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The presence of acyl-CoA hydrolase in rat brown-adipose-tissue peroxisomes

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The subcellular distribution of acyl-CoA hydrolase was studied in rat brown adipose tissue, with special emphasis on possible peroxisomal localization. Subcellular fractionation by sucrose-density-gradient centrifugation, followed by measurement of short-chain (propionyl-CoA) acyl-CoA hydrolase in the presence of NADH, resulted in two peaks of activity in the gradient: one peak corresponded to the distribution of cytochrome oxidase (mitochondrial marker enzyme), and another peak of activity coincided with the peroxisomal marker enzyme catalase. The distribution of the NADH-inhibited short-chain hydrolase activity fully resembled that of cytochrome oxidase. The substrate-specificity curve of the peroxisomal acyl-CoA hydrolase activity indicated the presence of a single enzyme exhibiting a broad substrate specificity, with maximal activity towards fatty acids with chain lengths of 3–12 carbon atoms. The mitochondrial acyl-CoA hydrolase substrate specificity, in contrast, indicated the presence of at least two acyl-CoA hydrolases (of short- and medium-chain-length specificity). The peroxisomal acyl-CoA hydrolase activity was inhibited by CoA at low (μM) concentrations and by ATP at high concentrations (> 0.8 mM). In contrast with the mitochondrial short-chain hydrolase, the peroxisomal acyl-CoA hydrolase activity was not inhibited by NADH.

INTRODUCTION

Various acyl-CoA hydrolases (thioesterases) have been described in different mammalian tissues and micro-organisms. Although the physiological functions of these enzymes are not clear, the apparently ubiquitous distribution of this enzyme group suggests an important role in fatty acid metabolism. It has been shown in rat liver that palmitoyl-CoA hydrolase activity is induced under conditions of increased fatty acid influx into the liver, which also results in the induction of mitochondrial and peroxisomal fatty acid oxidation capacity (Berge *et al.*, 1984*a*; Berge & Aarsland, 1985).

Results obtained by subcellular fractionation and enzyme purifications have demonstrated that palmitoyl-CoA hydrolase activity is present in rat liver microsomes and mitochondria (Berge & Farstad, 1979), cytosol (Berge *et al.*, 1981; Miyazawa *et al.*, 1981) and peroxisomes (Osmundsen *et al.*, 1980; Berge *et al.*, 1984*b*). The peroxisomal acyl-CoA hydrolase activity was found to be most active with palmitoyl-CoA as substrate (Osmundsen *et al.*, 1980; Berge *et al.*, 1984*b*). In addition, an acetyl-CoA hydrolase activity has been characterized from rat liver cytosol (Prass *et al.*, 1980) and mitochondria (Snoswell & Tubbs, 1978; Grigat *et al.*, 1979).

Brown adipose tissue (BAT) is an extremely active fatty acid-oxidation tissue. In BAT, acetyl-CoA hydrolase activity (Bernson, 1976) (which was later shown to be due to a short-chain acyl-CoA hydrolase activity; Alexson & Nedergaard, 1988) and medium- and long-chain acyl-CoA hydrolase activities have been demonstrated (Alexson & Nedergaard, 1988; Berge *et al.*, 1979). These enzyme activities, which are mainly localized in the mitochondria, were found to be induced during cold-acclimation (Berge *et al.*, 1979; Alexson *et al.*, 1989), a physiological situation where hypertrophy of the tissue occurs (Nedergaard *et al.*, 1980). During this hypertrophy of BAT, peroxisomes and peroxisomal β-oxidation are also induced (Nedergaard *et al.*, 1980), indicating an increased metabolic importance of these organelles. The present paper describes subcellular-fractionation experiments which demonstrate that BAT peroxisomes contain an acyl-CoA hydrolase. The substrate specificities and effects of nucleotides indicate that peroxisomes contain an acyl-CoA hydrolase that differs from the mitochondrial hydrolases, with respect to both substrate specificity and regulation.

EXPERIMENTAL

Animals and tissue fractionation

Adult Sprague–Dawley rats (obtained from Eklunds, Stockholm, Sweden) or Syrian hamsters (*Mesocricetus auratus*) were housed individually at +5 °C (8 h light/16 h dark) for at least 3 weeks. The interscapular BAT from 10–35 animals was pooled and homogenized in 0.25 M-sucrose, containing 3 mM-imidazole (pH 7.4). Routinely, a light-mitochondrial fraction was prepared as described by Alexson & Cannon (1984). Alternatively, the post-nuclear supernatant was centrifuged at 8500 *g* for 10 min to remove the heavy mitochondria. The mitochondrial supernatant was then centrifuged at 27000 *g* for 20 min, to obtain the light-mitochondrial fraction.

Abbreviation used: BAT, brown adipose tissue.

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The light-mitochondrial fractions were layered on top of linear sucrose density gradients containing 3 mM-imidazole, pH 7.4, 1 mM-EDTA and 0.1% ethanol (range 36–56% sucrose), and centrifuged at 45000 rev./min for 2 h in a Beckman VTi 50 vertical rotor; 2 ml fractions were collected from the bottom of the tube.

Enzyme assays

Acyl-CoA hydrolase was measured spectrophotometrically as described by Alexson & Nedergaard (1988). When the effect of added CoA was investigated, a radioactive assay was used with [1-^{14}C]octanoyl-CoA as substrate as described by Alexson & Nedergaard (1988) [except that the incubation medium did not contain 5,5′-dithiobis(nitrobenzoic acid)]. Catalase (EC 1.11.1.6) (Baudhuin *et al.*, 1964) and cytochrome oxidase (EC 1.9.3.1) (Leighton *et al.*, 1968) were measured as described in the references. Protein was measured as described by Bradford (1976).

RESULTS

Subcellular distribution

In order to study whether peroxisomes contain acyl-CoA hydrolase, the distributions of short-, medium- and long-chain hydrolase activities were investigated after fractionation of BAT homogenates. The protein and enzyme distribution after fractionation of a post-nuclear supernatant into heavy-mitochondrial, light-mitochondrial and supernatant fractions is shown in Table 1. The low recovery of cytochrome oxidase (48%) during the differential centrifugation is probably due to underestimation of the enzyme activity in the heavy- and light-mitochondrial fractions (as indicated by the high recovery in the gradient fractions, 196%). However, the low recovery of cytochrome oxidase does not interfere with the interpretation of the hydrolase distribution, as the recoveries of these enzyme activities were acceptable (82–105% in all cases except for C_{16} acyl-CoA hydrolase in the gradient fractions, where the recovery was 62%). Short- and medium-chain hydrolase activities were mainly found in the heavy-mitochondrial fraction, whereas the light-mitochondrial and supernatant fractions contained only 8–17% of the total hydrolase activities. The long-chain hydrolase activity showed a bimodal distribution, with about 47% of the activity in the heavy-mitochondrial fraction and about 41% in the supernatant fraction. Although the amount of the hydrolase activities was clearly lower in the light-mitochondrial fraction, compared with the amount of cytochrome oxidase, it was at least similar in magnitude to the percentage of the total protein found in the fraction (12–16% hydrolase activity compared with 11% protein). As the light-mitochondrial fraction was contaminated by peroxisomes and microsomes, it is possible that some of these hydrolase activities were in fact localized in peroxisomes. In order to study this possibility, the light-mitochondrial fraction was further fractionated by sucrose-density-gradient centrifugation.

BAT peroxisomes (micro-peroxisomes) have a lower density (they band at a density of about 1.22 g of sucrose/ml) than rat liver peroxisomes, which have a density of about 1.24 g/ml in a sucrose density gradient (Leighton *et al.*, 1968). BAT peroxisomes are therefore not completely separated from the mitochondria after fractionation. The amount of mitochondria is very high in BAT (40–50% of the cellular protein; Nedergaard *et al.*, 1983), and the amount of peroxisomes is rather low ($\leq$ 1% of the cellular protein; S. E. H. Alexson, unpublished work). The marker enzymes (catalase for peroxisomes and cytochrome oxidase for mitochondria) overlapped to some extent in the gradient. The relatively small proportion of peroxisomal protein present in the gradient appears as a shoulder in the gradient protein distribution (Fig. 1*b*). The distribution of the hydrolases, especially short- and medium-chain hydrolases, was similar to the distribution of protein (Figs. 1*c*, 1*f*, and 1*h*). The fractional activity of the hydrolases was several-fold higher than that of cytochrome oxidase in the peroxisomal fractions. Although we obtained similar results in four other experiments with both rat and hamster BAT, which clearly indicates a peroxisomal localization of these hydrolase activities, this was not conclusive, owing to the overlapping of peroxisomal and mitochondrial marker activities.

We have recently characterized short- and medium-chain acyl-CoA hydrolase activities of hamster and rat BAT (Alexson & Nedergaard, 1988; Alexson *et al.*, 1989). From those experiments it was concluded that the short- and medium-chain hydrolases found in rat and hamster BAT mitochondria are inhibited by NADH. In the present study, we have used this phenomenon as a diagnostic tool to investigate a possible peroxisomal localization of acyl-CoA hydrolase. In the present experiments, 750 μM-NADH inhibited about 90% of the short-chain hydrolase activity in the heavy-mitochondrial fraction, but only about 81% of the hydrolase activity in the light-mitochondrial fraction (Table 2). Furthermore, the distribution of the short-chain hydrolase activity, measured in the presence of 750 μM-NADH, changed

Table 1. Distribution of marker enzymes and acyl-CoA hydrolase activities after differential centrifugation of rat BAT homogenate

A postnuclear supernatant was prepared from 10.2 g of BAT, homogenized in 106 ml of 0.25 M-sucrose/3 mM-imidazole, pH 7.4, by centrifugation at 600 *g*. The post-nuclear supernatant was further fractionated to prepare the heavy- and light-mitochondrial and supernatant fractions (see the Experimental section). The total amount/enzyme activity in the postnuclear-supernatant fraction was: protein, 869 mg; catalase, 82.2 units; cytochrome oxidase, 197.7 units; C_3 hydrolase, 32.1 μmol/min; C_9 hydrolase, 32.8 μmol/min; C_{16} hydrolase, 23.6 μmol/min.

Enzyme	Distribution (%)			Recovery (%)
	Heavy-mitochondrial fraction	Light-mitochondrial fraction	Supernatant	
Protein	33.5	11.2	55.2	77.3
Catalase	23.0	52.1	24.9	145
Cytochrome oxidase	65.6	19.1	12.1	48.3
C_3 hydrolase	76.1	15.7	8.3	83.5
C_9 hydrolase	70.5	12.8	16.7	94.9
C_{16} hydrolase	46.6	12.2	41.2	87.6

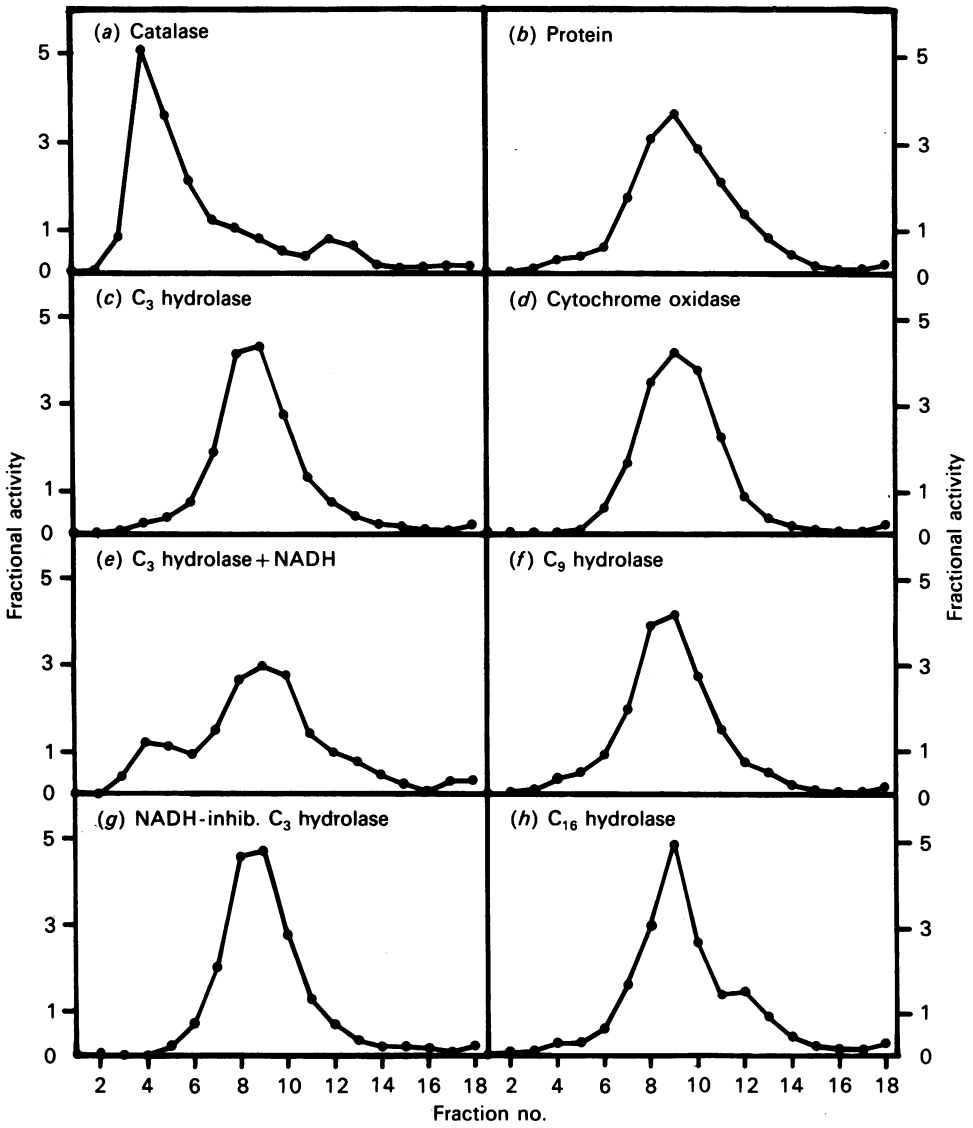

Fig. 1. Distribution of acyl-CoA hydrolase activities and marker enzymes after sucrose-density-gradient centrifugation of a light-mitochondrial fraction prepared from rat BAT

A light-mitochondrial fraction was layered on top of a sucrose density gradient and fractionated as described in the Experimental section. The enzymes (with recoveries) were: (*a*) catalase (68%), (*b*) protein (103%), (*c*) C_3-CoA hydrolase (89%), (*d*) cytochrome *c* oxidase (196%), (*e*) C_3-CoA hydrolase in the presence of 750 μM-NADH (105%), (*f*) C_9-CoA hydrolase (100%), (*g*) NADH-inhibitable C_3-CoA hydrolase (105%) and C_{16}-CoA hydrolase (62%). Fractional activity is the activity in each fraction divided by the total activity on the whole gradient and multiplied by the total number of fractions.

dramatically. Now only about 45% of the short-chain hydrolase activity (as compared with 76% in the absence of NADH) was found in the heavy-mitochondrial fraction, and the fraction of the total activity in the final supernatant fraction increased from 8 to 38% (Table 2). The distribution of the NADH-sensitive short-chain hydrolase, calculated as the difference between the activity measured in the absence and presence of NADH, showed about 83% of the activity in the heavy-mitochondrial fraction and only about 2% of the activity in the supernatant fraction.

When the distribution of NADH-insensitive (i.e. the activity remaining in the presence of NADH) short-chain hydrolase activity was investigated in the fractions obtained after sucrose-density-gradient centrifugation, it was found to be quite different from the total short-chain hydrolase distribution. Now two peaks of activity could clearly be observed, one peak corresponding to the mitochondria, but also another peak of activity corresponding to the peroxisomes (fractions 3–6, Fig. 1*e*). The short-chain hydrolase activity was not inhibited at all by NADH in fractions 3 and 4, whereas the inhibition was 38, 73, 83 and 86% in fractions 5–8 respectively. When the distribution of the NADH-sensitive short-chain hydrolase activity found in the gradient fractions was plotted, the distribution coincided with that of cytochrome oxidase (Fig. 1*g*). Both medium- and long-chain hydrolase activities showed similar distributions to the short-chain hydrolase, i.e. the main activity coinciding with the cytochrome oxidase distribution, but with a shoulder of activity in the region of the gradient where the peroxisomes were found (Figs. 1*f* and 1*h*).

Table 2. Distribution of NADH-sensitive and NADH-insensitive short-chain (C_3-CoA) hydrolase activities after differential centrifugation of a rat BAT postnuclear supernatant

The Table shows the distribution of short-chain acyl-CoA hydrolase activity, measured in the presence and absence of 750 μM-NADH, in the various fractions. The experiment and the different fractions are described in the legend to Table 1 and in the Experimental section.

Enzyme	Heavy-mitochondrial fraction	Light mitochondrial fraction	Supernatant	Recovery (%)
C_3 hydrolase (μmol/fraction)	20.4	4.2	2.2	83.5
C_3 hydrolase + 750 μM-NADH (μmol/fraction)	2.1	0.8	1.8	90.4
Inhibition by NADH (%)	89.6	81.4	17.7	–
NADH-sensitive C_3 hydrolase (% distribution)	82.6	15.5	1.9	82.2

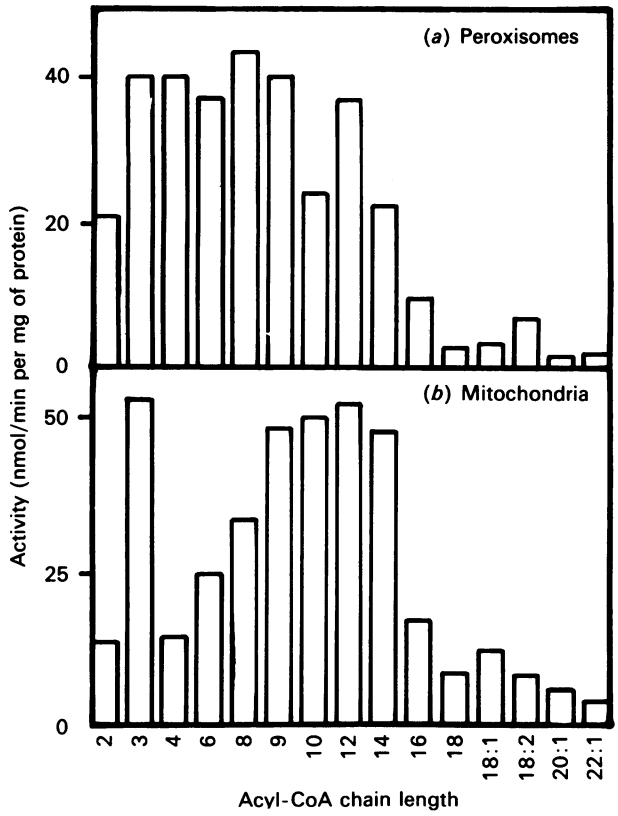

Fig. 2. Chain-length specificity of peroxisomal and mitochondrial acyl-CoA hydrolase activities

The substrate specificity was measured for (*a*) peroxisomal (fraction 4 from the sucrose-gradient centrifugation) and (*b*) mitochondria (fraction 8 from the sucrose-gradient centrifugation) acyl-CoA hydrolases. The activities were measured in the presence of 50 μM of the indicated acyl-CoAs.

Substrate specificity of the peroxisomal and mitochondrial acyl-CoA hydrolase activity

The substrate specificity was tested with acyl-CoAs with chain lengths of 2–22 carbon atoms. Fraction 4 from the gradient was used for the peroxisomal activity and fraction 8 for the mitochondrial activity. The peroxisomes in fraction 4 were calculated (based on the relative specific activity) to be purified about 100-fold. The substrate specificity of the peroxisomal hydrolase activity suggested the presence of a single hydrolase with broad substrate specificity for acyl-CoA, with a maximal activity towards C_3–C_{12} acyl-CoAs (Fig. 2*a*). The substrate-specificity curve obtained with the mitochondrial fraction demonstrated the presence of a short-chain hydrolase with maximal activity towards propionyl-CoA (C_3), and a medium-chain hydrolase with maximal activity towards C_9–C_{14} acyl-CoAs (Fig. 2*b*), in agreement with the previously reported specificity for mitochondrial hydrolase activity (Alexson *et al.*, 1989). The specific activity of the hydrolase activity towards medium-chain (C_9–C_{14}) acyl-CoAs was slightly higher in the mitochondria than in the peroxisomes (about 50 nmol/min per mg of protein in the mitochondria and 20–40 nmol/min per mg of protein in the peroxisomes). The specific activity of the mitochondrial hydrolase activity with stearoyl-, oleoyl, gadoleoyl- and erucoyl-CoA was about 2–4-fold higher than in peroxisomes, whereas the specific activities were similar with linoleoyl-CoA.

The activities of peroxisomal and mitochondrial acyl-CoA hydrolases were found to be very low with acetoacetyl-CoA or malonyl-CoA as substrates (Table 3).

Effect of nucleotides and ions on peroxisomal and mitochondrial acyl-CoA hydrolases

The effect of nucleotides and ions was tested on peroxisomal and mitochondrial acyl-CoA hydrolase activity. These results are summarized in Table 3. In agreement with earlier findings (Alexson *et al.*, 1989), NADH inhibited both mitochondrial short-chain (about 90% inhibition of C_3 acyl-CoA hydrolase activity) and medium-chain (about 40% inhibition of C_{10} acyl-CoA hydrolase activity) acyl-CoA hydrolase activity. The peroxisomal acyl-CoA hydrolase activity was found to be insensitive to NADH. Both the mitochondrial and the peroxisomal acyl-CoA hydrolases were inhibited by about 40% in the presence of 1.6 mM-ATP. Also, by the use of a radioactive assay, both peroxisomal and mitochondrial octanoyl-CoA hydrolase activity was found to be strongly inhibited by low (μM) concentrations of added CoA (Table 3). The approximate concentrations of added

Table 3. Effects of nucleotides and ions on the peroxisomal and mitochondrial acyl-CoA hydrolases

The effects of nucleotides and ions were tested on the acyl-CoA hydrolase activities found in the peroxisomal fraction (Fraction 5) and the mitochondrial fraction (Fraction 8) from the sucrose density gradient. Also the activities with acetoacetyl-CoA and malonyl-CoA were tested on these fractions. ND, not determined.

Additions	Activity (nmol/min per mg of protein)	
	Peroxisomes	Mitochondria
(*a*) With 50 μM-C_{10}-CoA		
None	46	50
+750 μM-NADH	46	30
+800 μM-ATP	39	ND
+1.6 mM-ATP	27	30
+100 μM-ADP	53	46
+500 μM-ADP	47	49
+2.5 mM-Ca^{2+}	40	57
+2.5 mM-Mg^{2+}	45	39
+500 μM-NADPH	45	ND
+800 μM-NAD^+	45	ND
(*b*) With 50 μM-[1-^{14}C]octanoyl-CoA		
None	54	45
+25 μM-CoA	32	19
+75 μM-CoA	14	12
(*c*) With 50 μM-C_3-CoA		
None	45	52
+750 μM-NADH	44	6
+100 μM-ADP	ND	49
50 μM-Acetoacetyl-CoA	16	8
50 μM-Malonyl-CoA	5	2

CoA causing 50% inhibition of peroxisomal and mitochondrial octanoyl-CoA hydrolase activities (at 50 μM C_8 acyl-CoA concentration) were 30 and 15 μM respectively. The effect of CoA was tested up to a concentration of 200 μM, which inhibited both peroxisomal and mitochondrial C_8 acyl-CoA hydrolase activities by about 87%. ADP, Ca^{2+} and Mg^{2+} were found to be without effect, or with only a slight effect, on both mitochondrial and peroxisomal acyl-CoA hydrolase activity. In addition, NAD^+ and NADPH had no effect on the peroxisomal acyl-CoA hydrolase activity.

DISCUSSION

These results suggest the presence of an acyl-CoA hydrolase activity in rat BAT peroxisomes. The localization of this hydrolase to peroxisomes is based on the distribution of NADH-sensitive and NADH-insensitive acyl-CoA hydrolase after fractionation of rat BAT homogenate by differential centrifugation, followed by further fractionation of a light-mitochondrial fraction in a sucrose density gradient. The results from the differential centrifugations showed that the NADH-sensitive short-chain hydrolase was localized mainly in the heavy-mitochondrial fraction.

Fractionation of the light-mitochondrial fraction by sucrose-density-gradient centrifugation revealed the presence of acyl-CoA hydrolase activity in peroxisomes. The peroxisomal localization of the acyl-CoA hydrolase activity became evident when the hydrolase activity in the gradient was measured in the presence of 750 μM-NADH.

The substrate specificity of the peroxisomal acyl-CoA hydrolase activity indicates the presence of a single enzyme with broad specificity for acyl-CoAs. The peroxisomal hydrolase activity was totally insensitive to NADH (in contrast with the mitochondrial activity). However, both peroxisomal and mitochondrial hydrolase activities were inhibited by added CoA at low concentrations. Also ATP inhibited hydrolase activities in both organelles, but at much higher concentrations. A separate CoA pool has been demonstrated in the matrix of isolated rat liver peroxisomes (Van Broekhoven *et al.*, 1981). Although no adenine or nicotinamide nucleotides could be detected in isolated rat liver peroxisomes (Mannaerts *et al.*, 1982), the peroxisomal membrane was shown to be permeable to various cofactors of β-oxidation (including CoA, NAD^+, ATP and carnitine) (Van Veldhoven *et al.*, 1987). The permeability of the peroxisomal membrane may indicate that some of these cofactors may leak out during isolation of peroxisomes.

The activities of cytosolic and mitochondrial acetyl-CoA hydrolases, as well as mitochondrial short- and medium-chain hydrolases, have been demonstrated to be influenced by nucleotides and/or ions (Prass *et al.*, 1980; Söling & Rescher, 1985; Alexson & Nedergaard, 1988). Palmitoyl-CoA hydrolase from rat liver mitochondria and an acyl-CoA hydrolase with broad substrate specificity, isolated from pig heart, have also been shown to be regulated by some ions and nucleotides (Berge & Døssland, 1979; Lee & Schulz, 1979). The lack of apparent regulation of the peroxisomal hydrolase activity by ions or nucleotides indicates that the peroxisomal hydrolase activity may be regulated by changes in intraperoxisomal concentrations of CoA. ATP appears less likely to be a regulator of the peroxisomal hydrolase activity, as relatively high concentrations are needed and so far ATP has not been detected in isolated peroxisomes.

The maximal activity of acyl-CoA hydrolase activity in isolated peroxisomes from BAT found in these experiments (about 50 nmol/min per mg of protein) is much lower than the activity of the β-oxidation system (about 160 nmol of NADH formed/min per mg of protein with lauroyl-CoA as substrate; S. E. H. Alexson, unpublished work). The formation of NADH gives an estimate of the number of β-oxidation cycles rather than the number of fatty acid molecules metabolized. Assuming that the peroxisomal β-oxidation system acts mainly as a chain-shortening system (Osmundsen *et al.*, 1980) catalysing two to three cycles of β-oxidation, the magnitudes of the hydrolase and β-oxidation may be similar.

Acyl-CoA hydrolase activity has previously been described in isolated rat liver peroxisomes (Osmundsen *et al.*, 1980; Berge *et al.*, 1984*b*). It was demonstrated that palmitoyl-CoA hydrolase activity was high (about 5 times that in isolated rat liver mitochondria). The activity with other substrates was found to be about 10 times lower. These peroxisomal acyl-CoA hydrolase activities were induced by clofibrate, as is the case with peroxisomal β-oxidation (Lazarow & de Duve, 1976). However, the induction of the peroxisomal acyl-CoA hydrolase activity was only seen if the peroxisome-enriched fraction (light-mitochondrial fraction) was obtained by increasing the centrifugal force in preparing this subcellular fraction (Berge *et al.*, 1984*b*).

The physiological function of acyl-CoA hydrolase activity in peroxisomes is not clear. It is possible that acyl-CoA hydrolase activity may regulate the extent of oxidation of different fatty acids. The peroxisomal β-oxidation system has been described to act mainly as a chain-shortening system (Osmundsen *et al.*, 1980). Acyl-CoA hydrolase may have an important function in the termination of chain-shortening of prostaglandins and long-chain polyunsaturated fatty acids, which can be β-oxidized by rat liver peroxisomes (Hiltunen *et al.*, 1986; Diczfalusy *et al.*, 1987; Hovik & Osmundsen, 1987; Schepers *et al.*, 1988; Diczfalusy & Alexson, 1988). Thus, during β-oxidation of long-chain acyl-CoAs it is conceivable that the chain-shortened fatty acids (with chain length of 16 carbon atoms or below) may be increasingly good substrates for the peroxisomal acyl-CoA hydrolase. It is possible that termination of β-oxidation is determined by competition between acyl-CoA oxidase and acyl-CoA hydrolase for the β-oxidation intermediates. Consequently, a pool of free CoA, available for the β-oxidation, may be maintainted (as the acyl-CoA hydrolase may become increasingly active at decreasing concentrations of free CoA). It is also possible that acyl-CoA hydrolase in peroxisomes may have a quantitative role in the regulation of fatty acid oxidation. Indeed it has been demonstrated that palmitoyl-CoA hydrolysis may be substantial during incubation of isolated peroxisomes with palmitoyl-CoA (Bartlett *et al.*, 1988).

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