Separate peroxisomal oxidases for fatty acyl-CoAs and trihydroxycoprostanoyl-CoA in human liver

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Abstract Fatty acyl-CoAs as well as the CoA esters of the bile acid intermediates di- and trihydroxycoprostanic acids are β-oxidized in peroxisomes. The first reaction of peroxisomal β-oxidation is catalyzed by acyl-CoA oxidase. We recently described the presence of two fatty acyl-CoA oxidases plus a trihydroxycoprostanoyl-CoA oxidase in rat liver peroxisomes (Schepers, L., P. P. Van Veldhoven, M. Casteels, H. J. Eyssen, and G. P. Mannaerts. 1990. *J. Biol. Chem.* **265:** 5242–5246). We have now developed methods for the measurement of palmitoyl-CoA oxidase and trihydroxycoprostanoyl-CoA oxidase in human liver. The activities were measured in livers from controls and from three patients with peroxisomopathies. In addition, the oxidase activities were partially purified from control livers by ammonium sulfate fractionation and heat treatment, and the partially purified enzyme preparation was subjected to chromatofocusing, hydroxylapatite chromatography, and gel filtration. In earlier experiments this allowed for the separation of the three rat liver oxidases. The results show that human liver, as rat liver, contains a separate trihydroxycoprostanoyl-CoA oxidase. In contrast to the situation in rat liver, no conclusive evidence was obtained for the presence of two fatty acyl-CoA oxidases in human liver. Our results explain why bile acid metabolism is normal in acyl-CoA oxidase deficiency, despite a severely disturbed peroxisomal fatty acid oxidation and perhaps also why, in a number of other cases of peroxisomopathy, di- and trihydroxycoprostanic acids are excreted despite a normal peroxisomal fatty acid metabolism.—**Casteels, M., L. Schepers, P. P. Van Veldhoven, H. J. Eyssen, and G. P. Mannaerts.** Separate peroxisomal oxidases for fatty acyl-CoAs and trihydroxycopro stanoyl-CoA in human liver. *J. Lipid Res.* 1990. **31:** 1865–1872.

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Peroxisomal β-oxidation consists of four consecutive reactions catalyzed by the following enzymes: acyl-CoA oxidase; the bifunctional protein displaying enoyl-CoA hydratase and β-OH-acyl-CoA dehydrogenase activity; and β-ketoacyl-CoA thiolase (1). Substrates for peroxisomal β-oxidation include medium-, long-, and very long-chain fatty acids, medium- and long-chain dicarboxylic fatty acids, the carboxyl side chains of the bile acid intermediates di- and trihydroxycoprostanic acid, and the carboxyl side chains of prostaglandins and certain xenobiotics (for reviews see 2 and 3). Although there is a considerable overlapping of the substrate spectra of peroxisomes and mitochondria, very long-chain fatty acids and di- and trihydroxycoprostanic acid are oxidized predominantly, if not exclusively, in peroxisomes. Peroxisomal β-oxidation of di- and trihydroxycoprostanic acid results in the formation of chenodeoxycholic and cholic acid, respectively (4).

It is not known whether all these β-oxidation substrates are oxidized by the same set of peroxisomal enzymes or by isoenzymes with different substrate preference as is the case in mitochondria (e.g., the three fatty acyl-CoA dehydrogenases). Very recently, we have demonstrated the presence of three acyl-CoA oxidases in rat liver peroxisomes: two fatty acyl-CoA oxidases that oxidize palmitoyl-CoA but not THC-CoA and a THC-CoA oxidase that does not oxidize palmitoyl-CoA (5). The enzymes could be distinguished on the basis of differences in pI value, binding to adsorption and affinity chromatography matrices, molecular mass and subunit composition, and inducibility by peroxisomes proliferators. The exact substrate spectrum of each enzyme is currently under study.

Disorders of peroxisomal β-oxidation are commonly characterized by an accumulation of very long-chain fatty acids and the excretion of di- and trihydroxycoprostanic acids instead of chenodeoxycholic and cholic acids (3). Some cases are known, however, in which abnormal bile acids are found in association with normal very long-chain fatty acids and vice versa (6, 7; R. Wanders, personal communication), suggesting that in the human liver differences may also exist between the peroxisomal β-oxidation of fatty acids and bile acid intermediates. We

Abbreviations: THC-CoA, CoA ester of 3α,7α,12α-trihydroxycoprostanic acid.

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therefore investigated the possibility that a separate THC-CoA oxidase is also present in human liver.

MATERIALS AND METHODS

Unlabeled THC-CoA and [26-^{14}C]THC-CoA were synthesized as described before (8) and stored as a freeze-dried powder at − 20°C. Stock solutions containing 1 mM unlabeled or labeled (sp act: 2.5 Ci/mol) THC-CoA were prepared in distilled water (pH 4.5–5). The stock solutions were divided into aliquots and stored at −20°C for a maximum period of 3 months.

Palmitoyl-CoA and Percoll were from Pharmacia, Uppsala, Sweden; N-ethylmaleimide and homovanillic acid were from Janssen Chimica, Beerse, Belgium; FAD and horseradish peroxidase (grade I, 250 U/mg) were from Boehringer Mannheim, West Germany. Bovine serum albumin, fraction V, was obtained from Sigma, St. Louis, MO, and was defatted according to Chen (9). All other chemicals were from Merck, Darmstadt, West Germany. Dialysis tubing with a molecular weight cutoff of 3500 was purchased from Spectrum Medical Industries Inc., Los Angeles, CA.

Liver homogenates

Human liver tissue was obtained *1*) at autopsy, *2*) as excess tissue after transplantation of livers from adult donors in children, or *3*) after partial hepatectomy for a liver tumor. A detailed description of the procedures was submitted to the University Hospital Ethics Committee, which granted its approval. All tissue was histologically examined in order to discard diseased or deteriorated material. Tissues were divided into small portions, frozen in liquid N_2, and stored at −60°C. Palmitoyl-CoA oxidase and THC-CoA oxidase were stable at this temperature.

Liver homogenates [5% (w/v)] were made in 10 mM inorganic pyrophosphate (PPi buffer), pH 9.0, containing 1 mM EDTA and 10 μM FAD, by means of five strokes each of a loose and tight-fitting plunger in a Dounce homogenizer. When indicated, homogenates were dialyzed against the same buffer at 4°C for 12 h. The homogenates were further diluted with the same buffer and used for the enzyme measurements (see below). Protein was determined according to the method of Peterson (10).

Acyl-CoA oxidase

Acyl-CoA oxidase was measured by following the production of H_2O_2 as described before (11) with the following modifications. The reaction mixture, 0.2 ml per assay, consisted of 50 mM Tris-HCl, pH 8.3, containing 12.5 μM defatted bovine serum albumin, 0.1 mg/ml horseradish peroxidase, 0.75 mM homovanillic acid, 10 μM FAD, and 62.5 μM palmitoyl-CoA or THC-CoA. The incubation was started by adding 0.05 ml of a 1% (w/v) liver homogenate in 10 mM PPi buffer, pH 9.0, containing 1 mM EDTA and 10 μM FAD. Incubations were carried out at 37°C. When no lag phase was present (dialyzed homogenates, partially purified enzyme preparations; see Results) incubation time was 20 min for palmitoyl-CoA oxidase and 60 min for THC-CoA oxidase. When a lag phase was present (freshly prepared homogenates) rates were taken between 10 and 20 min for palmitoyl-CoA oxidase and between 40 and 60 min for THC-CoA oxidase. Rates of H_2O_2 production in the absence of substrate were always subtracted. Both oxidase activities increased linearly with protein concentration up to 0.1 mg homogenate protein per assay. Catalase present in the homogenates did not interfere with the detection of H_2O_2 under our conditions, since preincubation of the homogenates in the presence of 5 mM sodium azide did not alter the observed rates of H_2O_2 production.

[^{14}C]THC-CoA oxidation

The reaction mixture, 0.2 ml per assay, consisted of 50 mM Tris-HCl, pH 8.3, containing 12.5 μM defatted bovine serum albumin, 10 μM FAD, 2.5 mM NAD^+, 62.5 μM CoA, 0.25 mM dithiothreitol, 2 mM KCN, and 62.5 μM [26-^{14}C]THC-CoA (sp act: 2.5 Ci/mol). The incubation was started by adding 0.05 ml of a 2% (w/v) liver homogenate in 10 mM PPi buffer, pH 9.0, containing 1 mM EDTA, 10 μM FAD, and 2 mM KCN. The reaction was terminated and the radioactive oxidation products were extracted as described before (8).

Subcellular fractionation

A portion of fresh tissue from a donor liver was homogenized in 0.25 M sucrose, 1 mM EDTA, pH 7.2, 1 mM DTT, 0.1% (v/v) ethanol, 5 μM FAD, and fractionated by differential centrifugation into an N-fraction, containing the nuclei and cell debris; an M-fraction enriched in mitochondria; an L-fraction enriched in lysosomes and peroxisomes; a P-fraction enriched in endoplasmic reticulum fragments; and an S-fraction containing the soluble components (12). The L-fraction was subfractionated in a self-generating Percoll density gradient (13). Marker enzymes were determined as described previously (13). The subcellular fractions, which were resuspended in homogenization buffer, and the Percoll gradient fractions were dialyzed for 12 h against 10 mM PPi buffer, pH 9, containing 1 mM EDTA and 10 μM FAD prior to the measurements of THC-CoA oxidase.

Partial purification of peroxisomal oxidases

Peroxisomal oxidases were partially purified as described before for rat liver (5). Briefly, whole liver homogenates, prepared in 10 mM PPi buffer, pH 9.0, containing 1 mM EDTA and 10 μM FAD, were sonicated in order to release the peroxisomal matrix proteins. The

released oxidases were further purified by means of ammonium sulfate precipitation and heat treatment in the presence of FAD. The final preparation was dissolved in PPi buffer and stored at −20°C for further use. The procedure resulted in an approximately 8-fold increase in the specific activity of both palmitoyl-CoA oxidase and THC-CoA oxidase.

Column chromatography

For chromatofocusing, aliquots of the partially purified oxidases were thawed, the storage buffer was replaced by a 25 mM ethanolamine buffer, adjusted to pH 9.4 with acetic acid, and a portion of the proteins was applied to a PBE 94 chromatofocusing column (12 × 0.9 cm; Pharmacia), equilibrated with the ethanol–acetic acid buffer, exactly as described before (5). The oxidases were eluted at a rate of 0.5 ml per min with a linear pH-gradient, generated by 25 mM Polybuffer (Pharmacia), adjusted to pH 6.0 with acetic acid. Fractions of 3 ml were collected in tubes containing 30 μl of 1 mM FAD, to prevent inactivation of the oxidases.

Aliquots of the partially purified (ammonium sulfate fractionation and heat treatment) oxidases dissolved in PPi buffer were also applied to a hydroxylapatite column (5 × 0.9 cm; Bio-Rad), equilibrated with 10 mM potassium phosphate buffer, pH 7.5, containing 10 μM FAD and 10% (v/v) ethylene glycol (5). Elution of the applied proteins occurred with a linear phosphate gradient containing FAD and ethylene glycol at a flow rate of 0.5 ml per min. Fractions of 0.95 ml were collected. The fractions of the hydroxylapatite column with the highest oxidase activities of interest were pooled, concentrated by dialysis against solid polyethylene glycol 20,000, and applied to a gel filtration column (Ultrogel AcA44 column; 80 × 1.6 cm, LKB), which was calibrated with catalase (mol wt 232,000), lactate dehydrogenase (mol wt 140,000), citrate synthase (mol wt 100,000), ovalbumin (mol wt 43,000), and soybean trypsin inhibitor (mol wt 21,500). The column was eluted with a 200 mM potassium phosphate buffer, pH 7.5, containing 10 μM FAD, 1 mM EDTA, and 10% (v/v) ethylene glycol at a rate of 10.5 ml/h. Fractions of 2.7 ml were collected.

RESULTS

Assay conditions for THC-CoA oxidase and palmitoyl-CoA oxidase in human liver homogenates

When THC-CoA oxidase was measured as a function of time in whole liver homogenates prepared from fresh or frozen tissue, a lag phase was observed during which no H_2O_2 formation could be detected (**Fig. 1a**). After this lag phase, H_2O_2 production proceeded linearly with time for at least 90 min. The lag phase was somewhat variable in length, differing from liver to liver, but it usually took approximately 30 min before linear rates of H_2O_2 production were obtained. With palmitoyl-CoA as the substrate, the lag phase was considerably shorter and lasted from 3 to 10 min, depending on the liver examined. Thereafter, H_2O_2 production was linear with time for approximately 20 min (Fig. 1b). A lag phase was no longer present when THC-CoA oxidase and palmitoyl-CoA oxidase were measured on homogenates that had been dialyzed for 12 h (Fig. 1) or on partially purified enzyme preparations obtained after ammonium sulfate fractionation and heat treatment (see Methods; data not shown). Palmitoyl-CoA oxidase lost some of its activity during dialysis; this was not the case for THC-CoA oxidase.

The above results suggest that freshly prepared homogenates contain low molecular weight substances that inhibit the oxidases and that are degraded during the lag phase or, more plausibly, that react with H_2O_2 and are inactivated during the lag phase, possibly by H_2O_2 itself. The following experiment supported the latter possibility. When liver homogenates were incubated with different amounts of uricase (which is not present in human liver) in the presence of a fixed uric acid concentration, a lag phase was observed at low uricase concentrations that resulted in H_2O_2 production rates comparable to those obtained with THC-CoA; at increasing uricase concentrations (higher H_2O_2 production rates) the lag phase became shorter and eventually disappeared at high concentrations (data not shown).

Rates of H_2O_2 production measured with unlabeled THC-CoA as the substrate equaled the rates of formation of radioactive oxidation products measured with radioactive THC-CoA, when the latter assay was performed under the same conditions as those used for the determi-

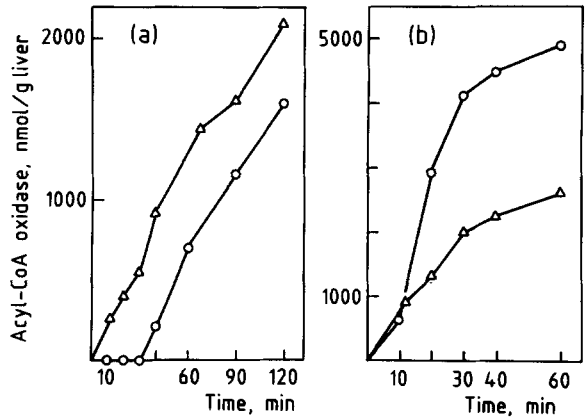

Fig. 1. Acyl-CoA oxidase activities in fresh and dialyzed homogenates from human liver. THC-CoA oxidase (panel a) and palmitoyl-CoA oxidase (panel b) were measured in freshly made homogenates (○) and in homogenates that had been dialyzed for 12 h (△) as described in the Methods section. Substrate concentration was 50 μM and the albumin concentration was 10 μM. Protein contents of fresh and dialyzed homogenates were equal.

nation of H_2O_2 (except that the cofactors for the further β-oxidation steps were also included; see Methods) (data not shown). In the radioisotopic assay, the end product of the whole β-oxidation sequence is measured. The results indicate that under the conditions of the experiment THC-CoA oxidase was limiting the activity of the whole sequence. Lag phases were observed with both assays. The lag phase found with the radioactive assay (10 min) was considerably shorter than that found with the assay in which H_2O_2 production was measured, again confirming that freshly prepared homogenates contain substances that react with H_2O_2. The lag phase in the radioactive assay is probably the result of the time needed to build up steady state concentrations of β-oxidation intermediates.

THC-CoA oxidase and palmitoyl-CoA oxidase activities were also measured versus increasing substrate concentrations (**Fig. 2**). Dialyzed homogenates were used and albumin was omitted from the reaction mixtures in order to avoid binding of palmitoyl-CoA and possibly of THC-CoA. At high concentrations palmitoyl-CoA caused substrate inhibition; this was perhaps also the case for THC-CoA, but only to a small extent. From the linear parts of the Lineweaver-Burk plots (Fig. 2, insets) an apparent K_m of 7 μM was calculated for THC-CoA oxidase and one of 62 μM for palmitoyl-CoA oxidase. It should be noted that, because of the impurity of the enzyme preparation, these apparent K_ms may only be crude reflections of the true K_ms of the pure enzymes.

In the next experiment the effect of increasing albumin concentrations was investigated on THC-CoA oxidase and palmitoyl-CoA oxidase, measured at a substrate concentration of 50 μM (**Fig. 3**). Albumin stimulated THC-CoA oxidase activity approximately 2-fold, except at high concentrations where the stimulatory effect was lost. At low concentrations, it barely affected palmitoyl-CoA oxidase; at higher concentrations it became inhibitory, possibly as a result of substrate binding. The effect of albumin on THC-CoA oxidase was also studied at substrate concentrations other than 50 μM (5, 10, 20, and 100 μM THC-CoA). Albumin stimulated the oxidase activity at all substrate concentrations tested (data not shown). The stimulatory effect was somewhat less at the lowest THC-CoA concentration. For every concentration, optimum rates were reached at an albumin concentration of 10 μM. The apparent K_m (11 μM) for THC-CoA oxidase, assayed in the presence of 10 μM albumin, differed only slightly from that obtained in the absence of albumin.

Triton X-100 inhibited both THC-CoA oxidase and palmitoyl-CoA oxidase in the presence or absence of albumin in a concentration-dependent fashion (data not shown). An inhibitory effect was already observed at a concentration as low as 0.02% (w/v). The effect of the detergent was studied since rupturing of the peroxisomal membranes might facilitate the access for peroxidase to the site of H_2O_2 production. It should be mentioned, however, that under the assay conditions (hypotonic and slightly alkaline buffer, frozen liver tissue), most peroxisomal membranes were probably already ruptured.

Subcellular distribution of THC-CoA oxidase

In rat liver THC-CoA oxidase is located in peroxisomes (5, 14) as is fatty acyl-CoA oxidase (1). Also in humans, fatty acyl-CoA oxidase is confined to peroxisomes (15). Pedersen and Gustafsson (4) and Kase and colleagues (16) demonstrated that the conversion of di- and trihydroxycoprostanic acid into bile acids is catalyzed by peroxisomal enzymes in rat liver (4) and also human liver (16), and Kase et al. (17, 18) showed that such conversion is deficient in patients with the Zellweger syndrome who lack recognizable peroxisomes (3). This indicates that, in the human, THC-CoA oxidase is also a peroxisomal activity. It was, therefore, not surprising to find that the subcellular distribution of THC-CoA oxidase coincided with that of the peroxisomal marker catalase after fractionation of a human liver homogenate by differential (**Fig. 4, A**) and isopycnic (Fig. 4, B) centrifugation, thereby

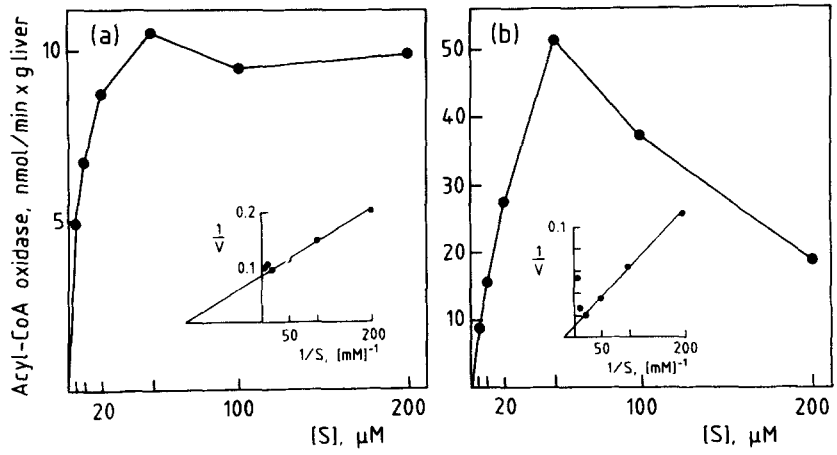

Fig. 2. Acyl-CoA oxidase activities versus increasing substrate concentrations. Dialyzed liver homogenates were incubated with increasing concentrations of THC-CoA (panel a) or palmitoyl-CoA (panel b) in the absence of albumin. The Lineweaver-Burk plots (insets) show an apparent K_m of 7 μM for THC-CoA oxidase and of 62 μM for palmitoyl-CoA oxidase.

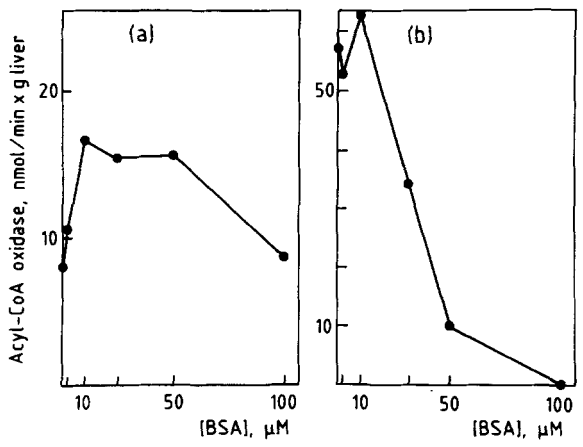

Fig. 3. Effect of albumin on acyl-CoA oxidase activities. Dialyzed liver homogenates were incubated with 50 μM THC-CoA (panel a) or palmitoyl-CoA (panel b) at different albumin concentrations.

confirming that the human enzyme activity is peroxisomal. In comparison with the rat, more catalase was found in the soluble fraction after differential centrifugation (Fig. 4, A). Normally, part of the catalase and other soluble matrix enzymes leaks from peroxisomes during homogenization. Human liver peroxisomes may be more fragile than the rat organelles or the preservation state of the donor liver used for fractionation may not have been optimum. Apparently, somewhat less THC-CoA oxidase leaked from the peroxisomes than catalase.

Acyl-CoA oxidase activities in human liver homogenates

Table 1 shows the activities of THC-CoA oxidase and palmitoyl-CoA oxidase, measured under optimum conditions, in livers from controls and in livers from three patients with a peroxisomal disease, two patients with the Zellweger syndrome, and one patient with X-linked adrenoleukodystrophy. Patients with the Zellweger syndrome lack recognizable peroxisomes and their peroxisomal β-oxidation of fatty acids and of bile acid intermediates is severely deficient (3). Patients with X-linked adrenoleukodystrophy lack a peroxisomal very long-chain fatty acyl-CoA synthetase, which causes an accumulation of very long-chain fatty acids in their tissues. Their peroxisomal β-oxidation enzymes and bile acid metabolism are normal (3). In agreement with the known defects, we found a severe decrease in the activities of THC-CoA oxidase and palmitoyl-CoA oxidase in the Zellweger patients, but normal values in the X-linked adrenoleukodystrophy patient.

Chromatofocusing of a partially purified preparation of peroxisomal oxidases

Rat liver contains two palmitoyl-CoA oxidases plus a THC-CoA oxidase. Upon chromatofocusing, one of the palmitoyl-CoA oxidases elutes around pH 8.3 and the other around pH 7.1. THC-CoA oxidase elutes in the same fractions as the second palmitoyl-CoA oxidase (5). In order to investigate whether human liver would also

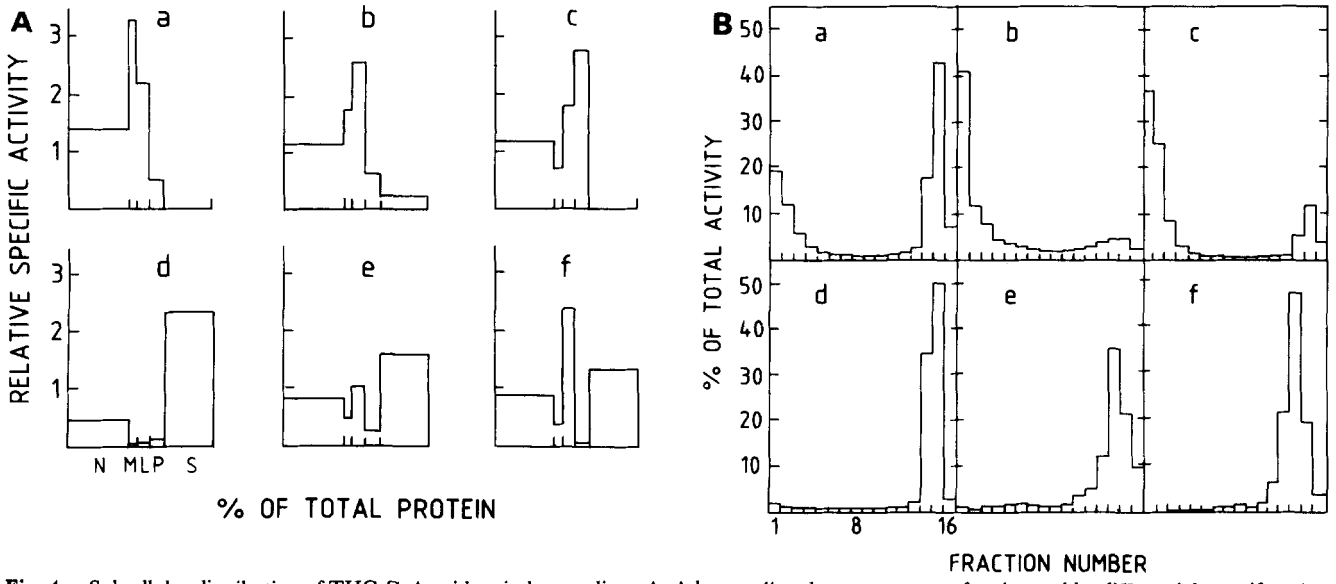

Fig. 4. Subcellular distribution of THC-CoA oxidase in human liver. A: A human liver homogenate was fractionated by differential centrifugation as described in Methods. Fractions were analyzed for glutamate dehydrogenase (marker for mitochondria; a), acid phosphatase (lysosomes; b), glucose-6-phosphatase (endoplasmic reticulum; c), lactate dehydrogenase (cytosol; d), catalase (peroxisomes; e), and THC-CoA oxidase (f). Results are expressed as relative specific activities versus cumulative percentage of protein. Recoveries for marker enzymes varied between 91 and 107%. Recovery for THC-CoA oxidase was 78%. B: The light mitochondrial fraction (L) was subfractionated on a Percoll gradient as described in Methods and the gradient fractions were analyzed for protein (a), acid phosphatase (b), glutamate dehydrogenase (c), glucose-6-phosphatase (d), catalase (e), and THC-CoA oxidase (f). Results are expressed as percentages of the total recovered gradient activity or content present in each fraction. Recoveries for protein and marker enzymes varied between 92 and 128%. Recovery for THC-CoA oxidase was 121%. Fractions 1 and 16 represent the fractions of highest and lowest density, respectively.

TABLE 1. Acyl-CoA oxidase activities in human liver homogenates

Patients	THC-CoA Oxidase	Palmitoyl-CoA Oxidase
	nmol H_2O_2/ min × g liver	
Control	16.77 ± 3.53	51.36 ± 9.07
Zellweger Syndrome	ND 2.45	ND ND
X-Linked adrenoleukodystrophy	14.65	37.65

H_2O_2 production was measured between 10 and 20 min with palmitoyl-CoA as the substrate and between 40 and 60 min with THC-CoA as the substrate. Freshly made homogenates were used. Results are expressed as means ± SE for four livers (controls) or as individual values (patients' livers). Ratios of palmitoyl-CoA oxidase to THC-CoA oxidase displayed relatively large fluctuations ranging from 1.8 to 6.5 in the control group; ND, not detectable.

possess two fatty acyl-CoA oxidases, we chromatofocused a partially purified preparation of peroxisomal oxidases. As shown in **Fig. 5**, palmitoyl-CoA oxidase eluted in two peaks, a larger one around pH 7.6 and a smaller one around pH 6.8. THC-CoA oxidase eluted in one peak around pH 6.7. The elution pattern of palmitoyl-CoA oxidase in human liver, as illustrated in Fig. 5, is reminiscent of that of the two fatty acyl-CoA oxidases of rat liver. However, it is premature to conclude from these data that human liver also contains two fatty acyl-CoA oxidases. In total, we performed six chromatofocusing experiments with three different partially purified oxidase preparations from one liver and three preparations from another liver. No small palmitoyl-CoA oxidase peak was observed in two preparations from one liver and in one preparation from the second liver. Since the original preservation state of the liver tissue or of parts of the tissue may not always have been optimum, it is possible that the smaller peak is the result of proteolytic damage to the oxidase. In the six chromatofocusing experiments, the peak activities eluted at pH 7.55 ± 0.08 (n = 6; larger palmitoyl-CoA oxidase peak), at pH 6.98 ± 0.06 (n = 3; smaller palmitoyl-CoA oxidase peak), and at pH 7.12 ± 0.13 (n = 6; trihydroxycoprostanoyl-CoA oxidase).

Effect of different additions on THC-CoA oxidase and palmitoyl-CoA oxidase

The chromatofocusing experiments suggested that the THC-CoA oxidase and palmitoyl-CoA oxidase activities are catalyzed by separate enzymes as is the case in rat liver. In a next series of experiments we investigated these enzyme activities (after partial purification) in a number of experimental conditions that were previously shown to differentially affect palmitoyl-CoA oxidase and THC-CoA oxidase in rat liver (5). As in rat liver, THC-CoA oxidase was strongly inhibited by N-ethylmaleimide and lithium chloride, whereas palmitoyl-CoA oxidase was only slightly affected. Contrary to the rat enzymes, zinc acetate inhibited the human palmitoyl-CoA oxidase whereas THC-CoA oxidase was slightly stimulated (data not shown). The different behavior of the human enzymes towards N-ethylmaleimide and salts again indicates that in human liver THC-CoA oxidase and fatty acyl-CoA oxidase are also distinct enzymes.

Hydroxylapatite column chromatography

In rat liver, THC-CoA oxidase could be separated from the palmitoyl-CoA oxidases by means of chromatography on hydroxylapatite. **Fig. 6** shows that, when a partially purified oxidase preparation from human liver was chromatographed on hydroxylapatite, a separation of the two activities was also obtained, clearly demonstrating that in the human, as in the rat, THC-CoA oxidase is a separate entity.

The fractions containing most of the THC-CoA oxidase activity and those containing most of the palmitoyl-CoA oxidase activity were pooled, concentrated, and loaded on a gel filtration column, and the molecular mass of the enzymes was estimated. Values of 138 kDa and 158.5 kDa were found for THC-CoA oxidase and palmitoyl-CoA oxidase, respectively.

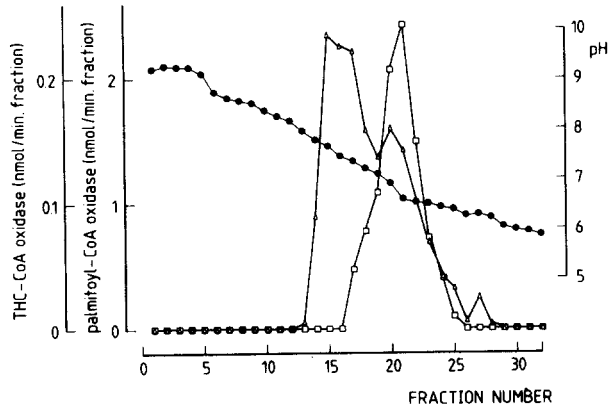

Fig. 5. Chromatofocusing of acyl-CoA oxidases. An aliquot of partially purified oxidases from human liver, containing 1.5 mU THC-CoA oxidase, 24.3 mU palmitoyl-CoA oxidase, and 4.2 mg protein, was applied to a chromatofocusing column and eluted with a linear pH-gradient. Palmitoyl-CoA oxidase (△), THC-CoA oxidase (□), and pH (●-●-●) were measured in all fractions. Recoveries for THC-CoA oxidase and palmitoyl-CoA oxidase were 63% and 68%, respectively. Protein recovery could not be calculated since the elution buffer interfered with the protein determination.

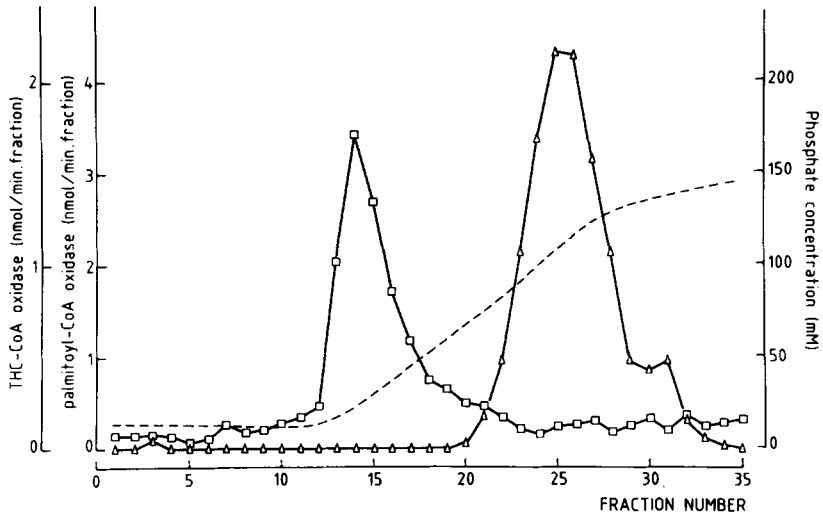

Fig. 6. Separation of palmitoyl-CoA oxidase and THC-CoA oxidase by hydroxylapatite chromatography. An aliquot of partially purified oxidases, containing 8.5 mU THC-CoA oxidase, 16.3 mU palmitoyl-CoA oxidase, and 9.1 mg of protein, was applied to a hydroxylapatite column and the proteins were eluted with a linear potassium phosphate gradient. Phosphate concentration (–), palmitoyl-CoA oxidase activity (△), and THC-CoA oxidase activity (□) were determined in the fractions. Recoveries for both oxidases were 85% and for protein 50%.

DISCUSSION

We recently discovered that rat liver peroxisomes contain two fatty acyl-CoA oxidases plus a separate THC-CoA oxidase. Since the possible occurrence of more than one fatty acyl-CoA oxidase and a separate THC-CoA oxidase might be of relevance for a better understanding of the molecular defects in a number of patients with peroxisomopathies, we investigated in more detail the acyl-CoA oxidases present in human liver.

The data establish that, as in the rat, THC-CoA and fatty acyl-CoAs are oxidized by distinct enzymes. This result explains why, in the case of fatty acyl-CoA oxidase deficiency, normal bile acids are found despite a severe disturbance of peroxisomal fatty acid oxidation (6). In addition, a few patients are known whose peroxisomal fatty acid oxidation appears to be normal but who excrete di- and trihydroxycoprostanic acids instead of chenodeoxycholic and cholic acids (7; R. Wanders, personal communication). This suggests that an isolated deficiency of THC-CoA oxidase may also occur.

Our experiments do not allow us to draw definitive conclusions about the occurrence of one or two fatty acyl-CoA oxidases in human liver. Although in some chromatofocusing experiments two palmitoyl-CoA oxidase peaks were observed, it cannot be excluded at this moment that these peaks were the result of proteolytic damage. The liver tissue used for the chromatofocusing experiments was taken from a donor liver that had been preserved in vitro for several hours and from a partial hepatectomy. In the latter case, the excised lobe had been ischemic for approximately 1 h due to the interruption of the local circulation during resection.

Human fatty acyl-CoA oxidase cross-reacts with antibodies raised against the inducible rat liver enzyme. By means of immunoblotting it has been shown that, similar to the inducible rat liver enzyme, the human enzyme consists of three polypeptide components: A (72 kDa), B (52 kDa), and C (21 kDa) (19, 20). In rat liver, components B and C are formed by post-translational proteolytic cleavage of component A and the enzyme exists as a mixture of A_2, B_2C_2, and ABC (21, 22). Our human enzyme preparations were not pure enough to determine with certainty their subunit composition as analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The estimated molecular mass of 158.5 kDa agrees with a subunit composition for the human fatty acyl-CoA oxidase that is similar to that of the inducible rat liver enzyme. The molecular mass of the human THC-CoA oxidase (138 kDa) is virtually the same as that of the rat liver enzyme (139 kDa) (5). Since the latter consists of two identical subunits of 69 kDa, the same seems to be true for the human enzyme.

At the start of our experiments, using assay conditions routinely in use in our laboratory for the determination of the rat liver enzymes, we encountered serious problems in measuring palmitoyl-CoA oxidase and especially THC-CoA oxidase in human whole liver homogenates. The initial lag phase, during which no H_2O_2 production could be detected, appeared to be the culprit. The presence of this lag phase, the length of which may vary from liver to liver, makes it necessary to measure the enzymes at multiple points over an extended period of time in order to ensure that optimum rates are taken. Attention must also be paid to homogenate dilution, since at low dilutions the lag phase may be considerably prolonged. The lag phases appear to be the result of the presence in freshly prepared homogenates of soluble compounds reacting with H_2O_2. Although we did not attempt to identify these compounds, it is our experience that reduced glutathione, CoASH, and ascorbic acid all decompose H_2O_2. Lag phases are of less importance in rat liver since the activities of the acyl-CoA oxidases are higher so that higher dilutions can be used. Perhaps rat

liver also contains lower concentrations of interfering substances.

In human whole liver homogenates we found THC-CoA oxidase activities of approximately 17 nmol per min per g of tissue, as measured under our conditions. This enzyme activity represents a 30-to 60-fold excess over the rate of bile acid synthesis reported in the literature (23).

As part of this investigation we measured THC-CoA oxidase and palmitoyl-CoA oxidase in the liver of *Bombina orientalis*. Some amphibians (the toad *Bombina orientalis*) and some reptiles (crocodiles and alligators) excrete mainly C_{27} bile acids such as di- and trihydroxycoprostanic acids (24). We found palmitoyl-CoA oxidase activity but not THC-CoA oxidase activity (M. Casteels, P. P. Van Veldhoven, and G. P. Mannaerts, unpublished results), suggesting that these lower vertebrates lack THC-CoA oxidase.

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REFERENCES

1. Hashimoto, T. 1987. Comparison of enzymes of lipid β-oxidation in peroxisomes and mitochondria. *In* Peroxisomes in Biology and Medicine. H. D. Fahimi, and H. Sies, editors. Springer-Verlag, Heidelberg. 97–104.
2. Mannaerts, G. P., and P. P. Van Veldhoven. 1990. The peroxisome: functional properties in health and disease. *Biochem. Soc. Trans.* **18:** 87–89.
3. Wanders, R. J. A., H. S. A. Heymans, R. B. H. Schutgens, P. G. Barth, H. van den Bosch, and J. M. Tager. 1988. Peroxisomal disorders in neurology. *J. Neurol. Sci.* **88:** 1–39.
4. Pedersen, J. I., and J. Gustafsson. 1980. Conversion of 3α, 7α, 12α-trihydroxy-5β-cholestanoic acid into cholic acid by rat liver peroxisomes. *FEBS Lett.* **121:** 345–348.
5. Schepers, L., P. P. Van Veldhoven, M. Casteels, H. J. Eyssen, and G. P. Mannaerts. 1990. Presence of three acyl-CoA oxidases in rat liver peroxisomes: an inducible fatty acyl-CoA oxidase, a non-inducible fatty acyl-CoA oxidase and a non-inducible trihydroxycoprostanoyl-CoA oxidase. *J. Biol. Chem.* **265:** 5242–5246.
6. Poll-Thé, B. T., F. Roels, H. Ogier, J. Scotto, J. Vamecq, R. B. Schutgens, R. J. Wanders, C. W. van Roermund, N. J. van Wijland, A. W. Schramm, J. M. Tager, and J-M. Saudubray. 1988. A new peroxisomal disorder with enlarged peroxisomes and a specific deficiency of acyl-CoA oxidase (pseudo-neonatal adrenoleukodystrophy). *Am. J. Hum. Genet.* **42:** 422–434.
7. Przyrembel, H., R. J. A. Wanders, C. W. T. van Roermund, R. B. H. Schutgens, G. P. Mannaerts, and M. Casteels. 1990. Di- and trihydroxycholestanoic acidemia with hepatic failure. *J. Inher. Metab. Dis.* **13:** 367–370.
8. Casteels, M., L. Schepers, J. van Eldere, H. J. Eyssen, and G. P. Mannaerts. 1988. Inhibition of 3α,7α,12α-trihydroxy-5β-cholestanoic acid oxidation and of bile acid secretion in rat liver by fatty acids. *J. Biol. Chem.* **263:** 4654–4661.
9. Chen, R. F. 1967. Removal of fatty acids from serum albumin by charcoal. *J. Biol. Chem.* **242:** 173–181.
10. Peterson, G. L. 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal. Biochem.* **83:** 346–356.
11. Van Veldhoven, P. P., and G. P. Mannaerts. 1985. Comparison of the activities of some peroxisomal and extraperoxisomal lipid-metabolizing enzymes in liver and extrahepatic tissues of the rat. *Biochem. J.* **227:** 737–741.
12. de Duve, C., B. C. Pressman, R. Giavetto, R. Wattiaux, and F. Appelmans. 1955. Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue. *Biochem. J.* **60:** 604–617.
13. Van Veldhoven, P. P., W. W. Just, and G. P. Mannaerts, 1987. Permeability of the peroxisomal membrane to cofactors of β-oxidation. Evidence for the presence of a pore-forming protein. *J. Biol. Chem.* **262:** 4310–4318.
14. Schepers, L., M. Casteels, J. Vamecq, G. Parmentier, P. P. Van Veldhoven, and G. P. Mannaerts. 1988. β-Oxidation of the liver carboxyl side chain of prostaglandin E_2 in rat liver peroxisomes and mitochondria. *J. Biol. Chem.* **263:** 2724–2731.
15. Bronfman, M., N. C. Inestrosa, and F. Leighton. 1979. Fatty acid oxidation by human liver peroxisomes. *Biochem. Biophys. Res. Commun.* **88:** 1030–1036.
16. Kase, B. F., K. Prydz, I. Björkhem, and J. I. Pedersen. 1986. In vitro formation of bile acids from di- and trihydroxy-5-β-cholestanoic acid in human liver peroxisomes. *Biochim. Biophys. Acta.* **877:** 37–42.
17. Kase, B. F., I. Björkhem, P. Hågå, and J. I. Pedersen. 1985. Defective peroxisomal cleavage of the C_{27}-steroid side chain in the cerebro-hepato-renal syndrome of Zellweger. *J. Clin. Invest.* **75:** 427–435.
18. Kase, B. F., J. I. Pedersen, B. Strandvik, and I. Björkhem. 1985. In vivo and in vitro studies on formation of bile acids in patients with Zellweger syndrome. Evidence that peroxisomes are of importance in the normal biosynthesis of both cholic and chenodeoxycholic acid. *J. Clin. Invest.* **76:** 2393–2402.
19. Tager, J. M., W. A. Ten Harmsen Van den Beek, R. J. A. Wanders, T. Hashimoto, H. S. A. Heymans, H. Van den Bosch, R. B. H. Schutgens, and A. W. Schram. 1985. Peroxisomal β-oxidation enzyme proteins in the Zellweger syndrome. *Biochem. Biophys. Res. Commun.* **126:** 1269–1275.
20. Suzuki, Y., T. Orii, and T. Hashimoto. 1986. Biosynthesis of peroxisomal β-oxidation enzymes in infants with Zellweger syndrome. *J. Inher. Metab. Dis.* **9:** 292–296.
21. Furata, S., T. Hashimoto, S. Miura, M. Mori, and T. Tatibana. 1982. Cell-free synthesis of the enzymes of peroxisomal β-oxidation. *Biochem. Biophys. Res. Commun.* **105:** 639–646.
22. Miura, S., M. Mori, M. Takiguchi, M. Tatibana, S. Furata, S. Miyazawa, and T. Hashimoto. 1984. Biosynthesis and intracellular transport of enzymes of peroxisomal β-oxidation. *J. Biol. Chem.* **259:** 6397–6402.
23. Carey, C., and M. J. Cahalane. 1988. Enterohepatic circulation. *In* The Liver. Biology and Pathobiology. I. M. Arias, W. B. Jakoby, H. Popper, D. Schachter, and D. A. Shafritz, editors. Raven Press, New York. 591–593.
24. Haslewood, G. A. D. 1967. Bile salt evolution. *J. Lipid Res.* **8:** 535–550.