Mitochondrial short-chain acyl-CoA dehydrogenase of human liver and kidney can function as an oxidase

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During an attempt to purify the peroxisomal acyl-CoA oxidases from human liver and kidney, we discovered a novel short-chain acyl-CoA oxidase, which was well separated from the known peroxisomal oxidases on various chromatographic columns. However, further experiments demonstrated that the novel oxidase is identical with the mitochondrial short-chain acyl-CoA dehydrogenase. (1) Subcellular fractionation revealed that the short-chain acyl-CoA oxidase is present in mitochondria and absent from peroxisomes. (2) The molecular mass (43 kDa) of the subunit of the purified oxidase was similar to that reported for the dehydrogenase. (3) The substrate spectrum of the oxidase was comparable with that described for the dehydrogenase. (4) On column chromatography, the oxidase and dehydrogenase activities co-eluted. Our results indicate that, in the absence of suitable electron acceptors, the short-chain acyl-CoA dehydrogenase is capable of transferring electrons directly to molecular oxygen, yielding potentially harmful H_2O_2. This raises the question as to whether the dehydrogenase might function as an oxidase in conditions in which the activity of the electron-transport chain is decreased, such as reperfusion after ischaemia.

INTRODUCTION

The first step of fatty acid β-oxidation is the desaturation of an acyl-CoA to a 2-*trans*-enoyl-CoA. In mitochondria this step is catalysed by an FAD-containing acyl-CoA dehydrogenase (for reviews, see [1,2]). The electrons resulting from the reaction are transferred from FAD to the electron-transferring flavoprotein, another FAD-containing protein, which donates electrons to the respiratory chain. Rat liver mitochondria contain four (short-, medium-, long- and very-long-chain) acyl-CoA dehydrogenases [3–8] that act on straight-chain acyl-CoAs and two (2-methyl-branched-chain acyl-CoA and isovaleryl-CoA) dehydrogenases that act on branched-chain acyl-CoAs. In peroxisomes the first step of β-oxidation is catalysed by an FAD-containing acyl-CoA oxidase [9,10] that transfers electrons directly to molecular oxygen, yielding H_2O_2, which is disposed of by another peroxisomal enzyme, catalase. Rat liver peroxisomes contain three acyl-CoA oxidases [11–13]: (1) an enzyme that oxidizes the CoA esters of medium-, long- and very-long-chain fatty acids and that is inducible by treatment of the animals with peroxisome proliferators (palmitoyl-CoA oxidase); (2) an enzyme that oxidizes the CoA esters of 2-methyl-branched-chain fatty acids and that is not inducible (pristanoyl-CoA oxidase); and (3) another non-inducible enzyme that oxidizes the CoA esters of the bile acid intermediates di- and tri-hydroxycoprostanic acids (trihydroxycoprostanoyl-CoA oxidase). The last enzyme is not found in extrahepatic tissues.

Human liver peroxisomes are also capable of oxidizing palmitoyl-CoA, pristanoyl-CoA and trihydroxycoprostanoyl-CoA [14,15]. As in the rat, palmitoyl-CoA and trihydroxycoprostanoyl-CoA are oxidized by separate enzymes [14]. In order to establish the presence of a separate acyl-CoA oxidase for 2-methyl-branched-chain fatty acids in human tissues, and in order to define the substrate specificities of the different human acyl-CoA oxidases, we separated human liver and kidney oxidases on various columns and measured the acyl-CoA oxidase activities in the eluates, using a variety of CoA esters as the substrates. Much to our surprise, we found during these experiments that an important portion of the short-chain acyl-CoA oxidase activity did not co-elute with the activities of palmitoyl-CoA oxidase, pristanoyl-CoA oxidase or trihydroxycoprostanoyl-CoA oxidase, suggesting that human tissues contain a separate short-chain acyl-CoA oxidase. However, purification of the enzyme and determination of its subcellular localization and substrate specificity demonstrated that the novel oxidase is identical with the mitochondrial short-chain acyl-CoA dehydrogenase, which in the absence of a suitable electron acceptor functions as an oxidase.

EXPERIMENTAL

Materials

Percoll, CoA and palmitoyl-CoA were from Pharmacia, Uppsala, Sweden. Glutaryl-CoA, butyryl-CoA, isobutyryl-CoA, valeryl-CoA, isovaleryl-CoA, hexanoyl-CoA, heptanoyl-CoA, octanoyl-CoA, decanoyl-CoA, Blue Dextran, cytochrome *c* (horse heart), phenazine methosulphate (PMS) and BSA were from Sigma, St. Louis, MO, U.S.A. The BSA was defatted as described by Chen [16]. Homovanillic acid, 4,4′-dithiodipyridine and benzamidine were from Janssen, Beerse, Belgium. 2,6-Dichloroindophenol (DCIP) was from Fluka, Buchs, Switzerland. FAD, β-galactosidase (*Escherichia coli*), urease S (jack bean), glutamate dehydrogenase (bovine liver), catalase (bovine liver), lactate dehydrogenase (pig heart), citrate synthase (pig heart), malate dehydrogenase (pig heart), uricase (pig liver) and peroxidase (grade II; 200 units/mg of solid) were from Boehringer, Mannheim, Germany. Dialysis tubing with a molecular-mass cut-off of 12000–14000 Da was purchased from Spectrum Medical Industries, Los Angeles, CA, U.S.A.

Abbreviations used: PMS, phenazine methosulphate; DCIP, 2,6-dichloroindophenol.

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Synthesis of lipids

Trihydroxycoprostanoyl-CoA [17], 2-methylpalmitoyl-CoA [12] and 2-methylhexanoyl-CoA [13] were synthesized as described in the references. The aqueous solutions of the CoA esters were standardized by measuring the A_{260} in 10 mM potassium phosphate buffer, pH 7.0 ($\epsilon = 15400$). No free CoA could be detected in these solutions by following the reaction with 4,4′-dithiodipyridine at 324 nm.

Preparation of homogenates and subcellular fractions

Human liver and kidney were obtained after partial hepatectomy or total nephrectomy for liver and kidney tumours respectively. All donors were adults. Approval was granted by the Institutional Ethics Committee. For enzyme purification, tissues were divided in small portions and stored at −80 °C until use. For subcellular-fractionation experiments, fresh liver and kidney were homogenized in 0.25 M sucrose/0.1 % (v/v) ethanol/1 mM EDTA, pH 7.2, and the homogenates were fractionated into nuclear (N), heavy mitochondrial (M), light mitochondrial (L), microsomal (P) and cytosolic (S) fractions as described before for rat liver [18]. The M-fraction, enriched in mitochondria and the L-fraction, enriched in peroxisomes, were subfractionated by isopycnic centrifugation in an iso-osmotic self-generating Percoll gradient [19].

Determination of marker enzymes and proteins

Marker enzymes (glutamate dehydrogenase, catalase, lactate dehydrogenase, acid phosphatase, glucose-6-phosphatase) and protein were determined as described previously [19].

Acyl-CoA oxidase measurements

Substrate-dependent H_2O_2 production in homogenates and subcellular fractions was measured by means of peroxidase-catalysed homovanillic acid dimerization [20,21], modified as follows. A portion of the homogenates or subcellular fractions (40 μl) diluted in homogenization medium was preincubated on ice with 10 μl of 100 μM FAD for 5–10 min in order to saturate the oxidase with cofactor. Reactions were then started by adding 0.2 ml of warm (37 °C) reaction mixture, consisting of 50 mM potassium phosphate buffer, pH 8.3, 0.75 mM homovanillic acid, 0.1 mg/ml peroxidase, 0.075 % (w/v) defatted BSA, 0.3 mM hexanoyl-CoA or 0.06 mM palmitoyl-CoA, 2-methylpalmitoyl-CoA or trihydroxycoprostanoyl-CoA. After incubation at 37 °C, reactions were terminated by adding 50 μl of 12 % (w/v) $HClO_4$. Incubation was terminated at several time points for homogenates, and rates of H_2O_2 production were calculated from the linear part of the curves. For subcellular fractions, reactions were terminated after 30 min. Denatured proteins were removed by centrifugation, and 0.2 ml of the supernatant was mixed with 2.8 ml of 0.5 M carbonate buffer, pH 10.7, containing 10 mM EDTA. After 10–15 min, fluorescence was measured (excitation 327 nm, slit 2 nm; emission 420 nm, slit 10 nm). Appropriate blanks, measured in the absence of substrate, were always performed. If necessary, fluorescence was corrected for substrate effects. Fluorescence readings were standardized by means of uric acid/uricase as described previously [20]. One enzyme unit (see legends to Figures) is defined as 1 μmol of H_2O_2 formed/min.

Partially purified oxidases and fractions from the columns were diluted in 10 mM potassium phosphate buffer (pH 7.5)/20 % (w/v) glycerol and assayed as described above. Reactions were terminated after 30 min.

Acyl-CoA dehydrogenase measurements

Acyl-CoA dehydrogenase activity was assayed spectrophotometrically with PMS and DCIP as intermediate and terminal electron acceptors, respectively, as described previously [3,6,7]. The incubation medium contained 0.1 M potassium phosphate buffer, pH 8.0, 1.5 mM PMS, 0.048 mM DCIP, 0.1 mM FAD and 0.1 mM acyl-CoA. The final volume was 0.5 ml. After preincubation of the enzyme in FAD-containing buffer for 5 min at 30 °C, PMS and DCIP were added and the reaction was started by addition of substrate. The reaction was carried out at 32 °C and bleaching of DCIP was followed at 600 nm.

Partial purification of human acyl-CoA oxidases

Human liver and kidney acyl-CoA oxidases were partially purified by heat treatment and $(NH_4)_2SO_4$ fractionation as described previously for rat liver [12].

Chromatographic separations

Protein chromatography was done on a Waters 600 system equipped with Teflon and PEEK tubing. The following columns were used: Protein PAK Glass HIC phenyl-5PW (8.0 mm × 75.0 mm) and DEAE-5PW (8.0 mm × 75.0 mm) (Nihon Waters Ltd., Tokyo, Japan), HiLoad Superdex 200 prep grade (1.6 cm × 60 cm) (Pharmacia Belga, Brussels, Belgium), and hydroxyapatite Bio-Gel HPHT (100 mm × 7.8 mm) (Bio-Rad, Richmond, CA, U.S.A.).

For hydrophobic chromatography on the phenyl column, a sample of the partially purified acyl-CoA oxidase preparation was thawed, diluted twice in 20 mM potassium phosphate buffer (pH 7.5)/0.6 M $(NH_4)_2SO_4$/10 μM FAD and dialysed overnight against the same buffer (buffer A). After centrifugation to remove undissolved material, a sample of the supernatant was injected on to the phenyl column, equilibrated in the same buffer, and the column was eluted at a flow rate of 0.8 ml/min. Bound proteins were eluted with a linear negative salt and positive ethylene glycol gradient [100–0 % buffer A; 0–100 % buffer B, containing 20 mM potassium phosphate, pH 7.5, 25 % (w/v) ethylene glycol and 10 μM FAD] over 45 min. Fractions (1.6 ml) were collected and analysed for conductivity and acyl-CoA oxidase activity, and their A_{280} was monitored. Fractions from the phenyl column containing the short-chain acyl-CoA oxidase activity were pooled, 0.1 vol. of 1 M potassium phosphate buffer, pH 8, was added, and the solution was adjusted to 50 % $(NH_4)_2SO_4$ saturation. After centrifugation, the pellet was dissolved in 200 mM potassium phosphate buffer, pH 7.5, containing 10 % ethylene glycol and 10 μM FAD, and dialysed overnight against the same buffer. The undissolved material was removed by centrifugation, and a sample of the supernatant was subjected to gel filtration on a HiLoad Superdex 200 column, equilibrated in the same buffer, and eluted at a flow rate of 1 ml/min. The A_{280} was monitored. Fractions (2 ml) were collected and assayed for acyl-CoA oxidase activity. The fractions from the gel-filtration column containing the highest short-chain acyl-CoA oxidase activity were pooled and concentrated by $(NH_4)_2SO_4$ precipitation. After centrifugation, the pellet was dissolved in 5 mM potassium phosphate buffer (pH 7.2)/1 mM EDTA (separation of the oxidases is better in the presence of EDTA)/10 μM FAD/10 % ethylene glycol, and dialysed overnight against the same buffer. The undissolved material was removed by centri-

fugation, and a portion of the supernatant was injected on to a hydroxyapatite column, equilibrated in the same phosphate buffer. After a wash of 5 min, the adsorbed oxidases were eluted with a phosphate gradient, increasing linearly from 5 to 200 mM potassium phosphate, pH 7.2, in the presence of 1 mM EDTA, 10 μM FAD and 10% ethylene glycol for 60 min. Elution rate was 0.6 ml/min; 1.8 ml fractions were collected and assayed for acyl-CoA oxidase activity and P_i, and their A_{280} was monitored.

The fractions from the hydroxyapatite column containing the highest activity of short-chain acyl-CoA oxidase were pooled and dialysed against 20 mM Tris/HCl buffer, pH 8.6, containing 20% (w/v) glycerol. A portion was injected on to a DEAE column, equilibrated in the same buffer, and the column was eluted at a flow rate of 1 ml/min. Bound proteins were eluted with a linear pH/salt gradient (0–100% buffer containing 20 mM Tris/HCl, pH 7.8, 0.25 M NaCl and 20% glycerol) over 40 min. Fractions (2 ml) were collected in tubes containing 20 μl of 1 mM FAD. The fractions were monitored at 210 nm, and analysed for acyl-CoA oxidase activity and conductivity.

SDS/PAGE

Electrophoresis in 10–20% (w/v) acrylamide gradient gels was done as described previously [11]. Gels were silver-stained as described by Guevara et al. [22].

RESULTS

Presence of a separate short-chain acyl-CoA oxidase in human liver and kidney

In order to establish the presence of several acyl-CoA oxidases with different substrate specificities in human tissues, we partially purified the oxidases from human liver and kidney by $(NH_4)_2SO_4$ fractionation and heat treatment. In a first screening, the partially purified preparations were loaded on various columns and the eluates were analysed for acyl-CoA oxidase activity with several acyl-CoA esters as the substrates. As an example, Figure 1 shows the elution pattern after hydrophobic chromatography of a partially purified oxidase preparation from liver on a phenyl column. 2-Methylpalmitoyl-CoA oxidase, which is identical with pristanoyl-CoA oxidase [12], was eluted in a single peak. Trihydroxycoprostanoyl-CoA oxidase co-eluted, as a single peak, with 2-methylpalmitoyl-CoA oxidase. Palmitoyl-CoA oxidase activity was eluted in a broad peak showing a shoulder that coincided with the fractions containing the highest 2-methylpalmitoyl-CoA oxidase and trihydroxycoprostanoyl-CoA oxidase activities. This result suggests that in the human, as in the rat [12,13], palmitoyl-CoA is oxidized by two enzymes: palmitoyl-CoA oxidase (peak) and pristanoyl-CoA oxidase (shoulder). Hexanoyl-CoA oxidase was eluted in three peaks: a first peak that was well separated from the palmitoyl-CoA oxidase, 2-methylpalmitoyl-CoA oxidase and trihydroxycoprostanoyl-CoA oxidase peaks; a second peak that co-eluted with the palmitoyl-CoA oxidase peak; and a third peak that co-eluted with the 2-methylpalmitoyl-CoA oxidase/trihydroxycoprostanoyl-CoA oxidase peak. These results suggest that (1) a separate short-chain acyl-CoA oxidase might exist in the human (first peak); (2) as is the case for rat palmitoyl-CoA oxidase (13), human palmitoyl-CoA oxidase shows some activity with hexanoyl-CoA (second peak); and (3) human pristanoyl-CoA oxidase is also slightly active towards hexanoyl-CoA (third peak); the last is not true for rat pristanoyl-CoA oxidase [13].

A significant portion of the hexanoyl-CoA oxidase activity could also be separated from palmitoyl-CoA oxidase, pristanoyl-CoA oxidase and trihydroxycoprostanoyl-CoA oxidase on hydroxyapatite and gel-filtration columns (results not shown). A similar separation of a portion of the hexanoyl-CoA oxidase activity from the other oxidase activities was obtained after chromatography of partially purified oxidase preparations from kidney (results not shown). Surprisingly, the latter preparations also contained significant trihydroxycoprostanoyl-CoA oxidase activity. In the rat, trihydroxycoprostanoyl-CoA oxidase activity cannot be detected in kidney or other extrahepatic tissues [12].

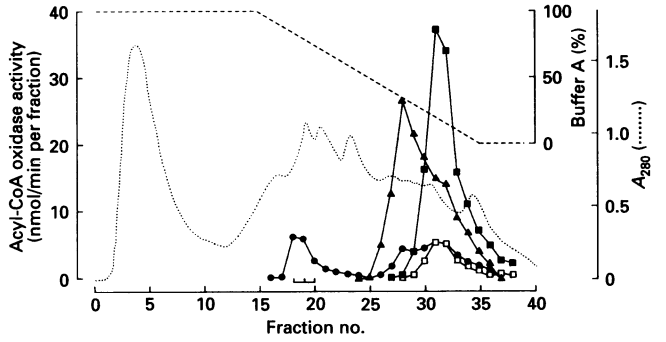

Figure 1 Separation of human liver acyl-CoA oxidases by hydrophobic chromatography on a phenyl column

A partially purified oxidase preparation obtained by heat treatment and $(NH_4)_2SO_4$ fractionation was diluted 2-fold in 20 mM potassium phosphate buffer (pH 7.5)/0.6 M $(NH_4)_2SO_4$/10 μM FAD, dialysed overnight against the same buffer, and 4 ml, containing 220.8 m-units of palmitoyl-CoA oxidase, 33.6 m-units of trihydroxycoprostanoyl-CoA oxidase, 181.2 m-units of 2-methylpalmitoyl-CoA oxidase and 90.4 m-units of hexanoyl-CoA oxidase, was injected on to the phenyl column, equilibrated with the same buffer. Bound proteins were eluted as described in the Experimental section. Fractions (1.6 ml) were collected and analysed for conductivity (% buffer A in the Figure), palmitoyl-CoA oxidase (▲; recovery 61%), trihydroxycoprostanoyl-CoA oxidase (□; recovery 61%), 2-methylpalmitoyl-CoA oxidase (■; recovery 74%) and hexanoyl-CoA oxidase (●; recovery 60%). For clarity, symbols were omitted when no activity was found in a fraction. Fractions 18–21 (horizontal bar) from five separate runs were pooled and subjected to gel filtration (see the text).

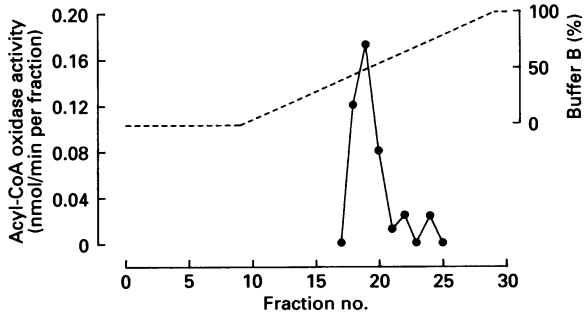

Figure 2 Chromatography of hepatic short-chain acyl-CoA oxidase on a DEAE anion-exchange column

The fractions most enriched in hexanoyl-CoA oxidase from the hydroxyapatite column (see the text) were combined and dialysed against 20 mM Tris/HCl buffer (pH 8.6)/20% glycerol. A 4.2 ml portion containing 1.8 m-units of hexanoyl-CoA oxidase was applied on to the DEAE column, equilibrated with the same buffer. Bound proteins were eluted with a linear pH/salt gradient as described in the Experimental section. Fractions (2 ml) were collected and analysed for conductivity (% buffer B in the Figure) and hexanoyl-CoA oxidase activity (●; recovery 25%).

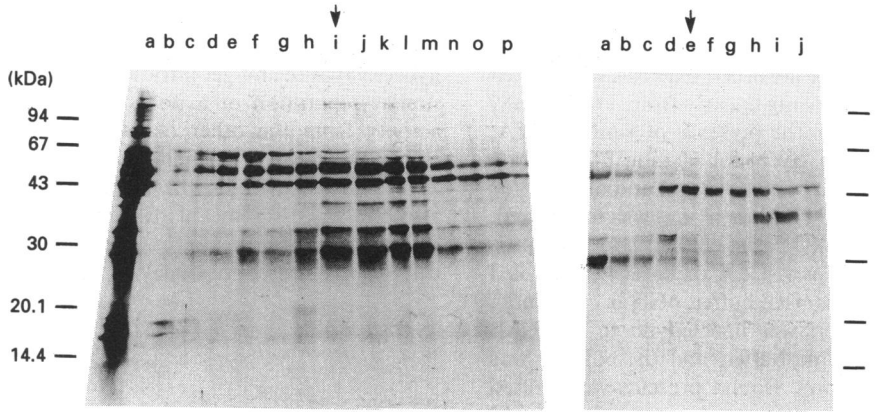

Figure 3 Analysis of column fractions by SDS/PAGE

Fractions from the hydroxyapatite and DEAE columns were analysed by SDS/PAGE. Positions of the molecular-mass markers (kDa) are shown at the left. Left panel: lane **a**, proteins contained in 20 μl of the preparation applied to the hydroxyapatite column; lanes **b–p**, proteins contained in 100 μl of fractions 10–24 respectively from the hydroxyapatite column. The arrow indicates the fraction containing the highest activity towards hexanoyl-CoA (fraction 17). Right panel: lanes **a–j**, proteins contained in 500 μl of fractions 15–24 from the DEAE column. The arrow indicates the fraction containing the highest activity towards hexanoyl-CoA (fraction 19).

Table 1 Substrate specificity of short-chain acyl-CoA oxidase

A partially purified acyl-CoA oxidase preparation from human liver was separated on a phenyl column, the fractions (18–21 of Figure 1) containing the short-chain acyl-CoA oxidase were pooled and acyl-CoA oxidase activity was measured on the pooled fractions with the CoA esters listed in the Table as the substrates. Final substrate concentrations are indicated in parentheses.

Substrate	Acyl-CoA oxidase activity (nmol/min per ml)
Butyryl-CoA (250 μM)	1.27
Isobutyryl-CoA (250 μM)	0.07
Glutaryl-CoA (250 μM)	0.1
Valeryl-CoA (250 μM)	2.94
Isovaleryl-CoA (250 μM)	0
Hexanoyl-CoA (250 μM)	2.98
2-Methylhexanoyl-CoA (250 μM)	1.72
Heptanoyl-CoA (250 μM)	1.52
Octanoyl-CoA (50 μM)	0.03
(250 μM)	0.13
Decanoyl-CoA (50 μM)	0
(250 μM)	0
Palmitoyl-CoA (50 μM)	0

Purification of the hepatic short-chain acyl-CoA oxidase

Subsequently, we attempted to purify the hepatic short-chain acyl-CoA oxidase by chromatography on various columns. Hydrophobic chromatography on a phenyl column was chosen as the first purification step (Figure 1). The fractions of the first hexanoyl-CoA oxidase peak were pooled, concentrated and subjected to gel filtration (results not shown). The molecular mass of the enzyme was estimated at 189 ± 5 kDa (mean $\pm$ S.E.M. for 4 experiments). The active fractions from the gel-filtration column were pooled, concentrated and applied to a hydroxyapatite column (results not shown). Finally, the active fractions from the hydroxyapatite column were pooled and loaded on a DEAE anion-exchange column (Figure 2). The fractions eluted from the hydroxyapatite and DEAE columns were also analysed by SDS/PAGE (Figure 3). In the fractions of highest purity only one major band with a molecular mass of 43 kDa was visible. Similar results were obtained for the short-chain acyl-CoA oxidase from kidney (results not shown).

The hexanoyl-CoA oxidase activities found in the second and third peaks from the phenyl column (Figure 1) invariably co-eluted from the columns (gel filtration, hydroxyapatite, DEAE) with palmitoyl-CoA oxidase and with pristanoyl-CoA oxidase respectively, confirming that these activities are side activities of palmitoyl-CoA oxidase and pristanoyl-CoA oxidase (results not shown).

Substrate-dependence and specificity of short-chain acyl-CoA oxidase

The substrate-dependence and specificity of the short-chain acyl-CoA oxidase were investigated in the pooled fractions containing the first hexanoyl-CoA oxidase peak from a phenyl column (see Figure 1).

Substrate-dependence was determined with hexanoyl-CoA as the substrate. After linear transformation of the curve in accordance with Lineweaver–Burk, an apparent K_m of 130 μM was calculated. Addition of albumin to the assay mixtures did not influence the kinetic parameters (results not shown).

The substrate specificity of the enzyme is shown in Table 1. The enzyme was most active towards hexanoyl-CoA and valeryl-CoA (C_5). Significant activity was also found with butyryl-CoA, heptanoyl-CoA, and the branched 2-methylhexanoyl-CoA. No or only insignificant activities were observed with the CoA esters of medium- (octanoic acid, decanoic acid) and long- (palmitic acid) chain fatty acids, short-chain dicarboxylic fatty acids (glutaric acid, C_5), the 2-methyl-branched isobutyric acid and the 3-methyl-branched isovaleric acid.

Subcellular distribution of hexanoyl-CoA oxidase

In the conviction that we had discovered a novel peroxisomal short-chain acyl-CoA oxidase, we wished to confirm the enzyme's peroxisomal localization by subcellular-fractionation experiments. Therefore, a kidney homogenate was fractionated by differential centrifugation in fractions enriched in nuclei, mitochondria (heavy mitochondrial fraction), peroxisomes and lyso-

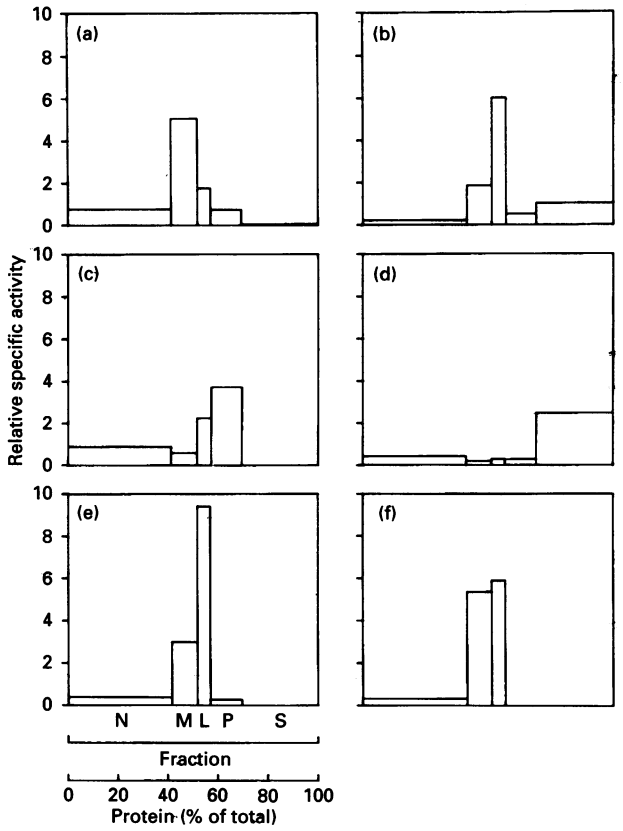

Figure 4 Subcellular distribution of hexanoyl-CoA oxidase in human kidney

Fresh human kidney was homogenized and fractionated by differential centrifugation into a nuclear (N), a heavy mitochondrial (M), a light mitochondrial (L), a microsomal (P) and a soluble (S) fraction, and marker enzymes, palmitoyl-CoA oxidase and hexanoyl-CoA oxidase were measured in each fraction. (**a**) Glutamate dehydrogenase (mitochondria); (**b**) catalase (peroxisomes); (**c**) glucose-6-phosphatase (endoplasmic reticulum); (**d**) lactate dehydrogenase (cytosol); (**e**) palmitoyl-CoA oxidase (peroxisomes); (**f**) hexanoyl-CoA oxidase. Results are expressed as relative specific activities versus percentage of total protein. Relative specific activity is defined as the percentage of total recovered activity present in a particular fraction divided by the corresponding percentage of total protein. Recoveries were between 79 and 113%.

somes (light mitochondrial fraction), endoplasmic reticulum, and soluble components. Figure 4 shows the relative specific activities of the marker enzymes in the different subcellular fractions. Surprisingly, the subcellular distribution of hexanoyl-CoA oxidase did not coincide with that of catalase and palmitoyl-CoA oxidase, two markers for the peroxisomal matrix. In the heavy mitochondrial fraction, the relative specific activity of hexanoyl-CoA oxidase was substantially higher than that of the peroxisomal markers, suggesting that part of the hexanoyl-CoA oxidase activity was associated with the mitochondria. The experiment was repeated once with kidney and once with liver, and each time very similar results were obtained (not shown). We therefore subfractionated a heavy and a light mitochondrial fraction from kidney on a self-generating Percoll gradient. Figure 5 shows the results obtained with the heavy mitochondrial fraction. Hexanoyl-CoA oxidase activity followed a bimodal distribution, but most of its activity coincided with that of the mitochondrial marker glutamate dehydrogenase, indicating a predominantly mitochondrial localization of the hexanoyl-CoA oxidase activity present in the heavy mitochondrial fraction. In contrast, palmitoyl-CoA oxidase was present almost completely in the catalase-containing gradient fractions. When the light mitochondrial fraction was subfractionated on a self-generating Percoll gradient, most of the hexanoyl-CoA oxidase activity was present in the fractions containing catalase and palmitoyl-CoA oxidase, and only a smaller part was present in the fractions containing glutamate dehydrogenase, demonstrating that the hexanoyl-CoA oxidase activity present in the light mitochondrial fraction is mostly peroxisomal (results not shown).

The hexanoyl-CoA oxidase activity present in the heavy mitochondrial fraction is due to the mitochondrial short-chain acyl-CoA dehydrogenase

Since the molecular mass (the native enzyme and its subunit) and the substrate specificity of our purified short-chain acyl-CoA oxidase agreed reasonably well with those of the short-chain acyl-CoA dehydrogenase reported by Finocchiaro et al. [7], we assumed that the mitochondrial hexanoyl-CoA oxidase activity was due to the short-chain acyl-CoA dehydrogenase and that the peroxisomal hexanoyl-CoA oxidase activity was a side activity of palmitoyl-CoA and pristanoyl-CoA oxidase. In order to prove this hypothesis, a liver homogenate was fractionated by differential centrifugation in fractions enriched in nuclei, mitochondria (heavy mitochondrial fraction), peroxisomes and lysosomes (light mitochondrial fraction), endoplasmic reticulum, and soluble components.

The heavy mitochondrial fraction was subjected to freeze–thawing and sonication followed by centrifugation, and the supernatant containing the released matrix proteins was loaded on a phenyl column. The column fractions were analysed for hexanoyl-CoA oxidase and hexanoyl-CoA dehydrogenase activity (Figure 6). Hexanoyl-CoA oxidase was eluted, as a single peak, in the region where the purified short-chain acyl-CoA oxidase was expected to be eluted, and virtually no activity was found in the region where the peroxisomal acyl-CoA oxidases are normally eluted (see Figure 1). The oxidase and dehydrogenase activities towards hexanoyl-CoA coincided, the dehydrogenase activity being approx. 20 times higher than the oxidase activity. The results indicate that (1) the purified acyl-CoA oxidase is of mitochondrial origin and (2) the oxidase activity is catalysed by the mitochondrial short-chain acyl-CoA dehydrogenase. Further evidence for these conclusions was obtained in the following experiments. The light mitochondrial fraction, prepared by differential centrifugation as described above, was subfractionated on a self-generating Percoll gradient (Figure 7), the fractions containing the mitochondria (glutamate dehydrogenase) and those containing the peroxisomes (catalase, palmitoyl-CoA oxidase) were separately pooled, and the mitochondrial and peroxisomal matrix proteins were extracted by freeze–thawing and sonication in hypotonic buffer followed by centrifugation. The released mitochondrial and peroxisomal matrix proteins were chromatographed on a phenyl column in two separate runs, and the eluates were analysed for hexanoyl-CoA oxidase, palmitoyl-CoA oxidase and 2-methylpalmitoyl-CoA oxidase activities. The combined results of these experiments again confirmed that the hexanoyl-CoA oxidase associated with the mitochondrial matrix proteins was eluted in the same fractions as the purified short-chain acyl-CoA oxidase (peak 1 of Figure 1) and the short-chain acyl-CoA dehydrogenase (see Figure 6), and that this separate short-chain acyl-CoA oxidase was absent from peroxisomal matrix proteins (results not shown).

Finally, a partially purified oxidase preparation from liver prepared by heat treatment and $(NH_4)_2SO_4$ fractionation was chromatographed on a phenyl column exactly as described for Figure 1, and acyl-CoA dehydrogenase activities were measured

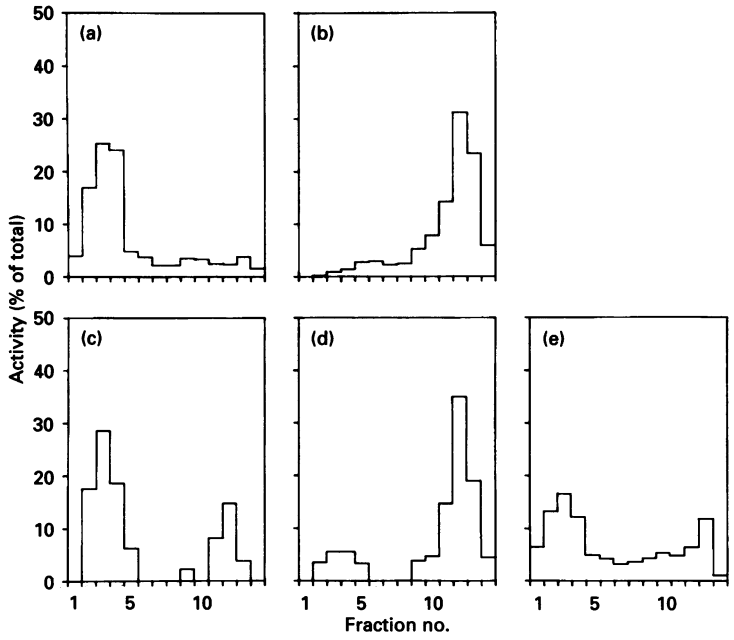

Figure 5 Subfractionation of a heavy mitochondrial fraction on a self-generating Percoll gradient

A heavy mitochondrial fraction, prepared by differential centrifugation and derived from 4 g of kidney, was subfractionated by isopycnic centrifugation in an iso-osmotic self-generating Percoll gradient. The gradient fractions were analysed for glutamate dehydrogenase (**a**), catalase (**b**), hexanoyl-CoA oxidase (**c**), palmitoyl-CoA oxidase (**d**) and protein (**e**). Results are expressed as percentage of total gradient activity or content present in each fraction numbered on the abscissa. Fractions 1 and 14 represent the fractions of highest and lowest density respectively. Recoveries were between 65 and 140%.

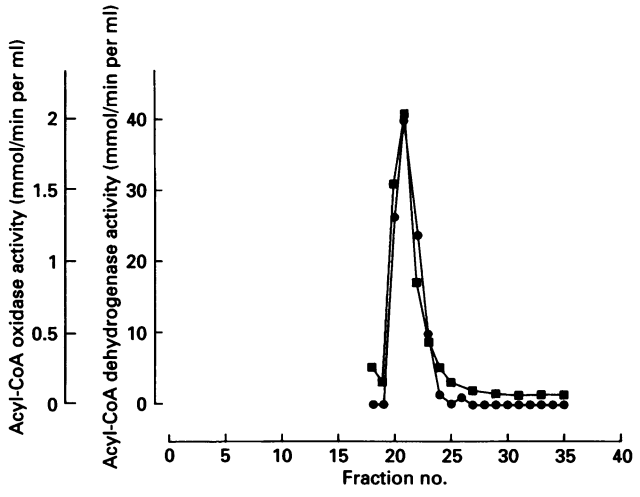

Figure 6 Hydrophobic chromatography of mitochondrial matrix proteins derived from human liver

Fresh human liver was homogenized and fractionated by differential centrifugation into a nuclear (N), a heavy mitochondrial (M), a light mitochondrial (L), a microsomal (P) and a soluble (S) fraction. In order to extract the matrix proteins, the heavy mitochondrial fraction was subjected to freeze–thawing and sonication followed by centrifugation, and the supernatant, containing the released matrix proteins, was dialysed overnight against 20 mM potassium phosphate buffer (pH 7.5)/0.6 M $(NH_4)_2SO_4$/10 μM FAD. A 4.8 ml portion of the dialysed supernatant containing 13.7 m-units of hexanoyl-CoA oxidase and 224 m-units of hexanoyl-CoA dehydrogenase was injected on to the phenyl column, equilibrated with the same buffer. Bound proteins were eluted as described in the Experimental section. Fractions (1.6 ml) were collected and analysed for hexanoyl-CoA oxidase (●; recovery 60%) and hexanoyl-CoA dehydrogenase activity (■; recovery 101%).

on the pooled fractions of the first hexanoyl-CoA oxidase peak and on those of the second and third peaks (containing the palmitoyl-CoA oxidase and 2-methylpalmitoyl-CoA oxidase activities). In the pooled first hexanoyl-CoA oxidase peak, acyl-CoA dehydrogenase was most active towards valeryl-CoA (460 nmol/min per ml), followed by hexanoyl-CoA (343 nmol/min per ml), 2-methylhexanoyl-CoA (250 nmol/min per ml) and butyryl-CoA (150 nmol/min per ml). As expected for the short-chain acyl-CoA dehydrogenase, no activity could be observed towards the CoA esters of medium- (octanoic, decanoic) and long- (palmitic) chain fatty acids. No acyl-CoA dehydrogenase activity was found in the pooled second and third peaks, indicating that the hexanoyl-CoA oxidase activity associated with these fractions is a side activity of the peroxisomal palmitoyl-CoA and pristanoyl-CoA oxidases.

DISCUSSION

This report demonstrates that the novel short-chain acyl-CoA oxidase that we originally thought to have purified is in fact the mitochondrial short-chain acyl-CoA dehydrogenase. Our conclusion is based on the following lines of evidence. (1) The short-chain acyl-CoA oxidase is present in mitochondria and absent from peroxisomes. (2) The substrate spectrum of the oxidase closely resembles that of the short-chain acyl-CoA dehydrogenase as reported by Finocchiaro et al. [7]. (3) On column chromatography the short-chain acyl-CoA oxidase was well separated from the peroxisomal acyl-CoA oxidases, but co-eluted with the mitochondrial short-chain acyl-CoA dehydrogenase. (4) The molecular mass of the subunit (43 kDa) of the purified oxidase is similar to that published by Tanaka's laboratory for the short-chain acyl-CoA dehydrogenase (41–42 kDa) [7,23]. Although the native molecular mass of the enzyme, as estimated by us

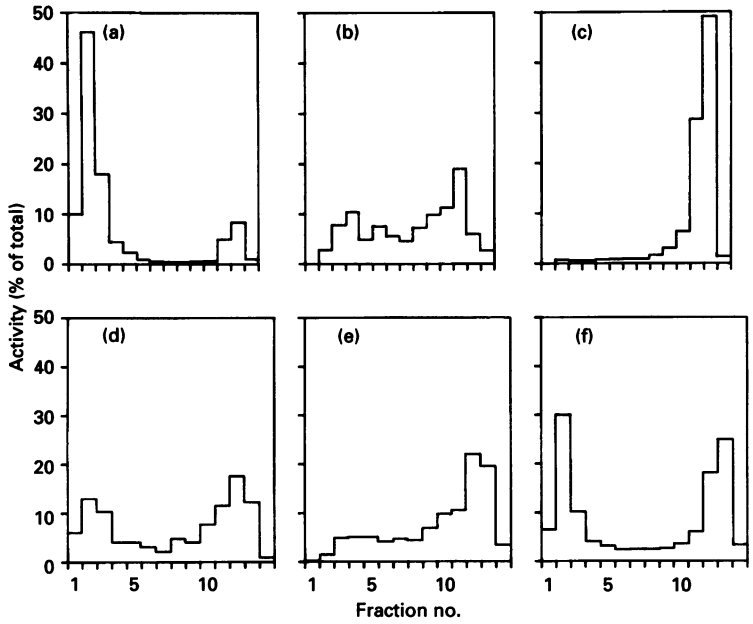

Figure 7 Subfractionation of a light mitochondrial fraction on a self-generating Percoll gradient

A light mitochondrial fraction, prepared by differential centrifugation and derived from 10 g of liver, was subfractionated by isopycnic centrifugation in an iso-osmotic self-generating Percoll gradient. The gradient fractions were analysed for glutamate dehydrogenase (**a**), catalase (**b**), glucose-6-phosphatase (**c**), hexanoyl-CoA oxidase (**d**), palmitoyl-CoA oxidase (**e**) and protein (**f**). Results are expressed as percentage of total gradient activity or content present in each fraction numbered on the abscissa. Fractions 1 and 14 represent the fractions of highest and lowest density, respectively. Recoveries were between 60 and 125%.

(189 kDa), is somewhat higher than the value reported by the laboratory of Tanaka (168 kDa) [7], we interpret the difference as being due to differences in experimental conditions.

Our results mean that in the absence of suitable electron acceptors human short-chain acyl-CoA dehydrogenase is capable of transferring electrons directly to molecular oxygen, albeit at lower rates than the reduction rates of the electron acceptors. Mitochondria contain four acyl-CoA dehydrogenases involved in the oxidation of straight-chain acyl-CoAs and two acyl-CoA dehydrogenases involved in the oxidation of branched-chain acyl-CoAs (see the Introduction). It remains to be investigated whether the other acyl-CoA dehydrogenases are also capable of functioning as oxidases when the proper electron acceptors are lacking. The observations that the subcellular distribution of palmitoyl-CoA oxidase was clearly peroxisomal and that virtually no palmitoyl-CoA dehydrogenase activity could be detected in column fractions displaying palmitoyl-CoA oxidase activity (G. Vanhove, P. P. Van Veldhoven and G. P. Mannaerts, unpublished work) suggest that at least the long- and very-long-chain acyl-CoA dehydrogenases, both of which are active towards palmitoyl-CoA, do not, or only poorly, function as oxidases.

The question remains as to whether our observations are of purely academic interest or whether they might have physiological or pathophysiological significance. Perhaps there might be implications for the pathogenesis of reperfusion injury after cardiac ischaemia. Reperfusion injury to the myocardium is believed to be caused, at least in part, by reactive oxygen species, and high fatty acid levels aggravate this injury [24]. After cardiac ischaemia, the activity of the electron-transport chain, which accepts electrons from the acyl-CoA dehydrogenases via the electron-transferring protein, is decreased [25]. Is it possible that the short-chain acyl-CoA dehydrogenase, which is present not only in liver and kidney but in other tissues as well [26], behaves as an oxidase under conditions of diminished electron-transport activity, thereby producing harmful amounts of H_2O_2?

Finally, during these studies the intriguing observation was made that trihydroxycoprostanoyl-CoA oxidase is present not only in human liver but also in human kidney. In the rat, trihydroxycoprostanoyl-CoA oxidase is absent from extrahepatic tissues. The significance of the enzyme's presence in human extrahepatic tissues is currently under study.

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