity of palmitoyl-CoA ligase whereas lignoceroyl-CoA ligase activity increased by 67% after 60 s of sonication. Similar results were observed when peroxisomal integrity was disrupted with detergent (octyl glucoside) or digitonin. The disruption of the integrity of peroxisomes with digitonin resulted in an increase in the activities of free catalase and lignoceroyl-CoA ligase by 70 and 50%, respectively, but it had no effect on the palmitoyl-CoA ligase activity (Figure 3). Disruption of peroxisomes with different concentrations of octyl glucoside increased the free catalase activity by approximately 2-fold, and a similar increase was observed in the activity of lignoceroyl-CoA ligase as compared to no change in the activity of palmitoyl-CoA ligase. This parallel appearance of a "latent pool" of lignoceroyl-CoA ligase activity with disruption of peroxisomal integrity as compared to no change in the activity of palmitoyl-CoA ligase suggests that the enzymatic site for palmitoyl-CoA is localized on the cytoplasmic surface while that for lignoceroyl-CoA ligase is on the luminal surface of the peroxisomal membrane.

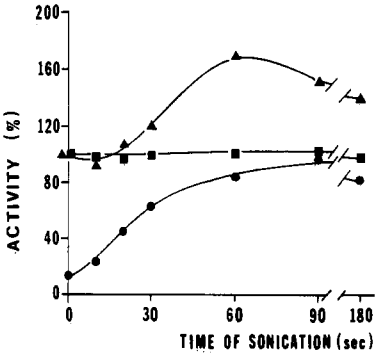


FIGURE 2: Effect of sonication on peroxisomal integrity and palmitoyl-CoA and lignoceroyl-CoA ligase activities. Purified peroxisomes (30 μg of protein) were sonicated for different periods of time in isotonic solution. Catalase (●), palmitoyl-CoA ligase (■), and lignoceroyl-CoA ligase (▲) activities were measured as described in the text. Free catalase, palmitoyl-CoA ligase, and lignoceroyl-CoA ligase in intact peroxisomes (without sonication) were considered as 100%. These results are the average of two independent determinations done in duplicate. Specific activities of palmitoyl-CoA ligase and lignoceroyl-CoA ligase are 17.6 ± 4.6 and 1.73 ± 0.70 nmol $h^{-1}$ (mg of protein)$^{-1}$, respectively.

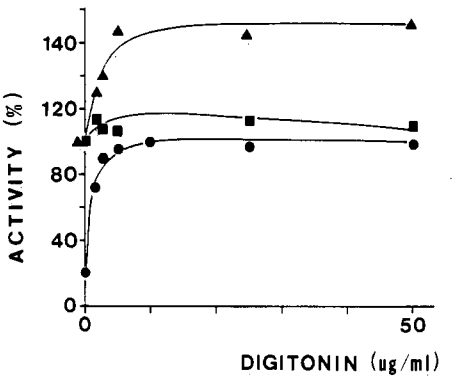


FIGURE 3: Effect of digitonin on peroxisomal integrity and palmitoyl-CoA and lignoceroyl-CoA ligase activities. Purified peroxisomes (30 μg of protein) suspended in 0.1 mL of 0.25 M sucrose were incubated with different amounts of digitonin for 1 h at 4 °C. Activities for catalase (●), palmitoyl-CoA ligase (■), and lignoceroyl-CoA ligase (▲) were measured as described in the text. Free catalase and palmitoyl-CoA ligase and lignoceroyl-CoA ligase in intact peroxisomes (in the absence of digitonin) were expressed as 100%. The results are an average of duplicates from two independent experiments.

*Effect of Trypsin on Acyl-CoA Ligase Activities in Intact and Disrupted Peroxisomes.* In this experiment, intact peroxisomes were disrupted with 0.1% Triton X-100 and were treated with trypsin (trypsin:peroxisomes ratio 1:20 by protein) for 30 min at 25 °C, and then trypsin was inhibited with soybean trypsin inhibitor. Acyl-CoA ligase activities were examined in the presence of 0.05% Triton X-100 in the assay medium to measure the total activities. Treatment with trypsin in both intact (in the absence of Triton X-100) and disrupted (Triton X-100 treatment) peroxisomes decreased palmitoyl-CoA ligase activity by 95%, suggesting that almost all of the palmitoyl-CoA ligase activity was accessible to trypsin in intact peroxisomes (Figure 4C). The lignoceroyl-CoA ligase activity in intact peroxisomes (without Triton X-100 treatment) decreased by only 32% (Figure 4B), and this corresponds to the disrupted peroxisomes in the preparation (Figure 4A). On

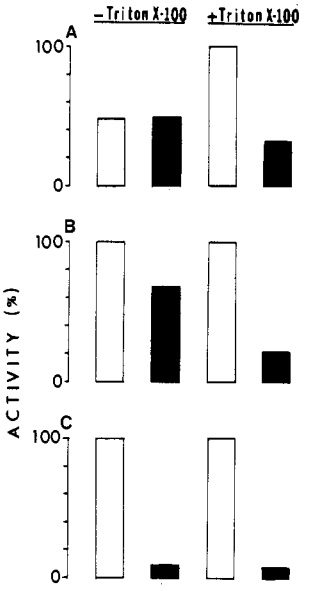


FIGURE 4: Effect of trypsin on peroxisomal palmitoyl-CoA and lignoceroyl-CoA ligase activities. The activities of catalase (A) were measured after trypsin treatment of intact (absence of Triton X-100) and disrupted (presence of Triton X-100) peroxisomes. Open bars represent control activities without trypsin treatment, and solid bars represent activities after trypsin treatment. Lignoceroyl-CoA ligase (B) and palmitoyl-CoA ligase (C) activities were measured in the presence of 0.05% Triton X-100 in the assay medium to measure the total activity. Purified peroxisomes suspended in 0.25 M sucrose were treated with trypsin (trypsin:peroxisome ratio 1:20 by protein) at 37 °C for 30 min in the presence or absence of 0.1% Triton X-100, and then the trypsin was inhibited by the addition of soybean trypsin inhibitor (trypsin:soybean trypsin inhibitor ratio 1:2 by protein). The results are the average of duplicates from two independent experiments.

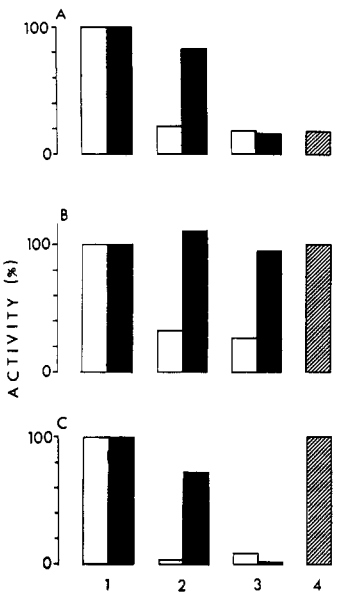


FIGURE 5: Effect of antibodies to palmitoyl-CoA ligase and peroxisomal membrane proteins on peroxisomal palmitoyl-CoA and lignoceroyl-CoA ligase activities. Lignoceroyl-CoA ligase (solid bars) and palmitoyl-CoA ligase (open bars) activities were examined in intact peroxisomes treated with antibodies to palmitoyl-CoA ligase and to PMP (A), peroxisomes disrupted with Triton X-100 after the antibody treatment (B), and subsequent treatment with antibodies following disruption of peroxisomes (C). Hatched bargraphs represent the percent of free catalase (4). Peroxisomes were treated with preimmune sera (1), antibodies to palmitoyl-CoA ligase (2), and antibodies to PMP (3). Peroxisomes were first treated with antibodies to palmitoyl-CoA ligase (antibody:peroxisome ratio 26.3 by protein) and to PMP (antibody:peroxisome ratio 27.6 by protein) for 1 h at 4 °C. The excessive antibodies were removed by 4 times dilution and sedimentation of peroxisomes by centrifugation at 18000 rpm for 20 min (A). Lignoceroyl-CoA and palmitoyl-CoA ligase activities were measured after suspension of the pallet in 0.25 M sucrose, pH 7.4. Part of the peroxisomes from different experiments in (A) was disrupted by treatment with Triton X-100 (0.05%) at 4 °C for 30 min, and acyl-CoA ligase activities were measured in these disrupted peroxisomes (B). Part of these disrupted peroxisomes was again treated with antibodies to palmitoyl-CoA ligase (antibody:peroxisome ratio 36.1 by protein) and to PMP (antibody:peroxisome ratio 37.8 by protein) for 1 h at 4 °C, and then palmitoyl-CoA and lignoceroyl-CoA ligase activities were measured as described in the text.

the other hand, trypsin decreased lignoceroyl-CoA ligase activity by 78% in a peroxisomal preparation disrupted with Triton X-100 (Figure 4B). The sensitivity of palmitoyl-CoA ligase activity to trypsin as compared to lignoceroyl-CoA ligase in the intact peroxisomes and the loss of lignoceroyl-CoA ligase activity only in the disrupted peroxisomes suggest that palmitoyl-CoA ligase is accessible to the trypsin in intact peroxisomes while lignoceroyl-CoA ligase is only accessible if peroxisomes are disrupted. This implies that palmitoyl-CoA is localized on the cytoplasmic surface whereas lignoceroyl-CoA ligase is located on the luminal surface of peroxisomal membranes.

*Effect of Antibodies on the Activities of Acyl-CoA Ligases in Intact and Disrupted Peroxisomes.* In this experiment, intact peroxisomes were first treated with antibodies to palmitoyl-CoA ligase or antibodies raised against PMP, and enzyme activities for palmitoyl-CoA and lignoceroyl-CoA ligase were measured after removal of excessive antibodies by centrifugation (Figure 5A). A part of the sedimented peroxisomes was disrupted by treatment with Triton X-100, and activities of palmitoyl-CoA and lignoceroyl-CoA ligases were measured in these disrupted peroxisomes (Figure 5B). Finally, a part of these disrupted peroxisomes was treated again with antibodies to palmitoyl-CoA ligase or to PMP, and enzyme activities for palmitoyl-CoA and lignoceroyl-CoA ligases were measured (Figure 5C).

Antibodies to palmitoyl-CoA ligase inhibited palmitoyl-CoA ligase by 76% but had little effect (18% inhibition) on lignoceroyl-CoA ligase activity in intact peroxisomes (Figure 5A). No "latent" pool of palmitoyl-CoA ligase activity appeared when these antibody-treated peroxisomes were disrupted with Triton X-100 (Figure 5B). Moreover, treatment of disrupted peroxisomes with antibodies to palmitoyl-CoA ligase resulted in almost complete inhibition of palmitoyl-CoA ligase activity (Figure 5C). The inhibition of palmitoyl-CoA ligase in intact peroxisomes and the lack of a "latent" pool of palmitoyl-CoA ligase activity in disrupted peroxisomes suggest that the enzymatic site for palmitoyl-CoA ligase is localized on the cytoplasmic surface of peroxisomes. The lack of effect of these antibodies on lignoceroyl-CoA ligase activity in intact and disrupted peroxisomes indicates that these antibodies do not react with lignoceroyl-CoA ligase.

Antibodies to PMP contain antibodies against both palmitoyl-CoA and lignoceroyl-CoA ligases because both of these enzymes are components of PMP. Treatment of intact peroxisomes with antibodies against PMP inhibited both palmitoyl-CoA and lignoceroyl-CoA ligase activities (Figure 5A). Disruption of PMP antibody-treated peroxisomes with Triton X-100 resulted in an increase of lignoceroyl-CoA ligase activity but had no effect on palmitoyl-CoA ligase activity (Figure 5B). The appearance of a "latent" pool of lignoceroyl-CoA ligase activity after disruption of antibody-treated peroxisomes (Figure 5B) and the inhibition of this activity following treatment with PMP antibodies in disrupted peroxisomes (Figure 5C) demonstrate that the enzymatic site for lignoceroyl-CoA ligase is localized on the luminal surface of the peroxisomal membrane. Inhibition of palmitoyl-CoA ligase

activity by both antibodies in intact peroxisomes (Figure 5A) indicates that the enzymatic site of palmitoyl-CoA ligase is localized on the cytoplasmic surface of the peroxisomal membrane.

## Discussion

Our results demonstrate that peroxisomal lignoceroyl-CoA ligase, like palmitoyl-CoA ligase (Krisan et al., 1980), is a constituent of the peroxisomal membrane and is not a matrix protein. The normal activity for palmitoyl-CoA ligase as compared to the deficient activity for lignoceroyl-CoA ligase in peroxisomes from X-ALD patients (Lazo et al., 1988) and the differential effect of detergents on these activities (Singh & Poulos, 1988) demonstrate that these two peroxisomal acyl-CoA ligase activities are two separate enzymes. Other cellular acyl-CoA ligase activities are found in mitochondria and microsomes. Cellular lignoceroyl-CoA ligase activity is present only in microsomes and peroxisomes whereas palmitoyl-CoA ligase is present in mitochondria, microsomes, and peroxisomes (Singh & Poulos, 1988; Lazo et al., 1988). The normal activity of microsomal lignoceroyl-CoA ligase compared to the deficient activity of peroxisomal lignoceroyl-CoA ligase (Lazo et al., 1988) and the differential effect of detergent on this activity in peroxisomes and microsomes (Singh & Poulos, 1988) suggest that the lignoceroyl-CoA ligases found in microsomes and peroxisomes are two separate enzymes. The palmitoyl-CoA ligases in mitochondria, microsomes, and peroxisomes have the same molecular mass (76 kDa), and polyclonal antibodies to the microsomal enzyme cross-react with this ligase in microsomes as well as in peroxisomes and mitochondria (Miyazawa et al., 1985; Singh et al., 1988).

The enzymatic site for palmitoyl-CoA ligase in microsomal membranes isolated from liver (Polokoff & Bell, 1978) and brain (Singh, 1985; Singh et al., 1985) has been determined to be on the cytoplasmic surface. Similar studies with peroxisomes have also shown the enzymatic site on the cytoplasmic surface (Mannaerts et al., 1982). However, in a study to immunocytochemically localize palmitoyl-CoA ligase, it was suggested that activation of fatty acid to acyl-CoA esters by this enzyme might occur inside the peroxisomes based on a higher density of gold particles on the matrical side of peroxisomal membranes (Yokota et al., 1987). Our results agree with the former observations (Mannaerts et al., 1982) indicating that the enzymatic site for palmitoyl-CoA ligase is on the cytoplasmic surface of peroxisomes. Therefore, some domains of the palmitoyl-CoA ligase polypeptide on the luminal side of the peroxisomal membrane may be easily accessible to antibodies to palmitoyl-CoA ligase. The enzymatic site of lignoceroyl-CoA ligase in microsomal membranes is on the cytoplasmic surface (Singh et al., 1985). Several lines of evidence reported in this study demonstrate that the enzymatic site of lignoceroyl-CoA ligase is on the luminal side of peroxisomal membranes. (1) Disruption of peroxisomal membranes by different techniques resulted in the release of a "latent" pool of catalase, a matrix enzyme, and as well as lignoceroyl-CoA ligase while palmitoyl-CoA ligase activity was unchanged. (2) Proteolytic enzyme treatment (trypsin) inhibited palmitoyl-CoA ligase activity but had no effect on lignoceroyl-CoA ligase activity in intact peroxisomes, and lignoceroyl-CoA ligase activity was only inhibited if peroxisomal integrity was disrupted before the trypsin. (3) Antibodies to palmitoyl-CoA ligase inhibited palmitoyl-CoA ligase activity in intact peroxisomes, and no "latent" pool of activity appeared when the integrity of antibody-treated peroxisomes was disrupted with detergent. The antibodies raised against PMP inhibited the activities of both palmitoyl-CoA and lignoceroyl-CoA ligases in intact peroxisomes. The disruption of PMP antibody-treated peroxisomes resulted in the appearance of a "latent" pool of only lignoceroyl-CoA ligase but not of palmitoyl-CoA ligase. The "latent" pool of lignoceroyl-CoA ligase activity was inhibited when detergent-disrupted peroxisomes were treated with PMP antibodies. These results clearly demonstrate that in the peroxisomal membrane the enzymatic site of palmitoyl-CoA ligase is on the cytoplasmic surface while for lignoceroyl-CoA ligase, the enzymatic site is located on the luminal surface.

Lignoceric acid is primarily and possibly exclusively $\beta$-oxidized in peroxisomes (Singh et al., 1984a) whereas lignoceroyl-CoA can be $\beta$-oxidized both in mitochondria and the peroxisomes (Lazo et al., submitted for publication). However, due to the absence of lignoceroyl-CoA ligase in mitochondria (Lazo et al., submitted for publication) and the instability of cytoplasmic lignoceroyl-CoA in vivo (Lazo et al., 1989), the $\beta$-oxidation of very long chain fatty acids may be restricted to peroxisomes. The peroxisomal $\beta$-oxidation enzymes (acyl-CoA oxidase, bifunctional enzyme, and acyl-CoA thiolase) are peroxisomal matrix constituents whereas the acyl-CoA ligases for both palmitic and lignoceric acids are peroxisomal membrane constituents. Therefore, the fatty acids must first be activated to acyl-CoA esters in peroxisomal membranes and then be transported to the matrix for their $\beta$-oxidation. The localization of the enzymatic site of palmitoyl-CoA ligase on the cytosolic surface suggests that palmitoyl-CoA is synthesized on the cytoplasmic surface and is then transported through the peroxisomal membrane for its $\beta$-oxidation in the matrix. On the other hand, the localization of the enzymatic site of lignoceroyl-CoA ligase on the luminal surface of peroxisomes implies that lignoceric acid is first transported through the peroxisomal membrane as free fatty acid and is then activated to lignoceroyl-CoA on the luminal surface of peroxisomal membranes before its $\beta$-oxidation in the matrix.

The activation and $\beta$-oxidation of lignoceric acid are deficient in peroxisomes from X-ALD patients but are not completely absent (Lazo et al., 1988). Residual activities in peroxisomes from X-ALD are derived from normal palmitoyl-CoA ligase activity (Lazo et al., 1989). Therefore, in X-ALD, lignoceroyl-CoA synthesized by palmitoyl-CoA ligase on the cytoplasmic surface of peroxisomes is transported through the peroxisomal membrane instead of free lignoceric acid. The lack of $\beta$-oxidation of lignoceric acid in peroxisomes from X-ALD could be due either to an abnormality in the transport of lignoceric acid through the membrane or to its activation by lignoceroyl-CoA ligase. However, the normal $\beta$-oxidation of lignoceroyl-CoA and the deficient activity of lignoceroyl-CoA ligase in peroxisomes from X-ALD (Lazo et al., 1988) would support the thesis that the defect in the $\beta$-oxidation of lignoceric acid in X-ALD is due to deficient activity of lignoceroyl-CoA ligase.

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