Identification and Purification of a Peroxisomal Branched Chain Fatty Acyl-CoA Oxidase*

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Isoprenoid (branched) fatty acids such as pristaneic acid can be degraded via β-oxidation in peroxisomes. We synthesized 2-methylpalmitoyl-CoA as a model substrate in order to study the first step of the peroxisomal β-oxidation of branched fatty acids, catalyzed by an acyl-CoA oxidase.

2-Methylpalmitoyl-CoA oxidase activity was found in rat liver homogenates. Subcellular fractionation demonstrated that the oxidase was confined to peroxisomes. 2-Methylpalmitoyl-CoA oxidase was also present in kidney and intestine. It was not induced in liver or in the extrahaepatic tissues by treatment of rats with peroxisome proliferators or by feeding diets containing excess isoprenoids. The enzyme was partially purified together with palmitoyl-CoA oxidase and trihydroxycoprostanoyl-CoA oxidase by heat treatment and ammonium sulfate fractionation of liver extracts. The partially purified preparation was chromatographed on various columns. 2-Methylpalmitoyl-CoA oxidase could be separated from the inducible (by peroxisome proliferators) palmityl-CoA oxidase and from trihydroxycoprostanoyl-CoA oxidase, but it always coeluted with the noninducible palmityl-CoA oxidase, recently described by us (Schepers, L., Van Veldhoven, P. P., Caeteels, M., Eysen, H. J., and Mannaerts, G. P. (1990) J. Biol. Chem. 265, 5242–5248). 2-Methylpalmitoyl-CoA oxidase was purified to near homogeneity in three chromatographic steps (anion exchange, hydroxyapatite, and gel filtration). Its apparent molecular mass is approximately 415 kDa, and it consists of identical subunits of approximately 70 kDa. The enzyme oxidized 2-methylpalmitoyl-CoA twice as rapidly as palmityl-CoA and pristanoyl-CoA as rapidly as palmityl-CoA, so that it can be considered as a branched fatty acyl-CoA oxidase. Since pristanoyl-CoA is one of its naturally occurring substrates we propose to name this enzyme pristanoyl-CoA oxidase.

EXPERIMENTAL PROCEDURES

Materials

Carbonyldimidazole and alumina were obtained from Fluka, Buchs, Switzerland. Percoll, prepacked desalting PD-10 columns, Sephadex LH-20, and CoA were purchased from Pharmacia, LKB Biotechnology Inc. Phytol (97%, racemic), 2,6,10-trimethyl-5,9-undecadiene-1-ol (98%), benzamidine, 4-aminoantipyrine, and homovanillic acid were from Janssen, Beerse, Belgium. Flavine adenine dinucleotide, 2,4,6-tribromo-3-hydroxybenzoic acid, Triton X-100, ferritin (horse spleen), β-galactosidase (Escherichia coli), urease S

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1 The abbreviation used is: CoA, coenzyme A.
Pristanoyl-CoA Oxidase

with cofactor and to inactivate interfering catalase activity. 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, 0.125 mM substrate. In addition, when palmitoyl-CoA oxidase was measured, the reaction mixture contained 0.65% (w/v) Triton X-100 and 0.075% (w/v) defatted albumin. To measure trihydroxycoprostanoyl-CoA oxidase, 0.3% (w/v) defatted albumin was added to the reaction mixture. After incubation at 37 °C, reactions were terminated by adding 50 μl of 12% (w/v) HClO4. 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 15 min, fluorescence was measured (excitation 392 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/catalase as described before (17).

When activities were measured in liver homogenates or subcellular fractions, reactions were terminated after 5 and 15 min (palmitoyl-, 2-methylpalmitoyl-, and pristanoyl-CoA as substrate) or 10 and 30 min (trihydroxycoprostanoyl-CoA), and the oxidation rate was calculated from the rise in fluorescence between the two time points in order to avoid interference of the initial lag phase (19). For column eluates, incubation times were prolonged to 10 min (palmitoyl-, 2-methylpalmitoyl-, and pristanoyl-CoA as substrate) and 30 min (trihydroxycoprostanoyl-CoA).

In some experiments, the (2-methyl)palmitoyl-CoA oxidase activity in subcellular fractions was measured by a colorimetric assay, based on the coupling of 2,4,6-tribromo-3-hydroxy-benzoic acid and 4-aminoantipyrine catalyzed by peroxidase in the presence of peroxide. Assay conditions were as described for the fluorometric method, except that volumes were increased 2-fold and homovanillic acid was replaced by 6.25 mM 2,4,6-tribromo-3-hydroxybenzoic acid and 1.25 mM 4-aminoantipyrine. Formation of the quinone-imine dye was followed at 511 nm. Although the extinction of the dye is pH-insensitive between pH 6.0 and 9.6, its formation is influenced by the pH being lower at more alkaline pHs. Therefore, the assay was standardized by means of glucose/glucose oxidase and peroxide. A molar extinction of 30,500 was used.

Partial Purification of Hepatic Acyl-CoA Oxidases

Rat liver (60 g) was homogenized by means of a Polytron for 3 min in 9 volumes of ice-cold 20 mM pyrophosphate buffer (pH 9.0), containing 5 mM benzamidine, 1 mM EDTA, 10 μM FAD, 50 μM L-1-tosylamido-2-phenylthyl chloromethyl ketone, 0.2 mM phenylmethylsulfonyl fluoride. The latter two inhibitors were added just before use to the homogenization buffer, from a 1000-fold concentrated stock solution prepared in ethanol. After homogenization, 1/100th volume of 1 mM KH2PO4 was added to decrease the pH to approximately 7.5, and the homogenate was centrifuged at 100,000 × g for 1 h. The supernatant was brought to 10% ammonium sulfate saturation and 25 μM FAD. When the salt was dissolved, the solution was placed in a water bath at 80 °C and slowly stirred. When the temperature reached 55 °C, the solution was kept at this temperature for 15 min in another water bath. The solution was quickly cooled and denatured proteins were removed by centrifugation at 10,000 × g for 10 min. The supernatant was brought to 45% saturation with ammonium sulfate, stirred for 15 min, and again centrifuged. The supernatant was discarded, and the pellet was dissolved in 20 mM potassium phosphate buffer (pH 7.5), 10 μM FAD (0.5 ml/g of initial liver) and stored in aliquots at −20 °C until use.

Chromatographic Separations

Protein chromatography was done on a Waters 600 system equipped with Teflon and polyethylene tubing. The two pumps used: Protein PAK Glass DEAE-5PW (80 × 7.5 mm) (Nihon Waters Ltd., Tokyo, Japan), hydroxyapatite Bio-Gel HPHT (100 × 7.8 mm) (Bio-Rad), Protein PAK Glass 300 SW (300 × 8 mm) (Nihon Waters Ltd.).

For ion-exchange chromatography, the partially purified preparation was thawed and centrifuged to remove undissolved material. An aliquot (2.5 ml) was then loaded on a PD10 desalting column,
equilibrated in 10 mM Tris-HCl buffer (pH 8.6), 20% (w/v) glycerol. The oxidases were eluted in 3 ml of equilibration buffer and a 2-ml portion was directly injected onto a DEAE column, equilibrated in the same buffer, and eluted at a flow rate of 1 ml/min. Bound proteins were eluted by means of a linear pH/salt gradient (0-100% buffer containing 10 mM Tris-HCl (pH 7.8), 0.2 M NaCl, 20% (w/v) glycerol) over 40 min. Fractions of 2 ml were collected in tubes containing 20 μl of 1 mM FAD and analyzed for oxidase activity.

Fractions from the DEAE column, containing 2-methylpalmitoyl-CoA oxidase activity, were pooled, 1/10th volume of 1 M potassium phosphate buffer (pH 7.5) was added, and the solution was brought to 50% ammonium sulfate saturation. After centrifugation, the pellet was dissolved in 10 mM potassium phosphate buffer (pH 7.5) containing 5 μM FAD. The undissolved material was removed by centrifugation, and a fraction was injected onto a hydroxylapatite 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 10 to 200 mM potassium phosphate (pH 7.5) in the presence of 5 μM FAD, for 50 min. The elution rate was 0.6 ml/min. Fractions of 1.8 ml were collected and assayed for oxidase activity and phosphate, while their absorbance was monitored at 210 nm.

The fractions from the hydroxylapatite column, containing the highest activity of 2-methylpalmitoyl-CoA oxidase were pooled and concentrated by means of ammonium sulfate precipitation. After centrifugation, the pellet was dissolved in 200 mM potassium phosphate buffer (pH 7.5) containing 10% (v/v) ethyleneglycol and 10 mM FAD. An aliquot (50 μl) was subjected to gel filtration on a Protein PAK 300 SW column at a flow rate of 0.5 ml/min. Absorbance was monitored at 210 nm. Fractions of 0.35 ml were collected and assayed for oxidase activity.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
Electrophoresis in 10-20% (w/v) acrylamide gradient gels was done as described before (5). Gels were silver-stained according to Guevara (20).

RESULTS
In the preceding paper (2) we demonstrated that peroxisomes are capable of oxidizing 2-methylpalmitate, and we mentioned that 2-methylpalmitoyl-CoA oxidase activity could be detected in whole liver homogenates. The optimum homogenate concentration in the 2-methylpalmitoyl-CoA oxidase assay was 0.1 mg of homogenate protein per ml; the optimum pH of the reaction, determined in different buffers, was 8.3 as is the case for palmitoyl-CoA oxidase and trihydroxycoprostanoyl-CoA oxidase (data not shown). Fig. 1 shows that, except for a short lag phase (see below), 2-methylpalmitoyl-CoA oxidase activity in whole liver homogenates was linear with time for approximately 25 min. For the sake of comparison, palmitoyl-CoA oxidase and trihydroxycoprostanoyl-CoA oxidase activities were also measured. Palmitoyl-CoA and 2-methylpalmitoyl-CoA oxidase displayed a short lag phase of approximately 2 min during which no H₂O₂ production could be detected. This lag phase is 3- to 4-fold longer for trihydroxycoprostanoyl-CoA oxidase. The lag phases are the result of the presence in liver homogenates of substances that scaveng H₂O₂ and that, in turn, are inactivated by H₂O₂ so that the lag phases last longer at lower H₂O₂ production rates (19). Fig. 2 shows the substrate dependency of 2-methylpalmitoyl-CoA oxidase. An apparent Vₚ₅₀ of 770 nmol/min × g of liver and an apparent Kₘ of 33 μM were calculated after linear transformation of the curve according to the Lineweaver-Burk method. The maximal activity of 2-methylpalmitoyl-CoA oxidase was only slightly lower than that of palmitoyl-CoA oxidase but approximately 4-fold higher than that of trihydroxycoprostanoyl-CoA oxidase (data not shown; see also Table I). Albumin inhibited 2-methylpalmitoyl-CoA oxidase activity at molar substrate:albumin ratios lower than 6, likely by binding the acyl-CoA ester. Triton X-100, which stimulates palmitoyl-CoA oxidase approximately 2-fold at a concentration of 0.02-0.04% (w/v), had only a slight stimulatory effect (±10%) at concentrations between 0.005 and 0.025% (v/v) and became inhibitory at higher concentrations (data not shown).

In a next series of experiments we wished to verify that 2-methylpalmitoyl-CoA oxidase is a peroxisomal enzyme. Rat liver homogenates were fractionated by differential centrifugation in fractions enriched in nuclei; mitochondria; peroxisomes and lysosomes; endoplasmic reticulum; and soluble components, respectively. Fig. 3 demonstrates that 2-methylpalmitoyl-CoA oxidase followed the subcellular distribution pattern of palmitoyl-CoA oxidase and catalase, two marker enzymes for the peroxisomal matrix. Less palmitoyl-CoA oxidase and 2-methylpalmitoyl-CoA oxidase activity than catalase was found in the soluble fraction, indicating that during homogenization of the liver the oxidases did not leak as easily from the peroxisomes as did catalase. When the peroxisome-enriched fraction, prepared by differential centrifugation, was subfractionated on a self-generating Percoll gradient, the distribution patterns of 2-methylpalmitoyl-CoA oxidase, palmitoyl-CoA oxidase, and catalase again coincided (Fig. 4), clearly demonstrating that 2-methylpalmitoyl-CoA oxidase is a peroxisomal enzyme.

Table I presents a limited tissue survey of palmitoyl-CoA oxidase, 2-methylpalmitoyl-CoA oxidase, and trihydroxycoprostanoyl-CoA oxidase, carried out in control rats and rats treated with the peroxisome proliferator fenofibrate. Palmitoyl-CoA oxidase activity was present in liver, intestine and kidney and was induced 16-, 2.5-, and 5-fold, respectively, by treatment of the rats with fenofibrate. Trihydroxycoprostanox-
Pristanoyl-CoA Oxidase

Table 1
Acyl-CoA oxidase activities in different tissues from untreated and fenofibrate-treated rats
Oxidase activities were measured in homogenates from liver, intestinal mucosa, and kidney. Results are expressed as means ± S.E. for three experiments.

| Substrate                  | Control          | Fenofibrate       |
|----------------------------|------------------|-------------------|
|                            | Liver            | Intestine         | Kidney             |
|                            | Liver            | Intestine         | Kidney             |
| Palmitoyl-CoA              | 915 ± 161        | 489 ± 36          | 217 ± 11           | 14,908 ± 508* | 1,118 ± 180 | 1,056 ± 39* |
| 2-Methylpalmitoyl-CoA      | 801 ± 56         | 95 ± 8            | 74 ± 11            | 867 ± 74    | 70 ± 18     | 66 ± 14     |
| Trihydroxycoprostanoyl-CoA | 219 ± 10         | ND*               | ND                 | 174 ± 10    | ND          | ND          |

* Statistically different from control with p < 0.005.
* ND, not detectable.

Fig. 3. Subcellular distribution of 2-methylpalmitoyl-CoA oxidase in rat liver. A rat liver homogenate was 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 and palmitoyl-CoA oxidase and 2-methylpalmitoyl-CoA oxidase were measured in each fraction. a, glutamate dehydrogenase (mitochondria); b, acid phosphatase (lysosomes); c, catalase (peroxisomes); d, glucose-6-phosphatase (endoplasmic reticulum); e, palmitoyl-CoA oxidase (peroxisomes); f, 2-methylpalmitoyl-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 82 and 128%.

Fig. 4. Subfractionation of a light mitochondrial fraction on a self-generating Percoll gradient. A light mitochondrial fraction, prepared by differential centrifugation and derived from 8 g of liver, was subfractionated by isopycnic centrifugation in an isosmotic self-generating Percoll gradient. The gradient fractions were analyzed for glutamate dehydrogenase (a), acid phosphatase (b), catalase (c), glucose-6-phosphatase (d), palmitoyl-CoA oxidase (e), 2-methylpalmitoyl-CoA oxidase (f), and protein (g). Results are expressed as percentage of total gradient activity or content present in each fraction numbered on the abscissa. Fractions 1 and 15 represent the fractions of highest and lowest density, respectively. Recoveries were between 87 and 114% except for acid phosphatase (61%).

for 2 weeks on different diets containing 0.2% (w/w) of different branched compounds: phytol, pristane, 2,6,10-trimethyl-5,9-undecadiene-1-ol, farnesol, α-tocopherol, and Annatto pigment. These compounds are partly converted in the organism to the corresponding carboxylic acids. A two-fold induction of hepatic palmitoyl-CoA oxidase was obtained after phytol, 2,6,10-trimethyl-5,9-undecadiene-1-ol and farnesol feeding but 2-methylpalmitoyl-CoA oxidase activity remained unchanged (data not shown).

Further evidence that 2-methylpalmitoyl-CoA is not oxidized by the (inducible) palmitoyl-CoA oxidase or by trihydroxycoprostanoyl-CoA oxidase was obtained in the following experiments. Trihydroxycoprostanoyl-CoA oxidase was severely inhibited by N-ethylmaleimide (Fig. 5A) and partially inhibited by LiCl (Fig. 5B). Palmitoyl-CoA oxidase was also partially inhibited by LiCl (Fig. 5B) but not by N-ethylmaleimide (Fig. 5A). 2-Methylpalmitoyl-CoA oxidase was inhibited only slightly by N-ethylmaleimide (Fig. 5A) or by LiCl (Fig. 5B).

The inducible and noninducible palmitoyl-CoA oxidases have been partially purified by others and by ourselves, respectively, by means of heat treatment of liver extracts in the
Pristanoyl-CoA oxidase was then exploited for the partial purification of the enzymes by heat treatment and ammonium sulfate fractionation as described under "Experimental Procedures." This procedure resulted in a 30- to 35-fold purification of 2-methylpalmitoyl-CoA oxidase, trihydroxycoprostanoyl-CoA oxidase, and palmitoyl-CoA oxidase as calculated from the specific activities of the enzymes in whole liver homogenates and in the partially purified preparations.

In function of the further purification of the oxidases, we also investigated the pH stability of 2-methylpalmitoyl-CoA oxidase. In the absence of FAD the enzyme was stable between pH 7 and 9. Below pH 7, the oxidase lost activity, even when FAD was present. Addition of glycerol, dimethyl sulfoxide, or ethylene glycol did not prevent inactivation. Between pH 9 and 10 inactivation could be prevented by FAD (data not shown).

At this point and before attempts were made to separate the oxidases by column chromatography we tested whether the CoA ester of the naturally occurring isoprenoid fatty acid pristanic acid would also be oxidized by a hepatic oxidase. Pristanoyl-CoA was not used in earlier experiments because we wanted to save our limited supply for the separation experiments. Pristanoyl-CoA oxidase activity was found in whole liver homogenates and in peroxisome-enriched fractions prepared by differential centrifugation. Fig. 7 shows that pristanoyl-CoA was oxidized in these enriched fractions at rates that were approximately 60% of those found with 2-methylpalmitoyl-CoA.

The subsequent part of this work was devoted to the separation of the different oxidases by means of chromatography on various columns.

Anion-exchange chromatography on a DEAE column was chosen as the initial step in the separation of the acyl-CoA oxidases. In the presence of 5 or 10 μM FAD the oxidases were not retained on the column. After removal of FAD by dialysis or by means of a desalting column, the major portion of the acyl-CoA oxidase activities bound to the column at a pH of 8.0 or higher. Glycerol was included in the elution buffer in order to prevent a loss of activity of trihydroxycoprostanoyl-CoA oxidase, which was not stable at alkaline pH in the absence of FAD.

Fig. 8 shows the separation of the oxidases on the DEAE column. While the retained palmitoyl-CoA oxidase activity eluted in two peaks, single peaks of activity were seen with presence of FAD (4, 5, 21). We, therefore, tested the heat lability of 2-methylpalmitoyl-CoA oxidase. Heating the oxidases at 55 °C in the presence of FAD inactivated trihydroxycoprostanoyl-CoA oxidase with a half-time of 18 min (Fig. 6), but palmitoyl-CoA oxidase and 2-methylpalmitoyl-CoA oxidase remained stable (data not shown). Trihydroxycoprostanoyl-CoA oxidase could be heated without loss of activity on condition that salt (e.g. sodium acetate, ammonium sulfate) was present (Fig. 6). The heat stability of the enzymes in the presence of FAD and salt (trihydroxycoprostanoyl-CoA oxidase) was then exploited for the partial purification of the enzymes by heat treatment and ammonium sulfate fractionation as described under "Experimental Procedures." This procedure resulted in a 30- to 35-fold purification of 2-methylpalmitoyl-CoA oxidase, trihydroxycoprostanoyl-CoA oxidase, and palmitoyl-CoA oxidase as calculated from the specific activities of the enzymes in whole liver homogenates and in the partially purified preparations.

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the enzyme was estimated at 415 ± 11 kDa (mean ± S.E.) for the four experiments, which is in agreement with our earlier estimation of the molecular mass of the noninducible palmitoyl-CoA oxidase (5).

The fractions eluting from the different columns were also analyzed by means of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 11). In the fractions of highest purity only one major band with a molecular mass of approximately 70 kDa was visible. This mass has previously also been attributed to the subunit of the noninducible palmitoyl-CoA oxidase (5).

After chromatography on hydroxylapatite the enzyme was purified nearly 450-fold as calculated from the specific activities of 2-methylpalmitoyl-CoA oxidase and pristanoyl-CoA oxidase in the starting homogenate and in the column fractions. At this step the yield of the enzyme was approximately 5%. Gel filtration did not result in a further increase in specific activity, possibly as a result of a slight loss of activity of the enzyme.

DISCUSSION

We recently described the presence of three acyl-CoA oxidases in rat liver: a fatty acyl-CoA (palmitoyl-CoA) oxidase that is induced by treatment of the animals with peroxisome proliferators and that has been purified before by others; a fatty acyl-CoA (palmitoyl-CoA) oxidase that is not induced; and trihydroxycoprostanoyl-CoA oxidase which oxidizes the CoA esters of the bile acid intermediates di- and trihydroxycoprostanic acids (5). The latter enzyme is not present in

trihydroxycoprostanoyl-CoA oxidase, 2-methylpalmitoyl-CoA oxidase, and pristanoyl-CoA oxidase. The latter two activities coeluted with the second palmitoyl-CoA oxidase peak.

When an enzyme preparation from a clofibrate-treated rat was injected on the column, the elution profile of the oxidases was similar to that observed with the preparation from an untreated rat. However, the first palmitoyl-CoA oxidase peak that was retained on the column was several-fold higher, whereas the height of the second peak remained unaltered (data not shown). This demonstrates that the palmitoyl-CoA oxidase activity eluting at low salt concentration is the inducible palmitoyl-CoA oxidase and that eluting at higher salt concentration is the noninducible palmitoyl-CoA oxidase. Analysis of the fractions containing the inducible palmitoyl-CoA oxidase by sodium dodecyl sulfate-polyacrylamide gel electrophoresis confirmed the presence of the 51- and 23-kDa subunits of the enzyme (4, 21) (data not shown). The order of elution of the inducible palmitoyl-CoA oxidase, trihydroxycoprostanoyl-CoA oxidase, and the noninducible palmitoyl-CoA oxidase is in agreement with previous chromatofocusing experiments from our laboratory (5).

In order to see whether the coeluting 2-methylpalmitoyl-CoA oxidase, pristanoyl-CoA oxidase, and noninducible palmitoyl-CoA oxidase activities reside with the same protein, the active fractions were pooled and further separated on hydroxylapatite. As evident from Fig. 9, all three activities again coeluted. The active fractions from the hydroxylapatite column were then subjected to gel filtration. Again coelution of the three enzymatic activities occurred on a Protein PAK 300 SW column (Fig. 10). The apparent molecular mass of

FIG. 8. Separation of hepatic acyl-CoA oxidases by anion-exchange chromatography. A partially purified oxidase preparation obtained by heat treatment and ammonium sulfate fractionation was brought to 10 mM Tris-HCl (pH 8.6), 20% (w/v) glycerol by means of a desalting column, and 2 ml containing 826 milliunits of palmitoyl-CoA oxidase, 129 milliunits of trihydroxycoprostanoyl-CoA oxidase, 669 milliunits of 2-methylpalmitoyl-CoA oxidase, and 286 milliunits of pristanoyl-CoA oxidase was loaded on a DEAE column equilibrated with the same buffer. Bound proteins were eluted by means of a linear pH/salt gradient as described under "Experimental Procedures." Fractions of 2 ml were collected and analyzed for protein, conductivity (% buffer B in the figure) and palmitoyl-CoA oxidase (□, recovery: 80%), trihydroxycoprostanoyl-CoA oxidase (●, recovery: 95%), 2-methylpalmitoyl-CoA oxidase (○, recovery: 80%), and pristanoyl-CoA oxidase (▲, recovery: 80%). Protein recovery was 110%. The palmitoyl-CoA oxidase activity eluting in the first fractions was not retained on the column. For the sake of clarity symbols were omitted when no activity was found in a fraction. Fractions 20 and 21 from six separate experiments (horizontal bar) were pooled and chromatographed on hydroxylapatite (Fig. 9).

FIG. 9. Chromatography of 2-methylpalmitoyl-CoA oxidase on hydroxylapatite. The fractions most enriched in 2-methylpalmitoyl-CoA oxidase from the DEAE column (see Fig. 8) were combined, precipitated with ammonium sulfate, and dissolved in 10 mM potassium phosphate buffer (pH 7.5), 5 μM FAD as described under "Experimental Procedures." 0.9 ml containing 231 milliunits palmitoyl-CoA oxidase, 742 milliunits 2-methylpalmitoyl-CoA oxidase, 356 milliunits pristanoyl-CoA oxidase, and 2.6 mg of protein was injected on a hydroxylapatite column equilibrated with the same buffer, and the oxidases were eluted with a linear phosphate gradient. Elution was monitored at 210 nm (dotted line), since the absorbance at 280 nm was very low, indicating a low content of aromatic amino acids for the oxidases under study. Fractions (1.8 ml) were analyzed for phosphate (dashed line), protein (not shown; recovery: 99%), palmitoyl-CoA oxidase (□, recovery: 72%), 2-methylpalmitoyl-CoA oxidase (○, recovery: 87%), and pristanoyl-CoA oxidase (▲, recovery: 75%). Fractions 16-19 (horizontal bar) were pooled and subjected to gel filtration (Fig. 10).
Pristanoyl-CoA Oxidase

Fig. 10. Gel filtration of 2-methylpalmitoyl-CoA oxidase. top, column calibration. The following marker proteins were used: β-galactosidase (540 kDa), urease (480 kDa), ferritin (440 kDa), glutamate dehydrogenase (320 kDa), catalase (322 kDa), aldolase (160 kDa), lactate dehydrogenase (140 kDa), citrate synthase (87 kDa), galactosidase (540 kDa), urease (480 kDa), ferritin (440 kDa), glutathione peroxidase (360 kDa), myoglobin (169 kDa), and carbonic anhydrase (29 kDa). The void volume ($V_v$) and total volume ($V_t$) were determined with blue dextran and FAD, respectively. The column fractions were analyzed for enzymatic activity using standard methods and for absorption at 620 nm (blue dextran), 280 nm (ferritin), 550 nm (cytochrome c after reduction with dithionite), or 450 nm (FAD). The regression coefficient of $V_t$ plotted versus log molecular mass was 0.98. In this experiment, the elution volume of 2-methylpalmitoyl-CoA oxidase, indicated by an open circle, corresponded to a molecular mass of 408 kDa. bottom, the fractions most enriched in 2-methylpalmitoyl-CoA oxidase from the hydroxylapatite column (see Fig. 9) were precipitated with ammonium sulfate and dissolved in 0.2 M potassium phosphate buffer (pH 7.5), 10 μM FAD, 1% (v/v) ethylene glycol, 50 μL containing 11.5 milliunits palmitoyl-CoA oxidase, 38.6 milliunits 2-methylpalmitoyl-CoA oxidase, and 16.9 milliunits pristanoyl-CoA oxidase, was injected on a Protein PAK 300 SW column equilibrated with the same buffer. Elution from the hydroxylapatite column (see Fig. 9) was precipitated with ammonium sulfate and dissolved in 0.2 M potassium phosphate buffer (pH 7.5), 10 μM FAD, 1% (v/v) ethylene glycol, 50 μL containing 11.5 milliunits palmitoyl-CoA oxidase, 38.6 milliunits 2-methylpalmitoyl-CoA oxidase, and 16.9 milliunits pristanoyl-CoA oxidase, was injected on a Protein PAK 300 SW column equilibrated with the same buffer. Elution from the hydroxylapatite column; lanes b–g, proteins contained in 22.5 μL of fractions 15–19 of the hydroxylapatite column; lane h, proteins contained in 5 μL of the preparation applied to the gel filtration column (derived from fractions 16–19 of the hydroxylapatite column); lanes i–l, proteins contained in 100 μL of fractions 18–21 of the gel filtration column. The position of the molecular weight markers, their mass expressed in kDa, is shown on the left side. The molecular mass of the subunit of 2-methylpalmitoyl-CoA oxidase was estimated at 69.4 ± 0.3 kDa (mean ± S.E. for four experiments). The 35-kDa band in lane h is an external contaminant not belonging to the enzyme preparation since it is not visible in the other lanes.

establishing that 2-methylpalmitoyl-CoA and pristanoyl-CoA are oxidized by the noninducible palmitoyl-CoA oxidase. The apparent molecular mass of the enzyme and that of its subunit are the same as those described earlier for the noninducible palmitoyl-CoA oxidase (5). The enzyme oxidizes 2-methylpalmitoyl-CoA twice as rapidly as palmitoyl-CoA and pristanoyl-CoA as rapidly as palmitoyl-CoA so that it can be considered as a branched chain acyl-CoA oxidase. Because one of its naturally occurring substrates is pristanoyl-CoA, we propose to call this enzyme pristanoyl-CoA oxidase.

Osumi and Hashimoto and their associates (22, 23) describe that rat liver contains two species of palmitoyl-CoA oxidase mRNA, which have the same number of nucleotides and which differ in their nucleotide sequence only in a small region. They are produced by alternative splicing of the transcript of a single gene. The nucleotide sequence of one of the two mRNA species corresponds to the amino acid sequence of the inducible palmitoyl-CoA oxidase but the translation product of the second species remains undetected. Although the molecular mass of the subunit of pristanoyl-CoA oxidase agrees with the nucleotide number of the splicing products of the palmitoyl-CoA oxidase gene, pristanoyl-CoA oxidase did not cross-react with polyclonal antibodies raised against the purified inducible palmitoyl-CoA oxidase, ruling out that pristanoyl-CoA oxidase is the translation product of the second mRNA species.²

Pristanoyl-CoA oxidase is present not only in liver but also in extrahepatic tissues. It is not induced by peroxisome pro-

²B. de Béthune, P. P. Van Veldhoven, and G. P. Mannerts, unpublished results.
lished results.

The physiological role of the enzyme is most probably the degradation of isoprenoid fatty acids and a number of other isoprenoids which can be converted to carboxylic acids via oxidation of a terminal methyl group. These isoprenoids may include the side chains of the fat-soluble vitamins but also dolichols, which most probably can be converted to dolicholic acids (25). Pristanoyl-CoA oxidase seems to be present also in human tissues. Wanders et al. (26) recently found pristanoyl-CoA oxidase activity in human liver, and we found that in human liver 2-methylpristanoyl-CoA oxidase activity is twice as high as palmityl-CoA oxidase activity. Poulos et al. (6) observed that pristanic acid accumulates in the plasma of patients with a generalized peroxisomal dysfunction (Zellweger syndrome, neonatal adrenoleukodystrophy and infantile Refsum’s disease), presumably as a result of a deficiency of pristanoyl-CoA oxidase. It might be worthwhile to look for a possible accumulation of other isoprenoid derivatives in these patients. Isolated deficiencies have been described for almost every peroxisomal β oxidation enzyme (27). The discovery of cases of isolated deficiency of pristanoyl-CoA oxidase would help to further clarify the physiological function of the enzyme and might reveal in how far a possible accumulation of isoprenoids contributes to the pathogenesis of the severe neurological and other abnormalities seen in patients with a generalized peroxisomal dysfunction. In this connection, our data also show that 2-methylpalmitate and 2-methylpristanate are easier to synthesize than pristanic acid, which are more difficult to synthesize. Our data also show that 2-methylpalmitate and 2-methylpristanate are easier to synthesize than pristanic acid, which are easier to synthesize than pristanic acid, and pristanoyl-CoA, which are easier to synthesize than pristanic acid, and pristanoyl-CoA, will be useful substrates for the detection of pristanoyl-CoA oxidase deficiencies in selected patients.

3-Methyl-substituted fatty acids cannot undergo β-oxidation, because the 3-methyl substitution prevents the 3-hydroxyacyl-CoA dehydrogenase reaction, the third step of the β-oxidation sequence. The first two steps are theoretically possible, however. We looked for 3-methylheptadecanoyl-CoA oxidase activity in rat liver but could not detect it, suggesting that pristanoyl-CoA oxidase is not active towards 3-methyl-substituted acyl-CoA esters.

One might wonder whether the fact that pristanoyl-CoA oxidase also displays palmityl-CoA oxidase activity would be advantageous or disadvantageous to the oxidation of branched fatty acids. Since straight chain fatty acids are in excess, they would inhibit the oxidation of branched chain fatty acids by competing for the enzyme. On the other hand, there is some evidence that in mitochondria the β-oxidation enzymes are associated with each other in multienzyme complexes in which the β-oxidation intermediates are channeled from one enzyme to the other without being released in the matrix (28). If this is the case in peroxisomes, the palmityl-CoA oxidase activity of pristanoyl-CoA oxidase might perhaps become a necessity since the oxidation of, for instance, pristanic acid involves three β-oxidation cycles of a 2-methyl-substituted acyl-CoA chain, in which propionyl-CoA is cleaved off, but also three β-oxidation cycles of a 4-methyl-substituted acyl-CoA chain in which acetyl-CoA is cleaved off. Finally, approximately one-third of the palmityl-CoA ox-

1 P. P. Van Veldhoven, G. Vanhove, and G. P. Mannaerts, unpublished results.

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