Presence of Three Acyl-CoA Oxidases in Rat Liver Peroxisomes

AN INDUCIBLE FATTY ACYL-CoA OXIDASE, A NONINDUCIBLE FATTY ACYL-CoA OXIDASE, AND A NONINDUCIBLE TRIHYDROXYCOPROSTANOYL-CoA OXIDASE

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Linda Schepers, Paul P. Van Veldhoven, Minne Casteels†, Hendrik J. Eyssen‡, and Guy P. Mannaerts§

From the Katholieke Universiteit Leuven, Afdeling Farmacologie, Campus Gasthuisberg, B-3000 Leuven, Belgium and the §Rega Instituut, Minderbroedersstraat, B-3000 Leuven, Belgium

Mammalian liver peroxisomes are capable of β-oxidizing a variety of substrates including very long chain fatty acids and the side chains of the bile acid intermediates di- and trihydroxycoprostanic acid. The first enzyme of peroxisomal β-oxidation is acyl-CoA oxidase. It remains unknown whether peroxisomes possess one or several acyl-CoA oxidases.

Peroxisomal oxidases from rat liver were partially purified by (NH₄)₂SO₄ precipitation and heat treatment, and the preparation was subjected to chromatography on hydroxylapatite and dye affinity matrices, and gel filtration. The column eluates were assayed for palmitoyl-CoA and trihydroxycoprostanoyl-CoA oxidase activities and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The results revealed the presence of three acyl-CoA oxidases: 1) a fatty acyl-CoA oxidase with a pI of 8.3 and an apparent molecular mass of 145 kDa. The enzyme consisted mainly of 52- and 22.5-kDa subunits and could be induced by clofibrate treatment; 2) a noninducible fatty acyl-CoA oxidase with a pI of 7.1 and an apparent molecular mass of 427 kDa. It consisted mainly, if not exclusively, of one polypeptide component of 71 kDa; and 3) a noninducible trihydroxycoprostanoyl-CoA oxidase with a pI of 7.1 and an apparent molecular mass of 139 kDa. It consisted mainly, if not exclusively, of one polypeptide component of 69 kDa.

Our findings are probably related to the recent discovery of two species of acyl-CoA oxidase mRNA in rat liver (Miyazawa, S., Hayashi, H., Hijikata, M., Ishii, N., Furuta, S., Kagamiyama, H., Osumi, T., and Hashimoto, T. (1987) J. Biol. Chem. 262, 8131–8137) and they probably also explain why in human peroxisomes the accumulation of very long chain fatty acids is not always accompanied by an excretion of bile acid intermediates and vice versa.
MATERIALS AND METHODS

Animals—Male Wistar rats weighing 120-150 g were maintained on a standard laboratory diet for 2 weeks. Clofibrate-treated rats were kept on the same diet containing 0.3% (v/w) clofibrate.

Partial Purification of Peroxisomal Oxidases—Rat liver and kidney homogenates were fractionated by differential centrifugation as described previously (11) to prepare a "1"-fraction enriched in peroxisomes and peroxisomal matrix proteins. After centrifugation at 100,000 g for 40 min, the oxidases present in the supernatant were concentrated by dialysis against solid polyethylene glycol 20,000. These fractions were applied either to dye affinity columns (Dyematrex, Amicon) or to a hydroxylapatite column (Bio-Rad), equilibrated with PPi buffer, pH 7.2, containing 25 mM FAD and 1 mM EDTA and stored at -20 °C for further use. Calculated on a protein basis, the above procedure resulted in an approximately 40-fold purification of palmitoyl-CoA oxidase or THC-CoA oxidase with respect to the homogenate.

Separation of Acyl-CoA Oxidases by Column Chromatography—For chromatofocusing, aliquots of the partially purified enzyme preparations were thawed, the storage buffer was replaced by 25 mM ethanolamine/acetate buffer, pH 9, by means of Sephadex G-25 chromatography and a portion of the proteins was applied to a PBE 94 chromatofocusing column (0.9 × 12 cm; Pharmacia LKB Biotechnology Inc.) equilibrated with ethanolamine/acetate acid buffer. The column was washed with 3 ml of equilibration buffer and the absorbed proteins were eluted at a rate of 0.5 ml/min by means of a pH gradient generated by 25 mM Polybuffer 96 (Pharmacia) adjusted to pH 6 with acetic acid. Fractions of 3 ml were collected in tubes containing 30 μl of 1 mM FAD. The fractions were dialyzed against 10 mM PPi buffer, pH 9, containing 10 μM FAD and 1 mM EDTA and stored at -20 °C for further use. Calculated on a protein basis, the above procedure resulted in an approximately 40-fold purification of palmitoyl-CoA oxidase and THC-CoA oxidase with respect to the homogenate.

Separation of Acyl-CoA Oxidases by Column Chromatography—For chromatofocusing, aliquots of the partially purified enzyme preparations were applied to a chromafocusing column (0.9 × 12 cm; Pharmacia LKB Biotechnology Inc.) equilibrated with polybuffer 96 (Pharmacia) adjusted to pH 6 with acetic acid. Fractions of 3 ml were collected in tubes containing 30 μl of 1 mM FAD. The fractions were dialyzed against 10 mM PPi buffer, pH 9, containing 10 μM FAD and 1 mM EDTA (storage in Polybuffer leads to inactivation of the oxidases) and protein, palmitoyl-CoA oxidase and THC-CoA oxidase were determined on the dialyzed fractions.

In some experiments the fractions containing the highest activity of palmitoyl-CoA oxidase or THC-CoA oxidase after chromatofocusing were pooled, dialyzed as described above, and, if necessary, concentrated by dialysis against solid polyethylene glycol 20,000. These fractions were applied either to dye affinity columns (Dyematrex, Amicon), equilibrated with PPi buffer, pH 7.2, containing 10 mM FAD and 10% (v/v) ethylene glycol. Elution of the proteins from the hydroxylapatite column was achieved with a phosphate gradient at a flow rate of 0.5 ml/min. The pooled and concentrated fractions from the chromatofocusing column were subjected to gel filtration on a Ultrogel AcA 44 column (1.6 × 80 cm; LKB), calibrated with catalase, lactate dehydrogenase, citrate synthase, ovalbumin, and soybean trypsin inhibitor or on a Protein PAK 300 SW column (0.8 × 30 cm; Nikon-Waters), calibrated with ferritin, catalase, lactate dehydrogenase, and citrate synthase.

Measurement of Acyl-CoA Oxidase Activity—Acyl-CoA oxidase activities were measured by following the production of H2O2 with palmitoyl-CoA or THC-CoA as the substrates (9, 10). Protein was measured according to the method of Peterson (12) with bovine serum albumin as standard.

RESULTS

Osumi, Hashimoto, and coworkers (7) reported the deduced amino acid sequence of the translation products of the two acyl-CoA oxidase mRNA species present in rat liver. We calculated the pl for each of the polyadenylates and found 8.6 for the first sequence, which corresponds to the purified induced enzyme, an 8.1 for the second sequence. Since the difference seemed reasonably large, we decided to chromatofocus a partially purified preparation of peroxisomal oxidases. When a preparation from a normal rat liver was used, we observed two separate peaks of palmitoyl-CoA oxidase activity

FIG. 1. Separation of partially purified palmitoyl-CoA oxidase and THC-CoA oxidase by chromatofocusing. 2 ml of a partially purified preparation of peroxisomal oxidases were separated on a chromatofocusing column as described under "Materials and Methods." A, palmitoyl-CoA oxidase; O, THC-CoA oxidase; pH. A, liver preparation from control animals, containing 450 milliunits of palmitoyl-CoA oxidase, 28.4 milliunits of THC-CoA oxidase and 1.3 mg of protein/ml. Recoveries after chromatofocusing were 60, 87, and 90%, respectively. B, liver preparation from clofibrate-treated animals, containing 2876 milliunits of palmitoyl-CoA oxidase, 450 milliunits of palmitoyl-CoA oxidase, 13.7 milliunits of THC-CoA oxidase and 3.4 mg of protein/ml. Recoveries were 71, 107, and 52%, respectively. C, kidney preparation from control animals, containing 575 milliunits of palmitoyl-CoA oxidase and 3.3 mg of protein/ml. Recoveries were 55 and 74%, respectively. D, kidney preparation from clofibrate-treated animals, containing 280 milliunits of palmitoyl-CoA oxidase and 2.3 mg of protein/ml. Recoveries after chromatofocusing were 60, 87, and 90%, respectively. B, liver preparation from clofibrate-treated animals, containing 2876 milliunits of palmitoyl-CoA oxidase, 450 milliunits of palmitoyl-CoA oxidase, 13.7 milliunits of THC-CoA oxidase and 3.4 mg of protein/ml. Recoveries after chromatofocusing were 60, 87, and 90%, respectively. B, liver preparation from clofibrate-treated animals, containing

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with a pI of 8.31 and 7.13, respectively (Fig. 1A).\(^2\) THC-CoA oxidase displayed a single peak that coincided with the palmitoyl-CoA oxidase peak at pH 7.1. When a preparation from a clofibrate-treated rat liver was focused, palmitoyl-CoA oxidase was no longer present in two peaks but a large single peak with a pI of 8.35 appeared (Fig. 1B). The activity at pH 8.3 was much larger in the liver from treated than from control rats, demonstrating that this activity represents the inducible enzyme. Except for a small shoulder at pH 7.1-7.2, there was little indication of a second palmitoyl-CoA oxidase in the liver from clofibrate-treated rats. It is probable, however, that the second enzyme was masked by the trailing edge of the large peak of inducible activity. In any case, the data show that the palmitoyl-CoA oxidase found at pH 7.1 was not induced by clofibrate treatment. Equally importantly, the fact that the enzyme with the lower pI was certainly not more abundant in the liver from treated rats, precludes the possibility that it was artifactually formed in vitro from the enzyme with the more alkaline pI and most probably also explains why the enzyme with the lower pI has been overlooked during earlier purification of the induced enzyme (3, 4).

THC-CoA oxidase was found in the treated liver in the same pH region as in the normal liver. Its activity was not increased after clofibrate treatment, confirming that the enzyme is not induced (10).

No THC-CoA oxidase was found in kidney (Fig. 1, C and D). The palmitoyl-CoA oxidase with the more alkaline pI was by far the major form of the enzyme in kidney from treated as well as from untreated animals. It is not clear whether the small shoulder at pH 7.1-7.3 (Fig. 1C) is an indication for the presence in kidney of a small amount of the second palmitoyl-CoA oxidase.

Fig. 2. Effect of different additions and storage conditions on palmitoyl-CoA oxidase and THC-CoA oxidase. The fractions with the highest palmitoyl-CoA oxidase and THC-CoA oxidase activities after chromatofocusing (fractions 12 and 21 of the experiment represented in Fig. 1A) were assayed in the presence of LiCl (A), in the presence of N-ethylmaleimide (NEM) (C), and after storage for 16 h at 4°C in the presence of the indicated percentage of ethylene glycol (D). \(\Delta\), Palmitoyl-CoA oxidase in fraction 12; O, palmitoyl-CoA oxidase in fraction 21; \(\Box\), THC-CoA oxidase in fraction 21. Results essentially similar to those of A, were obtained in the presence of NaCl and KCl. The effect of ethylene glycol was not due to an interference with the assay method. The increased activity of THC-CoA oxidase in the presence of ethylene glycol was due partly to an activation and partly to a stabilization of the enzyme. Palmitoyl-CoA oxidase was stable in the absence of ethylene glycol.

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\(^2\)For the sake of simplicity, the pH of the fraction in which a protein is eluted is denoted by the column pI value. This is not necessarily correct. Although a protein is eluted from the column when the pH of the surrounding buffer has reached the pI value of the protein, the buffer travels faster through the column than the protein so that the pH of the fraction in which the protein is recovered is somewhat lower than the true pI value. Osumi et al. (3) found a pI of 9.2 for the inducible fatty acyl-CoA oxidase after isoelectric focusing.
Finally, a complete separation of THC-CoA oxidase from palmitoyl-CoA oxidase was obtained when pooled fractions containing THC-CoA oxidase and the low pI fatty acyl-CoA oxidase from a chromatofocusing column were applied to a hydroxylapatite column, which was eluted by means of a discontinuous phosphate gradient (Fig. 3). THC-CoA oxidase eluted at the lower phosphate concentrations, whereas palmitoyl-CoA oxidase eluted at the higher concentrations.

Pooled fractions containing the inducible fatty acyl-CoA oxidase and those containing the noninducible fatty acyl-CoA oxidase and THC-CoA oxidase from a chromatofocusing column were also subjected to gel filtration. The apparent molecular masses of the enzymes were estimated at 145 kDa (inducible fatty acyl-CoA oxidase), 427 kDa (noninducible fatty acyl-CoA oxidase), and 139 kDa (THC-CoA oxidase) (data not shown).

The fractions eluted from the various columns were also analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Fig. 4 shows the results for the chromatofocusing experiment represented in Fig. 1A. In the control liver the inducible fatty acyl-CoA oxidase, which eluted around pH 8.3, consisted mainly of subunits with molecular masses of 52 and 22.5 kDa. A subunit with a molecular mass of 71 kDa was also present, but less abundant. A similar pattern was observed for the induced enzyme after clofibrate treatment (data not shown), confirming earlier reports by other laboratories (3, 4). The fractions, containing THC-CoA oxidase and the low pI fatty acyl-CoA oxidase showed the presence of a polypeptide of approximately 70 kDa (Fig. 4). Only small amounts of the 52- and 22.5-kDa subunits were found, suggesting that a 70-kDa polypeptide is the main component of THC-CoA oxidase as well as of the low pI fatty acyl-CoA oxidase. In kidney, where no THC-CoA oxidase was present and where little or no fatty acyl-CoA oxidase with low pI was found (see Fig. 1), no polypeptide with a molecular mass of approximately 70 kDa could be observed in the fractions eluting around pH 7.1. The fractions eluting around pH 8.3 contained polypeptides of 52 and 22.5 kDa and less abundantly the 71-kDa polypeptide as was the case for liver (data not shown). The fractions eluting from the green A or orange A columns (see above) contained a polypeptide with a molecular mass of approximately 70 kDa, whenever THC-CoA oxidase and/or palmitoyl-CoA oxidase activity was found (data not shown). Electrophoretic analysis of the fractions from the hydroxylapatite column confirmed that the main polypeptide component of the low pI fatty acyl-CoA oxidase as well as of THC-CoA oxidase is a polypeptide of approximately 70 kDa (Fig. 5). The molecular mass of the acyl-CoA oxidase component appears to be slightly larger than that of the THC-CoA oxidase component, 70.8 ± 0.75 and 69.4 ± 0.75 kDa, respectively (mean ± S.D. of four experiments). Our results confirm earlier reports about the apparent molecular mass and subunit composition of the inducible fatty acyl-CoA oxidase (3, 4). In addition, they indicate that the noninducible fatty acyl-CoA oxidase and THC-CoA oxidase are composed of six and two identical subunits, respectively.

**DISCUSSION**

Our experiments establish that rat liver contains three acyl-CoA oxidases: an inducible fatty acyl-CoA oxidase, which has already been purified (3, 4), a noninducible fatty acyl-CoA oxidase, and a noninducible THC-CoA oxidase. The separation of the three enzymes, as described above, will allow for the determination of the exact substrate specificity of each enzyme. It is attractive to believe that the two fatty acyl-CoA ox-
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The region where the two acyl-CoA oxidase sequences differ are the translation products of the two mRNA species recently discovered in rat liver (7, 8). If this is the case, the interesting situation ensues that during induction the rate of transcription of the single acyl-CoA oxidase gene is increased but that, most probably, the concentration of only one of the two alternatively spliced mRNAs is increased. The fact that the mRNA species for the inducible enzyme is more abundant than the other species in livers from animals treated with peroxisome proliferators (7, 8) would support the above contention. THC-CoA oxidase seems to be encoded by a separate gene. This conclusion is based on the smaller molecular mass of the polypeptide component and the sensitivity of the enzyme to N-ethylmaleimide. No cysteine residue is present in the region where the two acyl-CoA oxidase sequences differ (7).

Experiments are currently underway in our laboratory to verify whether the two fatty acyl-CoA oxidases correspond to the two mRNA species described by the laboratory of Osumi and Hashimoto (7, 8) and to determine which mRNA species increases its concentration on induction. Given that the two fatty acyl-CoA oxidases would correspond to the two mRNAs, an additional interesting question would emerge: why is the inducible enzyme readily cleaved and the noninducible one only slightly or not, despite the fact that both A polypeptides would have the same amino acid sequence at and in the vicinity of the cleavage site? Perhaps the limited difference in amino acid sequence at distance would render the cleavage site inaccessible. This limited difference in amino acid sequence and/or the fact that the 71-kDa subunit is not split, only slightly or not, despite the fact that both A polypeptides would have the same amino acid sequence at the cleavage site, would then also seriously influence the quaternary structure of the enzyme (6 x 71-kDa subunits instead of two (cleaved) 71-kDa subunits).

Our observations are also of interest with respect to human pathology. Children born with a deficiency of peroxisomal β-oxidation accumulate very long chain fatty acids in their tissues and excrete abnormal bile acids (di- and trihydroxycoprostanic acids instead of chenodeoxycholic and cholic acids), because peroxisomes are responsible for the β-oxidation of very long chain fatty acids and the carboxy side chain of bile acid intermediates (for a review, see Ref. 1). Recently, two cases of fatty acyl-CoA oxidase deficiency have been reported, in which there was an accumulation of very long chain fatty acids but no abnormal bile acids (16). This suggests that, as in rat liver, a separate THC-CoA oxidase is present in human liver. In addition, a patient is known who excretes abnormal bile acids, but who does not display an accumulation of very long chain fatty acids. This case suggests that a separate deficiency of THC-CoA oxidase can also occur. Whether the human liver possesses also two fatty acyl-CoA oxidases and what the consequences of a deficiency of one of the enzymes would be, remain to be studied.

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L Schepers, P P Van Veldhoven, M Casteels, H J Eyssen and G P Mannaerts

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