Studies on the Substrate Specificity of the Inducible and Non-Inducible Acyl-CoA Oxidases from Rat Kidney Peroxisomes

Ronald J.A. Wanders,*,2 Simone W. Denis,* and Georges Dacremont**

*Department of Clinical Biochemistry, University Hospital Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands; and **Department of Pediatrics, State University Ghent, B-9000 Ghent, Belgium

Received for publication, October 19, 1992

We have studied the substrate specificity of the inducible (acyl-CoA oxidase I) and non-inducible (acyl-CoA oxidase II) oxidases in peroxisome-enriched fractions from rat kidney. The two oxidases were separated by means of ion-exchange chromatography and shown to accept a variety of acyl-CoA esters as substrates, including lignoceryl-CoA, palmityl-CoA, lauroyl-CoA, caproyl-CoA, and trimethyltridecanoyl-CoA. Glutaryl-CoA was found to react exclusively with the inducible enzyme, and pristanoyl-CoA exclusively with the non-inducible enzyme. We conclude that under normal non-induced conditions both acyl-CoA oxidase I and II contribute to the oxidation of the various acyl-CoA esters with the exception of pristanoyl-CoA and glutaryl-CoA, although the extent to which each enzyme contributes to the oxidation was found to differ between the various acyl-CoA esters.

Mitochondria were long believed to be the sole site of fatty acid β-oxidation. Following the finding of Lazarow and de Duve (1) that rat liver peroxisomes are also capable of fatty acid β-oxidation, it has been established that non-mitochondrial fatty acid β-oxidation is much more widely distributed in nature than β-oxidation in mitochondria, which is mainly restricted to the animal kingdom.

Although the significance of a second β-oxidation system in peroxisomes was initially obscure, it is now clear that peroxisomes catalyze the β-oxidative chain-shortening of a distinct set of fatty acids and fatty acid derivatives (2). Indeed, peroxisomes are obligatory for the oxidation of very long chain fatty acids (3), di- and trihydroxycholestanoyl-CoA (18, 19). The latter enzyme is expressed in liver only, whereas the former two oxidases are present in multiple tissues. We (20) and others (21) have shown that the clofibrate non-inducible enzyme is not just a functional duplicate of the clofibrate inducible enzyme. Indeed, pristanoyl-CoA (2,6,10,14-tetramethylpentadecanoyl-CoA) was found to be a substrate for the non-inducible enzyme only (20, 21). We have studied the substrate specificity of the two acyl-CoA oxidases in more detail. The results are described in this paper.

MATERIALS AND METHODS

Materials—Acyl-CoA esters including glutaryl-CoA (1,5-pentadienyl-CoA), caproyl-CoA (hexanoyl-CoA, C6:0-CoA), lauroyl-CoA (dodecanoyl-CoA, C12:0-CoA), palmityl-CoA (hexadecanoyl-CoA, C16:0-CoA), and lignoceryl-CoA (tetracosanoyl-CoA, C24:0-CoA), homovanillic acid, horseradish peroxidase, bovine serum albumin (essentially fatty acid free), and flavine adenine dinucleotide were all obtained from Sigma (St. Louis, U.S.A.). Farnesol was purchased from Janssen Pharmaceuticals, Beerse, Belgium. Pristanoyl-CoA was synthesized as described below (20). The CoA ester of 4,8,12-trimethyltridecanoic acid was prepared as described below.

Synthesis of 4,8,12-trimethyltridecanoic acid and its CoA ester: 4,8,12-Trimethyltridecanoic acid was prepared from farnesol in an overall molar yield of 28%. Farnesol (15 g) was hydrogenated to hexahydrofarnesol (3,7,11-trimethyl-dodecanol) by platinum(IV) oxide and hydrogen (22) (yield 12.5 g hexahydrofarnesol). Hexahydrofarnesol was then converted to the methanesulfonate with methane

1 This work was financially supported by the Princess Beatrix Fund (The Hague, The Netherlands).

2 To whom correspondence should be addressed.
sulfonyl chloride and triethylamine (23) with a molar yield of 86%. The methanesulfonate was subsequently reacted with potassium cyanide in dry dimethylysulfoxide for 24 h at 75°C to yield the 4,8,12-trimethyltridecanonitrile (24), with a molar yield of 95%. Finally the nitrile was hydrolyzed with 1 M sodiumhydroxide in ethanol–water for 96 h at 85°C to give 4,8,12-trimethyltridecanoic acid.

The acid was purified by column chromatography on silica gel using hexane–ether (65:35, v/v) as eluent. Gas liquid chromatography-mass spectrometry of the methylester showed one homogeneous peak with molecular ion at m/z 270. Mass spectrometry of the unesterified acid: m/z 256 (M+, 1.6%), 85 (11.7%), 73 (28.6%), 71 (27.4%), 57 (45.7%), 43 (100%).

The CoA derivative of 4,8,12-trimethyltridecanoic acid was prepared by the method of Kawaguchi et al. (25) with the in situ formation of an acylimidazole and further reaction of the acylimidazole with CoA. The CoA-derivative was purified as follows. After removal of the tetrahydrofuran by evaporation under N₂, the CoA derivative was precipitated with 12% (w/v) HClO₄, washed subsequently with 2x2 ml of 0.8% (w/v) HClO₄, 2x0.75 ml of acetone, and 2x0.75 ml of diethylether. Finally, the precipitate was dissolved in H₂O and further purified by chromatography on a reversed phase C18 column with ethanol–water (45:55, v/v) as mobile phase. Fractions containing the CoA derivative were combined, the ethanol evaporated in a rotavapor and the aqueous solution lyophilized. Thin-layer chromatography of the solid in n-butanol/water/acetic acid (50:30:20, v/v/v) showed a single spot with R₂ 0.47. Hydrolysis in 0.1 M sodium hydroxide at 95°C yielded the starting products 4,8,12-trimethyltridecanoic acid and CoA.

Methods—Preparation of a peroxisome-enriched fraction from rat kidney and chromatographic separation of acyl-CoA oxidase I and II: Rat kidneys were finely minced and homogenized in a medium containing 250 mM sucrose, 2 mM EDTA, and 2 mM MOPS-NaOH (final pH 7.4). The resulting homogenate was subjected to differential centrifugation to prepare a light mitochondrial fraction enriched in peroxisomes from rat kidney by differential centrifugation conditions described elsewhere for the preparation of peroxisome-enriched fractions from rat liver (26). The final pellet was taken up in buffer A, containing 10 mM sodium pyrophosphate, 1 mM EDTA, and 0.01 mM FAD (final pH 9.0) followed by sonication (three cycles of 15 s with 45 s intervals at 4°C) and centrifugation (12,000 × gₜₚ, 10 min, 4°C).

An aliquot of the resulting supernatant was subjected to ion-exchange chromatography using a Mono-Q HR 5/5 column (Pharmacia, Uppsala, Sweden). The column was equilibrated with 10 mM Tris-HCl buffer (pH 8.6) and eluted by means of a linear pH/salt gradient (0-100% buffer containing 10 mM Tris-HCl (pH 7.8) and 0.2 M NaCl). Fractions of 2 ml were collected in tubes containing 20 μl of 1 mM FAD. Oxidase activity measurements were carried out immediately afterwards.

Acyl-CoA oxidase activity measurements: Acyl-CoA oxidase activity measurements were done essentially as described before, using a medium of the following standard composition: 50 mM MOPS-NaOH (pH 7.6), 1 mM homovanillic acid, 18 U/ml horseradish peroxidase, 0.1 mM NaN₃, and 0.01 mM FAD. Measurements were done in the absence and presence of bovine serum albumin at 5 or 10 μM. Reactions were started by addition of the acyl-CoA ester at desired concentrations. Fluorescence was followed at 30-s intervals for 10 min using a Cobas Bio Centrifugal Analyzer (excitation wavelength: 327 nm, emission filter: 410-490 nm), Hoffman-La Roche (Basel, Switzerland).

Calculation of the relative contribution of acyl-CoA oxidases I and I to the overall acyl-CoA oxidase activities: The contribution of acyl-CoA oxidase I and II to the overall acyl-CoA oxidase activity was calculated for caproyl-CoA (Fig. 4) using the kinetic parameters of Table I and the following Michaelis-Menten relationships:

\[ V_{1} = \frac{V_{\text{max,ox} I}}{1 + K_{\text{ox, I}}/[S]} \]

\[ V_{2} = \frac{V_{\text{max,ox} II}}{1 + K_{\text{ox, II}}/[S]} \]

with \( V_{\text{max,ox} I} = 227 \text{ pmol/min·mg protein} \), \( V_{\text{max,ox} II} = 75 \text{ pmol/min·mg protein} \), \( K_{\text{ox, I}} = 81 \text{ μM} \), and \( K_{\text{ox, II}} = 37 \text{ μM} \) (see Table I). It should be stressed that these \( V_{\text{max}} \)-values represent apparent \( V_{\text{max}} \)-values reflecting the maximal activities at saturating substrate concentration of the two oxidases I and II as present in rat kidney peroxisomes. Total oxidase activity at any given acyl-CoA concentration will then be:

\[ V_{\text{tot}} = V_{1} + V_{2} \]

The relative concentration of acyl-CoA oxidases I and II is then simply given by the value for \( V_{1}/V_{\text{tot}} \)× 100% and \( V_{2}/V_{\text{tot}} \)× 100%, respectively.

RESULTS

Chromatographic Separation of the Inducible and Non-Inducible Oxidases from Peroxisome-Enriched Fractions from Rat Kidney—Earlier studies have shown that the inducible and non-inducible acyl-CoA oxidases from rat liver have markedly distinct molecular properties including a profound difference in native molecular weight and pI. These properties allow easy separation of the two oxidases using either gelfiltration or ion-exchange chromatography (20, 21). Rather than using rat liver peroxisomes as a source of acyl-CoA oxidases, we decided to use rat kidney peroxisomes since rat liver peroxisomes also contain di/hydroxycholestanoyl-CoA oxidase in contrast to peroxisomes from rat kidney, which are devoid of this activity (18). After preparation of a peroxisome-enriched fraction from rat kidney by differential centrifugation, an extract was prepared and subjected to ion-exchange chromatography (see "MATERIALS AND METHODS").

Figure 1 shows the result of such an experiment. In this experiment 10.2 mg of a peroxisome-enriched fraction, subjected to sonication followed by centrifugation (see "MATERIALS AND METHODS"), was applied to DEAE ion-exchange chromatography and eluted by means of a linear pH/salt gradient [buffer 1: 10 mM Tris-HCl (pH 8.6), buffer 2: 10 mM Tris-HCl (pH 7.8) plus 0.2 mM NaCl]. The results show that some palmitoyl-CoA oxidase activity was not retained by the column, the retained activity eluting in two peaks. Pristanoyl-CoA oxidase activity, however, was found to elute in one peak. When the same experiment was repeated using a peroxisome-enriched fraction prepared from a clofibrate-treated rat, the first
Substrate Specificity of Acyl-CoA Oxidases in Rat Kidney

Peak of palmitoyl-CoA oxidase activity was found to be several-fold higher in contrast to the second peak, which remained unaltered. These results indicate that the first peak corresponds to the well-known inducible acyl-CoA oxidase, whereas the second corresponds to the recently identified non-inducible acyl-CoA oxidase, i.e., pristanoyl-CoA oxidase. The ion-exchange chromatography procedure described in Fig. 1 was found to show satisfactory recovery of both palmitoyl-CoA oxidase and pristanoyl-CoA oxidase activity, amounting to 65–100% of the total activity applied to the column for both oxidase activities. In this respect the ion-exchange chromatography procedure described in Fig. 1 is superior to our earlier described procedure using chromatofocusing (20), which showed a much lower recovery of activity, especially in the case of pristanoyl-CoA oxidase. The results of Fig. 1 closely correspond to earlier results (20, 21) in rat liver.

Top fractions corresponding to the inducible (acyl-CoA oxidase I) and non-inducible (acyl-CoA oxidase II) acyl-CoA oxidases were subsequently taken for substrate specificity studies as described below.

Substrate Specificity Measurements of Acyl-CoA Oxidases I and II—The results of substrate specificity studies are shown in Figs. 2 and 3. Acyl-CoA oxidase activity measurements were done in the absence and presence of bovine serum albumin at two different concentrations of 5 and 10 μM, respectively, since initial studies by Hashimoto and coworkers (16) have shown that some acyl-CoA esters may cause strong inhibition even at extremely low concentrations (see Fig. 9 in Ref. 16).

In the experiment of Fig. 2 a variety of saturated fatty acyl-CoA esters were tested as substrates for acyl-CoA oxidases I and II, including a very-long-chain acyl-CoA (lignoceroyl-CoA, C24:0-CoA), a long-chain acyl-CoA (palmitoyl-CoA, C16:0-CoA), a medium-chain acyl-CoA (lauroyl-CoA, C12:0-CoA), and two shorter-chain acyl-CoAs [caproyl-CoA, C6:0-CoA and glutaryl-CoA (1,5-pentanediol-CoA)].

The results of Fig. 2 (see also Table I) show that the two acyl-CoA oxidases accept each of the fatty acyl-CoA esters as substrate with the exception of glutaryl-CoA. The latter

![Fig. 1. Separation of the inducible and non-inducible acyl-CoA oxidases by ion-exchange chromatography of an extract prepared from a peroxisome-enriched fraction from rat kidney.](image)

A peroxisome-enriched fraction was prepared from rat kidney by density-gradient centrifugation followed by sonication and high speed centrifugation. The supernatant, containing 10.2 mg protein, was subjected to ion-exchange chromatography and subsequently eluted by means of a linear pH/salt gradient and palmitoyl-CoA oxidase and pristanoyl-CoA activities were measured in the eluted fractions. Palmitoyl-CoA oxidase activities (open symbols): total activity applied, 58.5 mU; recovery, 66%. Pristanoyl-CoA oxidase activity (closed symbols): total activity applied, 10.5 mU; recovery, 76%.

![Fig. 2](image)
TABLE I. Kinetic constants and apparent V_{max} -value for acyl-CoA oxidases I and II in peroxisome-enriched fractions from rat kidney. Values represent the mean of 3 independent experiments, including the one shown in Figs. 2 and 3 and were calculated from double-reciprocal plots. Apparent V_{max} -values are expressed in pmol/min·mg protein.

<table>
<thead>
<tr>
<th>Acyl-CoA ester studied</th>
<th>Acyl-CoA oxidase I</th>
<th>Acyl-CoA oxidase II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caproyl-CoA</td>
<td>81</td>
<td>227</td>
</tr>
<tr>
<td>Lauroyl-CoA</td>
<td>6.2</td>
<td>307</td>
</tr>
<tr>
<td>Palmitoyl-CoA</td>
<td>2.4</td>
<td>340</td>
</tr>
<tr>
<td>Lignoceryl-CoA</td>
<td>39</td>
<td>55</td>
</tr>
<tr>
<td>Trimethyltridecanoyl-CoA</td>
<td>6.3</td>
<td>47</td>
</tr>
<tr>
<td>Glutaryl-CoA</td>
<td>124</td>
<td>22</td>
</tr>
<tr>
<td>Pristanoyl-CoA</td>
<td>—</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 4. Relative contribution of acyl-CoA oxidases I and II to overall caproyl-CoA oxidase activity in rat kidney peroxisomal fractions. See text for details.

An important question is to what extent the two acyl-CoA oxidases contribute to the dehydrogenation of the various
Substrate Specificity of Acyl-CoA Oxidases in Rat Kidney

acyl-CoA esters under in situ conditions. To obtain information on this point we calculated the apparent $V_{\text{max}}$ values for acyl-CoA oxidases I and II, respectively, from Figs. 2 and 3. It should be stressed that the values obtained for $V_{\text{max, app}}$ represent the activities of the two oxidases in the initial rat kidney peroxisomal fraction as applied to the column for separation of the two acyl-CoA oxidases. When the results depicted in Table I are inspected it is clear that, with the exception of lignoceryl-CoA as substrate, the contribution of acyl-CoA oxidase I predominates over that of acyl-CoA oxidase II. Since the $K_m$-values of the two oxidases for lauroyl-CoA, palmitoyl-CoA and trimethyltridecanoyl-CoA are comparable, the relative contribution of acyl-CoA oxidases I and II to total acyl-CoA oxidation is the same irrespective of the concentration of lauroyl-CoA, palmitoyl-CoA, or trimethyltridecanoyl-CoA. This is not true for lignoceryl-CoA and caproyl-CoA, because the $K_m$-values of the two oxidases for each of the two acyl-CoA esters are different. Accordingly, the contribution of acyl-CoA oxidase activities I and II to total oxidation depends upon the concentration of lignoceryl-CoA and caproyl-CoA, respectively.

This is shown for caproyl-CoA in Fig. 4. The relative contribution of acyl-CoA oxidases I and II, respectively, can be calculated in a simple manner using the $K_m$ and the apparent $V_{\text{max}}$-values of Table I (see “MATERIALS AND METHODS” for details on the calculation procedure used). The results show that the contribution of acyl-CoA oxidase I to total activity increases with increasing concentrations of caproyl-CoA, whereas the reverse is true for acyl-CoA oxidase II. The same applies to lignoceryl-CoA with a major contribution of acyl-CoA oxidase II at lower acyl-CoA concentrations, which decreases with increasing substrate concentrations (not shown).

**DISCUSSION**

We have studied the substrate specificity of the inducible and non-inducible acyl-CoA oxidases in peroxisome-enriched fractions from rat kidney. The results show that all acyl-CoA esters investigated were substrates for both acyl-CoA oxidases I and II with the exception of pristanoyl-CoA, which reacts only with the non-inducible enzyme, and glutaryl-CoA, which is not a substrate for acyl-CoA oxidase II. These results are in good agreement with recent data from the group of Mannaerts (27) who studied the substrate specificities of the three acyl-CoA oxidases from rat liver.

It is remarkable that the branched-chain fatty acyl-CoAs pristanoyl-CoA and 2-methylpalmitoyl-CoA are not accepted as a substrate by the inducible acyl-CoA oxidase (20, 21, 26; see also Fig. 3), whereas another branched-chain fatty acyl-CoA, i.e., 4,8,12-trimethyltridecanoyl-CoA, reacts with both acyl-CoA oxidase I and II (Fig. 3). This is probably due to the position of the first methyl group at the carbon-2 position in pristanoyl-CoA and 2-methylpalmitoyl-CoA, but at the carbon-4 position in 4,8,12-trimethyltridecanoyl-CoA.

There is some uncertainty in the literature with regard to whether or not glutaryl-CoA is handled by a distinct oxidase. Vamecq and coworkers (28, 29) concluded that, at least in mice, glutaryl-CoA oxidase is distinct from the clofibrate-inducible palmitoyl-CoA oxidase, based on the differential effects of succinyl-CoA, isobutryryl-CoA, and especially Zn$^{2+}$ ions. Subsequent studies by Horie and coworkers (30), however, showed convincingly that, at least in the rat, glutaryl-CoA oxidase is identical with the clofibrate-inducible acyl-CoA oxidase. It remains to be established whether the different results are due to species differences or not.

The results in this paper further show that in general both acyl-CoA oxidases I and II contribute to the overall oxidation of acyl-CoA esters, although in most cases the contribution of acyl-CoA oxidase I exceeds that of acyl-CoA oxidase II, at least in rat kidney (Table I). Since the palmitoyl-CoA oxidase/pristanoyl-CoA oxidase activity ratio in kidney peroxisomes is comparable to the activity ratio in rat liver peroxisomes (20), the conclusions drawn can probably be applied directly to liver peroxisomes. It is obvious that under induced conditions, *e.g.*, by feeding clofibrate, the relative contribution of acyl-CoA oxidases I and II to total oxidation will shift greatly in favor of acyl-CoA oxidase I, except for pristanoyl-CoA, being an exclusive substrate for acyl-CoA oxidase II.

It will be important to carry out similar studies in human tissues, especially since several patients have been described with an acyl-CoA oxidase deficiency (31, 32). Although it has not yet been resolved definitively, there is probably a deficiency of acyl-CoA oxidase I only with normal activity of acyl-CoA oxidase II in these patients. This is concluded from the finding of elevated very-long-chain fatty acid levels, but normal pristanic acid levels in plasma from one of these patients (33), suggesting a deficient activity of acyl-CoA oxidase I only. If, indeed, acyl-CoA oxidase II were to be normally active in these patients, induction of this enzyme activity would be an effective means of reducing the accumulation of very-long-chain fatty acids in such patients. Whether the activity of acyl-CoA oxidase II can be induced in man, is not known at present.

The authors gratefully acknowledge Prof. G.P. Mannaerts and Dr. G. Vanhove for helpful discussions and Mr. G.J. Romeijn for preparation of the manuscript.

**REFERENCES**

Liss, New York