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Topography of very-long-chain-fatty-acid-activating activity in peroxisomes from rat liver

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We have investigated the localization of palmitoyl-CoA (hexadecanoyl-CoA) synthetase (EC 6.2.1.3) and cerotoyl-CoA (hexacosanoyl-CoA) synthetase in peroxisomes isolated from rat liver. Palmitoyl-CoA and cerotoyl-CoA synthetases, like acyl-CoA:dihydroxyacetone phosphate acyltransferase (EC 2.3.1.42), are present in the peroxisomal membrane. Trypsin treatment of intact peroxisomes led to the disappearance of both palmitoyl-CoA and cerotoyl-CoA synthetase activities but had little, if any, effect on L-α-hydroxy-acid oxidase (EC 1.1.3.15), D-amino acid oxidase (EC 1.4.3.3) or acyl-CoA:dihydroxyacetone phosphate acyltransferase. The latter three enzymes were inactivated if the trypsin treatment was preceded by disruption of the peroxisomes by sonication. These results show that the active site, or at least domains essential for the activity of cerotoyl-CoA synthetase, like that of palmitoyl-CoA synthetase, is located on the cytosolic face of the peroxisomal membrane.

INTRODUCTION

The peroxisomal β-oxidation system is involved in the oxidative chain-shortening of saturated long-chain fatty acids, saturated very-long-chain fatty acids, mono- and poly-unsaturated fatty acids and other compounds (see ref. [1] for a review). Peroxisomal β-oxidation, like that in mitochondria, proceeds via successive steps of dehydrogenation, hydratation, dehydrogenation and thiolytic cleavage. The enzymes involved, however, are different from their mitochondrial counterparts, except for the long-chain fatty acyl-CoA synthetase. This enzyme is identical to that present on the mitochondrial outer membrane and the membrane of the endoplasmic reticulum, as judged by several physicochemical, catalytic and immunological properties [2] and by restriction endonuclease mapping of the cDNA [3].

Mannaerts and coworkers [4] have shown that the catalytic site of palmitoyl-CoA (hexadecanoyl-CoA) synthetase (EC 6.2.1.3), or at least an essential domain of the enzyme, is located at the cytosolic face of the peroxisomal membrane. Hesler *et al.* [5] recently showed that the mitochondrial palmitoyl-CoA synthetase possesses one or more trypsin-sensitive sites on the inner surface of the membrane, in addition to proteinase-sensitive essential domains on the cytosolic surface. Nimmo [6], however, has reported that in intact mitochondria the palmitoyl-CoA synthetase is proteinase-resistant, which suggests that the enzyme is located at the inner surface of the mitochondrial outer membrane.

In recent years, evidence has been brought forward for the existence of a separate very-long-chain fatty acyl-CoA synthetase [7–12]. We [9] and others [10] have shown that this enzyme activity is present in peroxisomes and in the endoplasmic reticulum of rat liver, but not in mitochondria. However, Singh *et al.* [13] have recently reported that mitochondria from rat brain, in contrast to those from rat liver, contain lignoceroyl-CoA synthetase activity as well as the capacity to oxidize lignoceric acid.

The existence of a separate very-long-chain acyl-CoA synthetase has been questioned by Kishimoto and coworkers [14–16]. According to these authors, a single enzyme is responsible for the activation of long-chain and very-long-chain fatty acids, the substrate specificity being dependent upon the aggregation state of the enzyme as determined by its lipid environment. As pointed out by Nagamatsu *et al.* [14], early studies by Tanaka *et al.* [17] have shown that palmitoyl-CoA synthetase purified from rat liver microsomes is slightly reactive with behenic acid ($C_{22:0}$) and lignoceric acid ($C_{24:0}$).

As part of an ongoing study of the peroxisomal β-oxidation system, we have investigated the topographic organization of cerotoyl-CoA (hexacosanoyl-CoA) synthetase in the membranes of peroxisomes isolated from rat liver. The results described in this paper show that the active site of cerotoyl-CoA synthetase is located on the cytosolic face of the peroxisomal membrane. While our experiments were being performed, a paper by Lazo *et al.* [18] appeared in which the opposite conclusion was drawn. The possible reasons for this discrepancy are discussed in this paper.

MATERIALS AND METHODS

Isolation of peroxisomes from rat liver

Peroxisomes were prepared according to [19], with slight modifications [20]. The peroxisomes were collected from Nycodenz gradients and suspended in 10 vol. of buffer A [250 mM-sucrose, 2.5 mM-EDTA, 5 mM-Mops/NaOH and 0.1 % (v/v) ethanol (final pH 7.4)], sedimented by centrifugation and resuspended in a small volume (1–3 ml) of buffer A. The peroxisomal preparations were routinely more than 98 % pure (see [20]).

Treatment of peroxisomes with proteolytic enzymes

Portions of the peroxisomal fraction were incubated for 10 min at 25 °C in buffer A, either in the presence or in the absence of trypsin. Trypsin reactions were stopped by adding soybean trypsin inhibitor to 2 mg/ml. The samples were subsequently chilled and assayed for palmitoyl-CoA and cerotoyl-CoA synthetase activities within 1 h. The samples were stored at −80 °C and the other enzyme activities were measured within 1

Abbreviations used: DHAP-AT, acyl-CoA:dihydroxyacetone phosphate acyltransferase.

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week. Trypsin as well as trypsin inhibitor were dissolved in buffer A to prevent osmotic shock of the peroxisomes.

Analytical methods

The activities of palmitoyl-CoA synthetase and cerotoyl-CoA synthetase were measured as described for rat liver homogenates [21], except that the reaction medium contained additionally 250 mM-sucrose to prevent osmotic shock of the peroxisomes. Reactions were started by adding 20 μl of peroxisomal preparation in a final volume of 200 μl. The protein concentration was about 50 μg of protein/ml for cerotoyl-CoA synthetase measurements and about 1 μg of protein/ml for palmitoyl-CoA synthetase measurements. The reactions were terminated after 10 min by transferring a 150 μl portion of the incubation mixture to a glass tube containing 750 μl of Dole's reagent [22], 500 μl of n-heptane and 200 μl of 0.4 M-Mops/NaOH (pH 6.5). The lower (aqueous) layer was washed three times with 500 μl of n-heptane and the radioactivity in the final lower layer was determined. This procedure does not lead to loss of acyl-CoA esters, as tested by using authentic palmitoyl-CoA and cerotoyl-CoA (results not shown).

L-α-Hydroxy-acid oxidase (EC 1.1.3.15) was measured as described in [20]. D-Amino acid oxidase (EC 1.4.3.3) was measured as described in [20], except that D-alanine was used as substrate. Acyl-CoA:dihydroxyacetone phosphate acyltransferase (DHAP-AT; EC 2.3.1.42) was measured by the method of Davis & Hajra [23], as modified by Schutgens *et al.* [24]. Catalase (EC 1.11.1.6) was determined as described in [25].

Materials

Trypsin was purchased from Boehringer (Mannheim, Germany). Soybean trypsin inhibitor was obtained from Sigma (St. Louis, MO, U.S.A.). ATP and CoA were purchased from Boehringer. Nycodenz was obtained from Nycomed AS (Oslo, Norway). Radiochemicals were purchased from New England Nuclear (Dreieich, Germany). All other biochemicals were of the highest purity available.

RESULTS

Localization of the peroxisomal palmitoyl-CoA and cerotoyl-CoA synthetases

To determine the localization of the peroxisomal palmitoyl-CoA and cerotoyl-CoA synthetase activities, a suspension of purified peroxisomes was disrupted by sonication. In order to minimize aspecific binding of enzyme proteins to membranes, a medium containing 500 mM-KCl was used rather than buffer A alone. After centrifugation (100000 $g_{av.}$, 1.5 h), more than 78% of L-α-hydroxy acid oxidase activity was found in the supernatant, demonstrating that most of the matrix enzymes had been released. In accordance with its known localization in the peroxisomal membrane [26], DHAP-AT activity was found exclusively in the 100000 $g_{av.}$ pellet. The bulk of the palmitoyl-CoA and cerotoyl-CoA synthetase activities ($\geq$ 96%) were recovered in the 100000 $g_{av.}$ pellet as well, with only a little activity being detectable in the supernatant. These data suggest that the cerotoyl-CoA synthetase activity, like the palmitoyl-CoA synthetase activity [4], is associated with the peroxisomal membrane.

Transverse-plane topographical localization of the peroxisomal palmitoyl-CoA and cerotoyl-CoA synthetases

The topographical localization of the catalytic site (or essential domains) of palmitoyl-CoA synthetase and cerotoyl-CoA synthetase in the transverse plane of the peroxisomal membranes was studied in intact and sonicated peroxisomes by treatment with trypsin. In order to interpret the data of the transverse topographical studies, it is of great importance to check for the integrity of the peroxisomal membranes. In earlier studies the decrease in latency of catalase was used as an index for structural damage of peroxisomes [4,18]. Baudhuin [27], however, showed that repeated freeze–thawing of peroxisomes, which should lead to extensive and substantial damage of the membrane of the organelle, resulted in only a limited loss of catalase latency. We therefore decided not to use catalase latency as a parameter for peroxisomal integrity. Instead, we used the sensitivity of D-amino acid oxidase, L-α-hydroxy-acid oxidase and DHAP-AT activities to trypsin treatment as a marker for membrane integrity in the present study. D-Amino acid oxidase and L-α-hydroxy-acid oxidase are soluble enzymes located in the matrix of peroxisomes, as shown by Yokota *et al.* [28] in immuno-electron microscopical studies. DHAP-AT is an integral membrane protein whose active site is located exclusively at the inner surface of the peroxisomal membrane [29].

Figs. 1(*a*) and 1(*b*) demonstrate that the peroxisomes used in the transverse topographical studies were impermeable to trypsin. This is indicated by the fact that treatment with trypsin had little, if any, effect on the activities of the matrix enzymes L-α-hydroxy-acid oxidase and D-amino acid oxidase. In sonicated peroxisomes, however, trypsin treatment led to decreases of 95% in the activity of L-α-hydroxy-acid oxidase (Fig. 1*a*) and 85% in the activity of D-amino acid oxidase (Fig. 1*b*). The activity of DHAP-AT, which is a membrane-bound enzyme like the acyl-CoA synthetases, decreased by about 25% in intact peroxisomes at the lowest trypsin concentration (1 μg/mg of protein) and remained constant at higher concentrations of trypsin up to 1 mg of trypsin/mg of protein (Fig. 1*c*). In sonicated peroxisomes the DHAP-AT activity was maximally inactivated (by about 80%) at 1 mg of trypsin/mg of protein. Hardeman & van den Bosch [29] showed complete inactivation of DHAP-AT in sonicated peroxisomes at a trypsin concentration of approx. 1 mg of trypsin/mg of protein. The reason for this difference is not clear. Nevertheless, our results show that at least 75% of the isolated peroxisomes were not permeable to trypsin.

Fig. 1(*d*) shows the effect of trypsin treatment on the activity of palmitoyl-CoA synthetase using the same batches of intact and sonicated peroxisomes as in the experiments displayed in Figs. 1(*a*)–1(*c*). A 20% stimulation of palmitoyl-CoA synthetase activity was consistently observed at low trypsin concentrations (1 μg/mg of protein) in intact peroxisomes. This stimulation was absent when sonicated peroxisomes were incubated with trypsin (Fig. 1*d*). A 10–20% stimulation was also observed when low concentrations of proteinase K or Pronase were used (results not shown). The rapid inactivation of palmitoyl-CoA synthetase activity in both sonicated and intact peroxisomes is in accordance with previous results demonstrating that the active site for palmitoyl-CoA synthetase is on the cytoplasmic face of the peroxisomal membrane ([4]; see also [18]). Fig. 1(*e*) shows that trypsin treatment inactivated cerotoyl-CoA synthetase activity to the same extent irrespective of whether intact or sonicated peroxisomes were studied. This result demonstrates that the enzymic site for cerotoyl-CoA synthetase, like that for palmitoyl-CoA synthetase, is on the cytoplasmic surface of peroxisomes.

DISCUSSION

The enzymic site for palmitoyl-CoA synthetase has been determined to be on the cytoplasmic surface of rat liver microsomes [30], peroxisomes [4] and the mitochondrial outer membrane [5]. In all three subcellular fractions the active site, or essential domains, are accessible to hydrolysis by non-specific proteinases [4,5,30]. With regard to the very-long-chain fatty

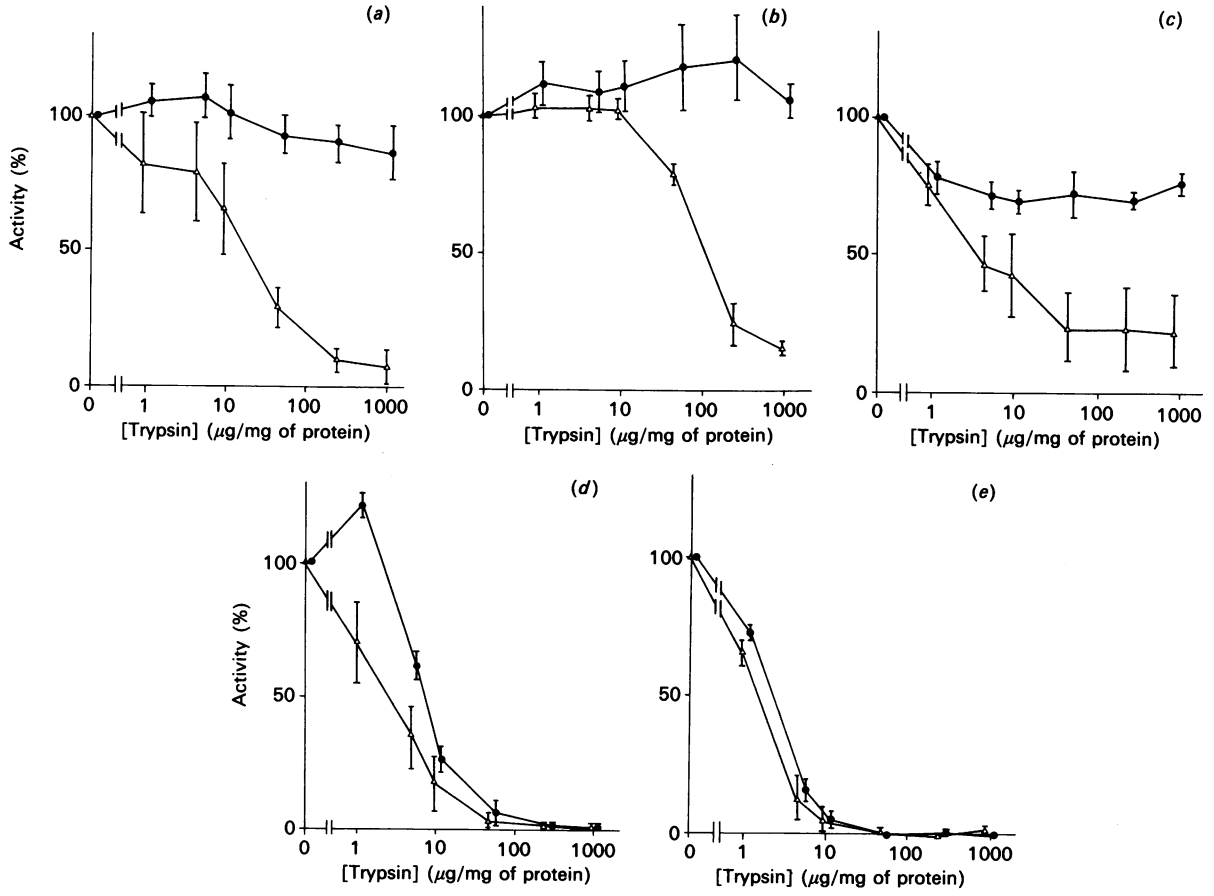

Fig. 1. Effect of trypsin on enzyme activities in purified peroxisomes

Portions of peroxisomes, suspended in buffer A (see the Materials and methods section) at a protein concentration of 1 mg/ml, were treated with trypsin (10 min, 25 °C) at the indicated concentrations. The peroxisomes were either sonicated (3 × 15 s at 80 W) (△) before incubation with trypsin or were kept intact (without sonication) (●). The enzymes assayed were: (*a*) L-α-hydroxy-acid oxidase, (*b*) D-amino acid oxidase, (*c*) acyl-CoA:dihydroxyacetone phosphate acyltransferase, (*d*) palmitoyl-CoA synthetase, and (*e*) cerotoyl-CoA synthetase. Palmitoyl-CoA and cerotoyl-CoA synthetase activities were assayed within 1 h of trypsin treatment. The peroxisomes were stored at −80 °C and the other enzyme activities were measured within 1 week. Enzyme activities in the absence of trypsin were expressed as 100%. The results are the means ± S.E.M. of 3–4 separate experiments. Specific activities of palmitoyl-CoA synthetase and cerotoyl-CoA synthetase were 52.6 ± 1.6 and 0.65 ± 0.08 nmol/min per mg of protein respectively. The specific activities of the other enzymes were: L-α-hydroxy-acid oxidase, 28.5 ± 3.5 nmol/min per mg of protein; D-amino acid oxidase, 48.1 ± 12.6 nmol/min per mg of protein; acyl-CoA:dihydroxyacetone phosphate acyltransferase, 0.87 ± 0.06 nmol/min per mg of protein.

acyl-CoA synthetase activity (using lignoceric acid as substrate), Singh *et al.* [7] have shown that in microsomal membranes the enzymic site is located at the cytoplasmic surface. Our results demonstrate that the catalytic site of the peroxisomal cerotoyl-CoA synthetase, like that of palmitoyl-CoA synthetase [4], is on the cytosolic side of the peroxisomal membrane. Incubation of intact peroxisomes with trypsin completely inactivated both palmitoyl-CoA synthetase and cerotoyl-CoA synthetase activities. On the other hand, trypsin treatment had no effect on D-amino acid oxidase, L-α-hydroxy-acid oxidase and DHAP-AT activities. The latter enzyme activities were severely (DHAP-AT) or almost completely (L-α-hydroxy-acid oxidase and D-amino acid oxidase) inactivated by trypsin in disrupted peroxisomes. In contrast, Lazo *et al.* [18] reported that the active site of lignoceroyl-CoA synthetase was on the luminal side of peroxisomes. In their study, trypsin treatment (50 μg/mg of protein, incubation for 30 min at 37 °C) had only a small effect on lignoceroyl-CoA synthetase activity in intact peroxisomes. Lignoceroyl-CoA synthetase activity was only inhibited if peroxisomal integrity was disrupted with Triton X-100. Our results, however, demonstrate complete inhibition of cerotoyl-CoA synthetase (50 μg of trypsin/mg of protein, incubation for 10 min at 25 °C) not only in disrupted but also in intact peroxisomes. The reason for this discrepancy is unclear (see below). Furthermore, Lazo *et al.* [18] identified a 'latent pool' of lignoceroyl-CoA synthetase activity by using different techniques to disrupt the peroxisomal membrane. However, the extent to which lignoceroyl-CoA synthetase activity increased with these various techniques differed markedly; the activity increased by about 70% after 60 s of sonication, by 50% after digitonin permeabilization, and by as much as 200% after disruption of peroxisomes with octyl glucoside [18]. These findings are not easy to reconcile with the general assumption, supported by the results of Baudhuin [27] and van Veldhoven *et al.* [31], that rat liver peroxisomes, when isolated, are permeable to low-molecular-mass components. In our studies we were not able to detect a 'latent pool' of cerotoyl-CoA synthetase activity. Upon sonication, the free catalase activity increased about 5-fold, whereas the cerotoyl-CoA synthetase activity remained the same (W. Lageweg, J. M. Tager & R. J. A. Wanders, unpublished work). Sonication of trypsin-pretreated intact peroxisomes also did not result in an increase in cerotoyl-CoA synthetase activity (results not shown).

A major difference between our experimental set-up and that

of Lazo *et al.* [18] is that we have systematically tried to avoid the use of detergents to break up the peroxisomal membrane, whereas Lazo *et al.* [18] used Triton X-100 in various experiments as well as in the assay medium. Our preference for using ultrasonic disruption rather than detergents to break up the peroxisomal membrane was inspired by earlier work of Mannaerts *et al.* [4], who showed that the 4-fold stimulation of palmitoyl-CoA synthetase activity caused by Triton X-100 observed by Krisans *et al.* [32] (which led the latter authors to conclude that palmitoyl-CoA synthetase was located at the luminal site of peroxisomes) was related to the physical state of the palmitate rather than to the latency of the enzyme. In this respect, it is worthwhile to mention that 0.025% (w/v) Triton X-100 caused a 4-fold stimulation of peroxisomal cerotoyl-CoA synthetase activity [33]. Furthermore, we [33] and others [11] have shown that detergents such as Triton X-100 affect the two synthetase activities differently. Thus some of the results presented by Lazo *et al.* [18] may have been caused or influenced by the Triton X-100 used in the experiments.

Another major difference between our results and those of Lazo *et al.* [18] concerns the specific activities of palmitoyl-CoA synthetase and cerotoyl-CoA synthetase. Indeed, the specific activities reported by Lazo *et al.* [18] for peroxisomal palmitoyl-CoA and cerotoyl-CoA synthetase (0.29 ± 0.08 and 0.029 ± 0.01 nmol/min per mg of protein for palmitic acid and lignoceric acid activation respectively) are about 200-fold and about 25-fold lower respectively than those reported in this study (52.6 ± 1.6 and 0.65 ± 0.08 nmol/min per mg of protein for palmitic acid and cerotic acid activation respectively). The values we have found clearly resemble those of Singh & Poulos [11], who measured values of 29.0 and 1.29 nmol/min per mg of protein for stearic acid and lignoceric acid activation respectively. Accordingly, the significance of the results reported by Lazo *et al.* [18] can be questioned.

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