Evidence that peroxisomal acyl-CoA synthetase is located at the cytoplasmic side of the peroxisomal membrane

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1. Subfractionation by isopycnic density-gradient centrifugation in self-generating Percoll gradients of peroxisome-rich fractions prepared by differential centrifugation confirmed the presence of acyl-CoA synthetase in peroxisomes. Peroxisomes did not contain nicotinamide or adenine nucleotides other than CoA. 2. The gradient fractions most enriched in peroxisomes were pooled and the peroxisomes sedimented by centrifugation, resulting in a 50-fold-purified peroxisomal preparation as revealed by marker enzyme analysis. 3. Palmitate oxidation by intact purified peroxisomes was CoA-dependent, whereas palmitoyl-CoA oxidation was not, demonstrating that the peroxisomal CoA was available for the thiolase reaction, located in the peroxisomal matrix, but not for acyl-CoA synthetase. This suggests that the latter enzyme is located at the cytoplasmic side of the peroxisomal membrane. 4. Additional evidence for this location of peroxisomal acyl-CoA synthetase was as follows. Mechanical disruption of purified peroxisomes resulted in the release of catalase from the broken organelles, but not of acyl-CoA synthetase, indicating that the enzyme was membrane-bound. Acyl-CoA synthetase was not latent, despite the fact that at least one of its substrates appears to have a limited membrane permeability, as evidenced by the presence of CoA in purified peroxisomes. Finally, Pronase, a proteinase that does not penetrate the peroxisomal membrane, almost completely inactivated the acyl-CoA synthetase of intact peroxisomes.

We have previously demonstrated that rat liver peroxisomes possess their own CoA pool (Van Broekhoven *et al.*, 1981). This CoA appears to be available for the thiolase reaction during peroxisomal β -oxidation of fatty acids, but not for the activation of fatty acids by the peroxisomal acyl-CoA synthetase. A possible explanation is that, unlike the enzymes of the β -oxidation sequence (Appelkvist & Dallner, 1980; Hüttinger *et al.*, 1980), the acyl-CoA synthetase is not located in the peroxisomal matrix, but at the cytoplasmic aspect of the peroxisomal membrane. The data presented below indicate that this is the case.

During our studies on the localization of the acyl-CoA synthetase, we have also looked for the presence in peroxisomes of cofactors for fatty acid activation and oxidation other than CoA, namely adenine and nicotinamide nucleotides. [Throughout and text the term 'adenine nucleotides' refers to the sum of AMP, ADP and ATP, and excludes CoA.] No indication for their presence was found.

Experimental

Animals

Male Wistar rats weighing 200 g were kept on a regular chow diet containing 0.3% clofibrate for 2 weeks. Clofibrate, a hypolipidaemic agent, causes the proliferation of peroxisomes in rodent liver and enhances the capacity of the peroxisomal fatty acid oxidation system severalfold (Lazarow & de Duve, 1976).

Cell fractionation and purification of peroxisomes

Liver homogenates (25%, w/v) prepared in 0.25 M-sucrose / 5 mM-dithiothreitol / 0.1% ethanol (homogenization solution) were fractionated by differential centrifugation as described previously (Mannaerts *et al.*, 1979). The λ -fraction, which is mostly enriched in lysosomes and peroxisomes (Leighton *et al.*, 1968), was subfractionated by centrifugation in self-generating Percoll gradients (Osmundsen & Neat, 1979). An iso-osmotic stock

solution of Percoll (Pharmacia) was made by mixing 9 vol. of Percoll with 1 vol. of 2.5 M-sucrose, and this stock solution was diluted 3.3-fold with homogenization solution made 2 mM in Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.2, and 1 mM in EDTA. The diluted Percoll solution will be designated hereafter as 30% (iso-osmotic) Percoll.

A sample (2 ml) of a λ -fraction in homogenization solution containing peroxisomes from 8 g of liver was loaded on top of 32 ml of 30% Percoll and centrifuged at 4°C for 30 min at 60 000 g in a MSE 8 × 50 ml fixed-angle rotor. The gradient was collected in 12 fractions of equal volume, starting from the bottom. Fractions 6–11, containing 80–90% of the catalase (peroxisomes) and approx. 50% of the glucose 6-phosphatase (microsomal fraction) of the gradient, were pooled, diluted 5-fold with homogenization solution to lower the Percoll concentration, and the peroxisomes were sedimented under the same centrifugation conditions as the original λ -fraction. This centrifugation step resulted in the recovery in a loosely packed pellet of 60–70% of the peroxisomes present in the pooled fractions, whereas only 10% of the contaminating microsomal fraction co-sedimented. The supernatant was discarded and the pellet resuspended in homogenization solution to a final protein concentration of approx. 2 mg/ml (purified peroxisomes).

Enzyme assays

Marker enzymes and protein were determined as described previously (Mannaerts *et al.*, 1979; Debeer *et al.*, 1979; Thomas *et al.*, 1980). Catalase, a marker enzyme for the peroxisomal matrix that shows latency, was assayed by the method of Baudhuin *et al.* (1964). Total catalase activity was measured after pretreatment of the enzyme source with 10 mg of Triton X-100/ml for 2 min to disrupt the peroxisomal membranes. Free catalase activity refers to the activity measured in 0.25 M-sucrose in the absence of detergent. Free catalase expressed as the percentage of total catalase found in a particular preparation was taken as an index for the structural integrity of the peroxisomes of that preparation.

Approximately half of the catalase in liver homogenates is soluble and can be measured as free catalase. It is still a matter of debate whether part of this catalase is truly soluble in the intact cell or whether it is entirely released from peroxisomes during homogenization and/or centrifugation. Electron micrographs of peroxisomes present in λ -fractions (Leighton *et al.*, 1975) or in our purified peroxisomal preparation (results not shown) show significant matrix extraction. Nevertheless, the organelles are surrounded by a continuous and apparently intact membrane, and catalase present in these fractions is latent. Assuming that all catalase

was originally present in peroxisomes, it could be calculated that our purified peroxisomes had lost approx. 60% of their matrix components, compared with 50% matrix extraction in peroxisomes of the original λ -fractions.

Because of the presence of extraperoxisomal catalase, regardless of its origin, it is necessary to use the total sedimentable catalase activity of the liver homogenates in calculations of the peroxisomal yield of purified preparations (see Table 2).

The distribution of acyl-CoA synthetase in the Percoll gradients was measured by the method of Polokoff & Bell (1975). In experiments with purified peroxisomes acyl-CoA synthetase was measured by the method of Krisans *et al.* (1980), modified as follows. The reaction mixture consisted of 150 mM-Tris/HCl, pH 7.4, 8 mM-MgCl₂, 2.5 mM-ATP, 0.6 mM-CoA, 3.7 mM-dithiothreitol, 0.3 mM-[1-¹⁴C]-palmitate (sp. radioactivity 1 μ Ci/ μ mol), 130 mM-sucrose, 0.05% ethanol, 0.05% Triton X-100, and contained approx. 15 μ g of peroxisomal protein/ml. To study the latency of acyl-CoA synthetase, the enzyme was measured in the above assay mixture in the presence and absence of Triton and in the presence of various amounts of albumin as indicated in Fig. 3. In the absence of detergent and in the presence of sufficient albumin to counteract the detergent effect of the palmitate, the assay mixture preserved the structural integrity of the peroxisomes, as evidenced by measurements of catalase latency.

Measurements of adenine and nicotinamide nucleotides

Adenine nucleotides (sum of ATP plus ADP plus AMP) (Lust *et al.*, 1981) and nicotinamide nucleotides (sum of NAD⁺ plus NADH) (Passonneau & Lowry, 1970) were determined by enzymic cycling methods. The concentration of adenine nucleotides found after cell fractionation was low in comparison with values obtained previously in freeze-clamped livers (Debeer *et al.*, 1974). Since the values of freshly prepared homogenates were equally low, the loss of adenine nucleotides occurred mainly during the initial homogenization period.

Experiments on purified peroxisomes

Fatty acid oxidation. Peroxisomal fatty acid oxidation was measured as the generation of acid-soluble radioactivity from [1-¹⁴C]palmitate or [1-¹⁴C]palmitoyl-CoA at a palmitate or palmitoyl-CoA:albumin molar ratio of 1.67 (Mannaerts *et al.*, 1979). The assay mixtures contained approx. 150 μ g of peroxisomal protein/ml.

Mechanical disruption. A suspension of purified peroxisomes in homogenization solution (1 mg of protein/ml) was homogenized with an Ultra-Turrax homogenizer in ice for various times. The treated

organelles were centrifuged for 45 min at 100 000g. Catalase and acyl-CoA synthetase activities were measured in the supernatants and pellets.

Pronase treatment. A suspension of purified peroxisomes in homogenization solution (2mg of protein/ml) was incubated at 30°C for various times with 1.85 or 3.7 µg of Pronase (from *Streptomyces griseus*; Boehringer)/mg of peroxisomal protein. At the end of the incubation the mixtures were diluted 15-fold with ice-cold homogenization solution to quench the proteolytic activity, and acyl-CoA synthetase and free and total catalase were measured.

Results

Distribution of acyl-CoA synthetase, adenine and nicotinamide nucleotides after equilibrium density centrifugation of peroxisome-rich subcellular fractions

λ -Fractions, which are mostly enriched in lysosomes and peroxisomes (Leighton *et al.*, 1968), were subfractionated by centrifugation in self-generating Percoll gradients (Fig. 1). Table 1 shows the activities of marker enzymes in the starting homogenates, the composition of the λ -fractions and the recoveries of marker enzymes in the gradients.

Mitochondria (glutamate dehydrogenase) and lysosomes (acid phosphatase) were collected with the bottom fractions of the gradient, whereas peroxisomes (catalase) were found in the top fractions. Microsomal fractions (glucose 6-phosphatase) displayed a bimodal distribution.

Krisans *et al.* (1980) demonstrated the presence of acyl-CoA synthetase activity not only in the microsomal fraction and mitochondria, but also in peroxisomes. Our results confirm these findings. Approximately half of the acyl-CoA synthetase activity was found in the bottom fractions containing the mitochondria and part of the microsomal fraction, while the other half was present in the top fractions containing the peroxisomes and the remaining microsomal fraction (Fig. 1). Comparison of the distributions of acyl-CoA synthetase, catalase and glucose 6-phosphatase in these top fractions clearly shows that acyl-CoA synthetase activity is associated not only with the microsomal fraction, but with the peroxisomes as well.

The distribution of adenine nucleotides and of NAD⁺ plus NADH closely followed that of the mitochondria, and no indication was found for their presence in peroxisomes. These findings strongly suggest that, except for CoA (Van Broekhoven *et al.*, 1981), peroxisomes do not possess a separate pool of these nucleotides.

Fig. 1. Subfractionation of a peroxisome-enriched subcellular fraction (λ) by equilibrium density centrifugation in a self-generating Percoll gradient

The composition of the λ -fraction and recoveries of marker enzymes in the gradients are shown in Table 1. Bottom to top fractions are drawn from left to right on the abscissa. Results are normalized as described by Leighton *et al.* (1968). Abbreviations: GDH, glutamate dehydrogenase; G6Pase, glucose 6-phosphatase; acid Phase, acid phosphatase; adenine nucleotides = sum of ATP + ADP + AMP; nicotinamide nucleotides = sum of NAD⁺ plus NADH.

Table 1. *Absolute activities of marker enzymes in whole homogenate, composition of λ -fractions and recoveries of marker enzymes after equilibrium density centrifugation of λ -fractions in 30% Percoll gradients*

Gradients were those of Fig. 1. Results are expressed as means $\pm$ S.E.M. for the numbers of experiments indicated in parentheses. A unit of activity is defined as 1 μ mol of substrate transformed/min except for catalase, where the unit is that defined by Baudhuin *et al.* (1964).

	Absolute activity (units/g of liver)	λ -fraction (% of homogenate)	Recovery from gradient (%)
Protein (5)	205 $\pm$ 14†	10.3 $\pm$ 0.6	109 $\pm$ 4
Catalase (5)	72 $\pm$ 3.7	27.9 $\pm$ 2.1	98 $\pm$ 3
Glutamate dehydrogenase (5)	201 $\pm$ 21	16.2 $\pm$ 1.0	105 $\pm$ 4
Glucose 6-phosphatase (5)	22.2 $\pm$ 0.9	11.3 $\pm$ 1.4	118 $\pm$ 7
Acid phosphatase (1)	12.8	49.1	82
Acyl-CoA synthetase (2)	2.4	17.1	108
Adenine nucleotides (3)	1.6 $\pm$ 0.6‡	7.1 $\pm$ 0.3	116 $\pm$ 7
Nicotinamide nucleotides (1)*	0.71‡	13.5	100

* Nicotinamide nucleotides were also determined on three 25% Percoll gradients. Like 30% gradients these gradients only indicated the presence of nicotinamide nucleotides in mitochondria.

† mg/g.

‡ μ mol/g.

Table 2. *Comparison of the composition of a λ -fraction and a purified peroxisomal pellet*

Results are expressed as percentages of total homogenate activity and relative specific activity. Relative specific activity is defined as the percentage of total activity present in the λ -fraction or peroxisomal pellet divided by the corresponding percentage of total protein. Results are means $\pm$ S.E.M. for the numbers of experiments indicated in parentheses.

	λ -fraction		Purified peroxisomal pellet	
	% of total homogenate	Relative sp. activity	% of total homogenate	Relative sp. activity
Protein (4)	11.16 $\pm$ 2.1	—	0.48 $\pm$ 0.09	—
Catalase (3)	23.1 $\pm$ 1.6	2.1	12.4 $\pm$ 0.75	25.8
Sedimentable catalase (3)	48.8 $\pm$ 3.2	4.4	26.2 $\pm$ 1.66	54.6
Urate oxidase (1)	45.6	4.1	22.8	47.5
Glutamate dehydrogenase (2)	23.3	2.1	0.33	0.69
Glucose 6-phosphatase (4)	10.4 $\pm$ 2.1	0.9	0.53 $\pm$ 0.06	1.10
Acid phosphatase (2)	45.8	4.1	1.93	4.0
Acyl-CoA synthetase (2)	17.2	1.5	2.31	4.8

Preparation of purified peroxisomes

Gradient fractions 6–11, containing 80–90% of the catalase activity, were pooled, diluted with iso-osmotic sucrose, and the peroxisomes were sedimented by centrifugation. Table 2 shows the composition of the resulting pellet, which is compared with the corresponding λ -fractions that were loaded on the gradients. The marker-enzyme content of the pellet reveals that it is a 50-fold purified peroxisomal preparation with high yield.

Krisans *et al.* (1980) calculated that peroxisomes, mitochondria and microsomal fractions from livers of rats treated with clofibrate contain 12, 13 and 75% respectively of the liver's total palmitoyl-

CoA synthetase activity. From these data and the marker-enzyme content of the peroxisomal pellet, it can be calculated that 88% of the palmitoyl-CoA synthetase activity of this pellet could be due to the peroxisomes themselves; 1% would be mitochondrial and 11% microsomal. The corresponding values for a λ -fraction are 35, 18 and 47%.

Fatty acid oxidation by purified peroxisomes

Purified peroxisomes were used to compare the oxidation of palmitoyl-CoA and palmitate. In agreement with earlier experiments (Mannaerts *et al.*, 1979), and with the existence of a separate CoA pool in peroxisomes, palmitoyl-CoA oxidation was not stimulated by CoA. However, the rate of

palmitate oxidation, which in the presence of CoA equalled that of palmitoyl-CoA, was suppressed by more than 90% in the absence of the cofactor (results not shown). Since the calculated intra-peroxisomal CoA concentration (0.9 mM) (Van Broekhoven *et al.*, 1981) is many-fold higher than

the exogenous CoA concentration needed for half-maximal oxidation of palmitate (0.02 mM) (Shindo & Hashimoto, 1978), these results indicate that the peroxisomal CoA is available for the thiolase reaction during the β -oxidation of fatty acids, but not for their activation. A possible explanation is that, unlike the β -oxidation enzymes, the acyl-CoA synthetase is not located in the peroxisomal matrix, but is associated with the cytoplasmic side of the peroxisomal membranes.

Since in the presence of CoA and ATP, palmitate and palmitoyl-CoA were oxidized at almost equal rates (see above), the data also demonstrate that the synthetase activity is sufficient to allow near-maximal oxidation rates. From enzyme-activity measurements Krisans *et al.* (1980) reached a similar conclusion.

Localization of the peroxisomal acyl-CoA synthetase

In a first experimental approach we sought confirmation for a membrane localization of the acyl-CoA synthetase. Therefore a suspension of purified peroxisomes was subjected to mechanical disruption by Ultra-Turrax treatment for various times. The treated organelles were then sedimented by centrifugation. As shown in Fig. 2, after 20 s of mechanical disruption more than 80% of the catalase was found in the supernatant, demonstrating that most of the matrix enzymes had been released. In agreement with a membrane localization, the acyl-CoA synthetase activity sedimented with the broken organelles.

A localization at the cytoplasmic side of the peroxisomal membrane implies that the acyl-CoA

Fig. 2. Effect of mechanical disruption on release of catalase and acyl-CoA synthetase from purified peroxisomes

A suspension of purified peroxisomes was disrupted by Ultra-Turrax treatment for various times and the treated organelles were sedimented by centrifugation. Enzyme release is expressed as the percentage of total enzyme activity that was recovered in the supernatant. Results are means for two experiments.

Fig. 3. Effect of Triton X-100 on acyl-CoA synthetase activity (a) and on latency of catalase (b) in purified peroxisomes. Acyl-CoA synthetase and free catalase were measured in purified peroxisomes versus increasing palmitate:albumin molar ratios (increasing unbound palmitate concentrations) in the presence (●) or absence (○) of 0.5 mg of Triton X-100/ml. ∞, Absence of albumin. Results are means for four experiments.

Fig. 4. Inactivation of acyl-CoA synthetase activity of purified peroxisomes by treatment with Pronase

Purified peroxisomes were incubated with Pronase for various times, diluted 15-fold to quench the proteolytic activity and acyl-Co synthetase was measured (a). At the end of the incubations free catalase activity was also measured (b). ●, Absence of Pronase; ■, 1.85 µg of Pronase/mg of peroxisomal protein; ▲, 3.70 µg of Pronase/mg of protein. Results are means for two experiments.

synthetase cannot show latency. However, Krisans *et al.* (1980) found that peroxisomal acyl-CoA synthetase activity was stimulated by Triton X-100 and concluded that the enzyme was latent. Fig. 3 shows that, although Triton X-100 completely abolished the latency of catalase (Fig. 3b), the detergent did not stimulate the acyl-CoA synthetase when the enzyme was assayed in the presence of albumin (Fig. 3a). In the absence of albumin, a condition used by Krisans *et al.* (1980), Triton caused a 4-fold stimulation. However, when portions of the substrate mixtures for the synthetase assay were counted for radioactivity, it was observed that in the absence of albumin and Triton only 23% of the radioactivity was recovered as compared with the substrate mixtures containing albumin and/or Triton. In the absence of albumin, Triton may prevent the adsorption of unbound palmitate on glass or plasticware (Suzue & Marcel, 1972) or may influence the distribution of unbound palmitate in the solution. Pande (1981) has described that palmitoylcarnitine in solution largely leaves the bulk phase to segregate at the water/air and water/apolar interphases. A similar phenomenon appears to occur in palmitoyl-CoA solutions (Barden & Cleland, 1969). Thus the stimulation by Triton of the acyl-CoA synthetase in the absence of albumin appears to be related to the physical state of the palmitate rather than to the latency of the enzyme. Moreover, in the absence of albumin catalase latency was already completely abolished by the detergent effects of palmitate itself, so that Triton did not have any additional effect.

Finally, treatment with Pronase, a proteinase that cannot penetrate the peroxisomal membrane, almost completely inactivated the acyl-CoA synthetase activity (Fig. 4a) without concomitant structural damage to the peroxisomes, as shown by the small decrease in catalase latency (Fig. 4b). These results indicate that the catalytic site, or at least essential domains, of the acyl-CoA synthetase are located at the cytoplasmic aspect of the peroxisomal membrane. Incubation alone (30°C) in the absence of Pronase also resulted in a partial inactivation of the enzyme, which appeared to stabilize after a 10 min incubation period. The reason for this inactivation is unknown.

Discussion

Several lines of evidence indicate that the peroxisomal acyl-CoA synthetase is located at the cytoplasmic aspect of the peroxisomal membrane. (1) Mechanical disruption of peroxisomes results in the release of matrix enzymes (catalase), but not of acyl-CoA synthetase, indicating that the latter enzyme is membrane-bound. (2) Peroxisomal CoA appears to be available for the thiolase reaction, located in the peroxisomal matrix, but not for the acyl-CoA synthetase. (3) Acyl-CoA synthetase is not latent, despite the fact that the membrane permeability for at least one of its substrates (CoA) seems to be limited, as evidenced by the existence of a CoA pool in purified peroxisomes. (4) Acyl-CoA synthetase is almost completely inactivated by proteinase treatment of intact peroxisomes.

Although our purified peroxisomes were still contaminated to a minor extent with microsomal fraction, which contain most of the liver's acyl-CoA synthetase activity (Krisans *et al.*, 1980), it could be calculated that the contribution of the microsomal acyl-CoA synthetase was too small (11%) to invalidate or even to distort our conclusions on the localization of the peroxisomal enzyme.

The present experiments were performed on livers of rats pretreated with clofibrate, since this drug causes the proliferation of peroxisomes and also enhances total as well as peroxisomal acyl-CoA synthetase activity 2–3-fold (Krisans *et al.*, 1980). However, the proliferated peroxisomes appear to differ from normal peroxisomes. Although the capacity of the peroxisomal β -oxidation system increases roughly to the same extent as the number and volume fraction of the peroxisomes, catalase activity increases only slightly, and the activities of other enzymes such as D-amino acid oxidase, L- α -hydroxy acid oxidase and urate oxidase may even decrease (Leighton *et al.*, 1975). Thus, strictly, our conclusions only apply to proliferated peroxisomes. It seems highly unlikely, however, that the membrane localization of acyl-CoA synthetase would differ in normal and proliferated organelles.

The localization of the acyl-CoA synthetase at the external surface of the peroxisomal membrane implies that not the fatty acid but its CoA ester is the species that has to cross the membrane. A molecular mechanism is not known, but Appelkvist & Dallner (1980) provided evidence that a carrier may be involved. Since the inward transport of acyl-CoA molecules would result in the net accumulation of CoA within the peroxisome, it is tempting to postulate a carrier that might exchange external long-chain acyl-CoA against internal short-chain acyl-CoA molecules, which form the terminal stage of peroxisomal β -oxidation (Lazarow, 1978), or perhaps against free CoA. Such a carrier mechanism would be analogous to the mitochondrial acyl-carnitine : carnitine exchange.

The absence of nicotinamide nucleotides from purified peroxisomes and the finding that NAD⁺ strongly stimulates the β -oxidation of fatty acids by intact peroxisomes (Mannaerts *et al.*, 1979) indicate that nicotinamide nucleotides can cross the peroxisomal membrane, possibly by a carrier-mediated mechanism. If this is the case, NADH produced during β -oxidation does not need to be re-oxidized within the peroxisome, but the peroxisomal redox state could be governed by the redox state of the cytosolic nicotinamide nucleotides.

The absence of adenine nucleotides from peroxisomes may indicate that they too can penetrate the

peroxisomal membrane. However, since the peroxisomal acyl-CoA synthetase appears to be located at the cytoplasmic side of the peroxisomal membrane, there seems to be no need for the presence of adenine nucleotides in the peroxisomal matrix.

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