Identification and characterization of the 2-enoyl-CoA hydratases involved in peroxisomal β-oxidation in rat liver

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In this study we attempted to determine the number of 2-enoyl-CoA hydratases involved in peroxisomal β-oxidation. We therefore separated peroxisomal proteins from rat liver on several chromatographic columns and measured hydratase activities on the eluates with different substrates. The results indicate that rat liver peroxisomes contain two hydratase activities: (1) a hydratase activity associated with multifunctional protein 1 (MFP-1) (2-enoyl-CoA hydratase/Δ^3,Δ^2-enoyl-CoA isomerase/L-3-hydroxyacyl-CoA dehydrogenase) and (2) a hydratase activity associated with MFP-2 (17β-hydroxysteroid dehydrogenase/D-3-hydroxyacyl-CoA dehydrogenase/2-enoyl-CoA hydratase). MFP-1 forms and dehydrogenates L-3-hydroxyacyl-CoA species, whereas MFP-2 forms and dehydrogenates D-3-hydroxyacyl-CoA species. A portion of MFP-2 is proteolytically cleaved, most probably in the peroxisome, into a 34 kDa 17β-hydroxysteroid dehydrogenase/D-3-hydroxyacyl-CoA dehydrogenase and a 45 kDa D-specific 2-enoyl-CoA hydratase. Finally, the results confirm that MFP-1 is involved in the degradation of straight-chain fatty acids, whereas MFP-2 and its cleavage products seem to be involved in the degradation of the side chain of cholesterol (bile acid synthesis)

INTRODUCTION

Peroxisomes are the subcellular site of β-oxidation of very-long-chain fatty acids, 2-methyl-branched fatty acids (e.g. pristanic acid and the synthetic 2-methylpalmitic acid) and the bile acid intermediates di- and trihydroxycoprostanic acids [1–3]. In rat liver the first step of peroxisomal β-oxidation is catalysed by three separate acyl-CoA oxidases: palmitoyl-CoA oxidase [4,5], pristanoyl-CoA oxidase [6] and trihydroxycoprostanoyl-CoA oxidase [7], involved in the oxidation of the CoA esters of straight-chain fatty acids, 2-methyl-branched fatty acids and the bile acid intermediates respectively [8]. In addition we have shown that the second (2-enoyl-CoA hydratase) and third (3-hydroxyacyl-CoA dehydrogenase) reactions of peroxisomal β-oxidation are catalysed by two separate multifunctional proteins (MFPs) [9,10]. MFP-1 (78 kDa) displays 2-enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and Δ^3,Δ^2-enoyl-CoA isomerase activity [11,12]. The enzyme specifically dehydrogenates L-3-hydroxy stereoisomers and shows homology with the hydratases and 3-hydroxyacyl-CoA dehydrogenases present in mitochondria. MFP-2 (79 kDa) displays 2-enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 17β-hydroxysteroid dehydrogenase activity. The enzyme specifically dehydrogenates D-3-hydroxy stereoisomers [10] and shows homology with the D-specific peroxisomal β-oxidation MFP from yeast [13]. MFP-2 is identical with an enzyme originally described as pig endometrial 17β-oestradiol dehydrogenase IV [14] and consists of an N-terminal short-chain alcohol dehydrogenase domain, harbouring the 17β-hydroxysteroid dehydrogenase activity [15] and the D-3-hydroxyacyl-CoA dehydrogenase activity [10], followed by a 2-enoyl-CoA hydratase domain and finally a C-terminal domain that shows homology with sterol carrier protein 2 [15]. After importation into the peroxisome, a portion of MFP-2 is proteolytically cleaved, giving rise to an active homodimeric enzyme (subunit approx. 40 kDa by SDS/PAGE) that consists of the N-terminal 17β-hydroxysteroid/3-hydroxyacyl-CoA dehydrogenase domain of MFP-2 [10]. Rat liver peroxisomes also contain a 45 kDa (gel electrophoresis) polypeptide that cross-reacts with antiserum raised against MFP-2 [10], suggesting that this polypeptide might represent the C-terminal hydratase/sterol carrier protein 2 domains of MFP-2.

The naturally occurring 3-hydroxyacyl-CoA intermediate in the β-oxidation of straight-chain fatty acids has the L-configuration [16,17] indicating that MFP-1 is involved in the degradation of straight-chain fatty acids. The physiological 3-hydroxyacyl-CoA intermediate in bile acid synthesis was originally reported to be 24*R*,25*S*-varanoyl-CoA in which the 24*R* hydroxyl group corresponds to a D-3-hydroxyl group in the carboxy side chain [18,19]. The physiological 3-hydroxyacyl-CoA intermediate in bile acid synthesis was originally reported to be 24*R*,25*S*-varanoyl-CoA in which the 24*R* hydroxyl group corresponds to a D-3-hydroxyl group in the carboxy side chain [18,19]. The designation of this isomer, based on reports by three different groups [20–22], was recently changed to 24*R*,25*R*. The D-stereospecificity of MFP-2, together with the fact that MFP-2 specifically dehydrates and dehydrogenates the 24*R*,25*R* stereoisomer of varanoyl-CoA [10], indicates that MFP-2 is not involved in the β-oxidation of straight-chain fatty acids but in that of the side chain of cholesterol (bile acid synthesis). The stereochemical configuration of the 3-hydroxy intermediate in the β-oxidation of 2-methyl-branched fatty acids remains unknown; it is therefore currently impossible to conclude which of the two MFPs is involved in the metabolism of the branched fatty acids.

The presence of three acyl-CoA oxidases and three 3-hydroxyacyl-CoA dehydrogenases (MFP-1, MFP-2 and its N-terminal cleavage product) raises the question of whether hydratases besides the two MFPs described above occur in

Abbreviation used: MFP, multifunctional protein.

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peroxisomes. Indeed, three research groups reported the presence of so-called peroxisomal hydratases 2, also known as D-3-hydroxyacyl-CoA dehydratases. A first protein, described as an 80 kDa homodimer consisting of 44 kDa subunits, was purified by Li et al. [23]. Malila et al. [24] described a 62 kDa homodimer (subunit 33.5 kDa) and Cook et al. [25] purified a protein of 150 kDa consisting of 78, 71 and 47 kDa polypeptides after gel electrophoresis. All three enzymes specifically catalysed the hydration of a 2-*trans*-enoyl-CoA to a D-3-hydroxyacyl-CoA.

The aim of the present work was to determine the exact number of hydratases present in peroxisomes and to elucidate how closely (some of) the additional hydratases would be related to the two MFPs.

EXPERIMENTAL

Materials

CoA, acetoacetyl-CoA and palmitoyl-CoA were from Pharmacia Benelux. Nycodenz was from Nycomed. Acyl-CoA oxidase (from *Arthrobacter* sp.), crotonase (from bovine liver), octanoyl-CoA and crotonyl-CoA were from Sigma. 3-Hydroxyacyl-CoA dehydrogenase (pig heart) was obtained from Boehringer Mannheim. C_{18} cartridges (500 mg; 3 ml) were from Varian Benelux.

Animals

Livers from control and clofibrate-treated rats were used as described previously [9].

Synthesis and purification of the substrates

2-*trans*-Octenoyl-CoA and 2-*trans*-hexadecenoyl-CoA were synthesized as described previously [10]. The four stereoisomers of varanoyl-CoA were obtained as described by Dieuaide-Noubhani et al. [10]. Racemic 3-hydroxy-2-methylpalmitoyl-CoA was prepared as described previously [9]. D-3-Hydroxyoctanoyl-CoA was synthesized by incubating octanoyl-CoA (200 μM) in 50 mM Tris/HCl, pH 8, with acyl-CoA oxidase (25 m-units/ml) and with purified rat liver MFP-2 (30 m-units/ml octenoyl-CoA hydratase). After 20 min of incubation, the CoA esters were partly purified by applying the reaction mixture to a C_{18} cartridge and eluting the esters with methanol [10]. After evaporation of the methanol, the residue was dissolved in 50 mM potassium phosphate buffer, pH 5.3, (buffer A) and injected on an analytical reverse-phase column (Econosphere C_{18}, 150 mm × 4.6 mm, pore size 80 Å, bead size 5 μm; Alltech). The D-3-hydroxyoctanoyl-CoA and the octenoyl-CoA were separated with a gradient of acetonitrile (15–22 %, v/v) in buffer A, at a flow rate of 1 ml/min. The acetonitrile was evaporated under nitrogen and the sample containing the pure D-3-hydroxyoctanoyl-CoA was applied to a C_{18} cartridge to remove the buffer salt.

Purification and subfractionation of peroxisomes

Peroxisomes were purified and subfractionated into matrix proteins (S1 fraction), peripheral membrane proteins (S2 fraction) and integral membrane proteins (fraction P2) as described previously [9].

Chromatographic purification of the 2-enoyl-CoA hydratases

Protein chromatography was done on an inert Waters 600 system. The following columns were used: protein PAK Glass SP-5PW (8.0 mm × 75 mm), protein PAK Glass HIC phenyl-5PW (8.0 mm × 75 mm), both from Nihon Waters Ltd, and alkyl-Superose HR 5/5 from Pharmacia.

The peroxisomal peripheral-membrane protein fraction (S2) was concentrated against solid poly(ethylene glycol) (20 kDa) and dialysed overnight against 20 mM potassium phosphate, pH 7.0. After centrifugation the protein solution was applied to a cation-exchange sulphopropyl column eluted with a phosphate gradient as described previously [9]. The flow rate was 0.8 ml/min and fractions of 1.6 ml were collected.

Fractions from the sulphopropyl column (fractions 13–19 and fractions 20–25 in the experiment presented in Figure 1) were pooled, concentrated, dialysed overnight against 20 mM potassium phosphate buffer (pH 7.5)/0.6 M $(NH_4)_2SO_4$ (buffer A) and injected to a phenyl column equilibrated with the same buffer. The bound proteins were eluted by a linear negative salt and a positive ethylene glycol gradient [100–0 % buffer A; 0–100 % buffer B containing 20 mM potassium phosphate, pH 7.5 and 30 % (w/v) ethylene glycol] over 50 min at a flow rate of 0.8 ml/min. Fractions of 1.6 ml were collected.

Enzyme assays

Varanoyl-CoA dehydrogenase [9] and acetoacetyl-CoA reductase [26] activities were measured as described previously. Hydratase activities were measured as the hydration of 2-*trans*-enoyl-CoAs for straight-chain fatty acids or in the reverse direction (dehydration) for varanoyl-CoA and 3-hydroxy-2-methylpalmitoyl-CoA as described previously [10]. D-3-Hydroxyacyl-CoA dehydratase activity (substrate D-3-hydroxyoctanoyl-CoA) was measured indirectly by monitoring NADH production in a reaction mixture containing crotonase (conversion of the 2-octenoyl-CoA, formed via dehydration of D-3-hydroxyoctanoyl-CoA, to L-3-hydroxyoctanoyl-CoA) and L-3-hydroxyacyl-CoA dehydrogenase (dehydrogenation of the L-3-hydroxyoctanoyl-CoA). The reaction mixture contained 50 mM Tris/HCl, pH 8.0, 1 mM NAD^+, 60 mM hydrazine, pH 8.0, 50 mM KCl, 0.05 % defatted BSA, 25 μM D-3-hydroxyoctanoyl-CoA, crotonase (2 units/ml) and L-3-hydroxyacyl-CoA dehydrogenase (40 μg/ml). The reaction was started by the addition of 25 μl of enzyme solution, diluted in 0.125 M Tris/HCl, pH 8.0, to the incubation mixture, in a total volume of 250 μl. NADH formation was followed at 340 nm and corrected for substrate-independent NADH formation and for NADH production due to D-hydroxyacyl-CoA dehydrogenase activity. Normally, at the dilution used to measure D-dehydratase activity, the D-dehydrogenase activity was responsible for less than 10 % of the NADH production.

SDS/PAGE and immunoblotting

SDS/PAGE, silver staining of the gels and immunoblotting were performed as described previously [9,10].

N-terminal sequencing of the 45 kDa polypeptide

Fractions corresponding to peak D (see Figure 3) of octenoyl-CoA hydratase activity from the phenyl column were pooled, dialysed against 20 mM potassium phosphate buffer, pH 7, and concentrated by dialysis against poly(ethylene glycol) (20 kDa). Proteins were precipitated by adding 0.1 vol. of 100 % (w/v) trichloroacetic acid and were collected by centrifugation. The pellet was redissolved in 20 μl of SDS-containing sample buffer and loaded on a minigel containing 10 % (w/v) polyacrylamide [27]. Proteins were further electroblotted on a Pro Blott membrane (Applied Biosystems) in 0.1 M Tris/borate buffer, pH 8.0

[28], and the pattern was revealed by staining with Amido Black. The band corresponding to the 45 kDa enoyl-CoA hydratase was excised and subjected to automated Edman degradation by using a 477A model sequenator equipped with a 120A model phenylthiohydantoin amino acid analyser (Applied Biosystems).

RESULTS

Subperoxisomal distribution of 2-enoyl-CoA hydratase activities

Highly purified peroxisomes were subfractionated into matrix, peripheral membrane and integral membrane proteins. Table 1 shows that most of the hydratase and dehydratase activities were found in the protein fraction that behaves as peripheral membrane proteins. Similar results have previously been obtained for the 3-hydroxyacyl-CoA dehydrogenase activities [10]. As for the varanoyl-CoA stereoisomers, dehydratase activity was found only with the 24*R*,25*R* and 24*S*,25*S* stereoisomers.

Separation of the 2-enoyl-CoA hydratases

The peripheral-membrane protein fraction was used for the further separation of the hydratases. After dialysis and concentration, the proteins were applied to a sulphopropyl column and eluted with a linear gradient of potassium phosphate (Figure 1). The highest hydratase activities were obtained with crotonyl-CoA and octenoyl-CoA as the substrates. Crotonyl-CoA hydratase activity was eluted in one large peak (fractions 20 and 21) that also showed octenoyl-CoA and hexadecenoyl-CoA hydratase activities and 3-hydroxy-2-methylhexadecanoyl-CoA and 24*S*,25*S*-varanoyl-CoA dehydratase activities. Octenoyl-CoA hydratase activity was eluted in two peaks: the peak described above (fractions 20 and 21) and an earlier peak (fractions 13–19) that also displayed 24*R*,25*R*-varanoyl-CoA dehydratase activity but little crotonase activity. The substrate spectrum of the early peak corresponds to that of the purified MFP-2 [9,10], whereas that of the second peak (fractions 20 and 21) corresponds to that of the purified MFP-1 [9]. SDS/PAGE confirmed the presence of MFP-2 (79 kDa) in fractions 15–20 and the presence of MFP-1 (78 kDa) in fractions 17–22 (Figure 2). A smaller peak of activity was seen in fraction 18 with crotonyl-CoA, 3-hydroxy-2-methylhexadecanoyl-CoA and hexadecenoyl-CoA as the substrates (Figure 1). This peak, which did not correspond to a particular band on SDS/PAGE, might be the result of the simultaneous presence of MFP-1 and MFP-2 (Figure 2).

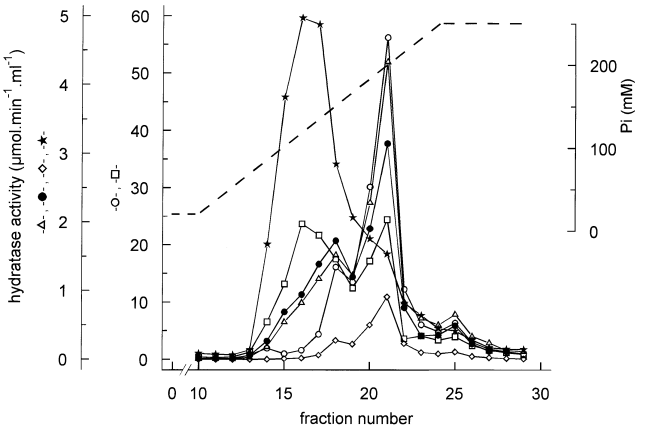

Figure 1 Separation of hepatic peroxisomal 2-enoyl-CoA hydratases from untreated rats by cation-exchange chromatography

A 15.2 ml portion of the peripheral-membrane protein fraction (S2), containing 21 mg of protein and derived from 180 g of rat liver, was concentrated approx. 4-fold and dialysed overnight against buffer A. After removal of particulate material by centrifugation, the protein solution contained 1230 units of crotonase, 1410 units of octenoyl-CoA hydratase, 68 units of hexadecenoyl-CoA hydratase, 70 units of 3-hydroxy-2-methylpalmitoyl-CoA dehydratase, 168 units of 24*R*,25*S*-varanoyl-CoA dehydratase and 29 units of 24*S*,25*S*-varanoyl-CoA dehydratase. A sample of 1.6 ml was injected on the sulphopropyl column, equilibrated with buffer A. The bound proteins were eluted by means of a linear phosphate gradient (20–250 mM, broken line). Fractions of 1.6 ml were collected and 2-enoyl-CoA hydratase activities were assayed with crotonyl-CoA (○, recovery 60%), octenoyl-CoA (□, recovery 53%), hexadecenoyl-CoA (●, recovery 61%), 3-hydroxy-2-methylpalmitoyl-CoA (△, recovery 81%), 24*R*,25*S*-varanoyl-CoA (★, recovery 60%) and 24*S*,25*S*-varanoyl-CoA (◇, recovery 80%). Fractions 11–19 and fractions 20–22 from two runs were pooled separately and used for further purification. Symbols are omitted where no activity was found in a fraction.

Fractions 11–19 and 20–22 from the sulphopropyl column were separately pooled and each of the pooled fractions was chromatographed on a phenyl column. The hydratase activities of fractions 20–22 from the sulphopropyl column were again co-eluted in a single peak (Figure 3, upper panel) that corresponded to the presence of MFP-1 on SDS/PAGE and immunoblotting (results not shown). The hydratase activities of fractions 11–19 from the sulphopropyl column were eluted in four peaks

Table 1 Release of enoyl-CoA hydratase activity after subfractionation of purified peroxisomes

Purified peroxisomes from rat liver were subfractionated into matrix proteins (S1), peripheral membrane proteins (S2) and integral membrane plus core proteins (P2) as described previously [9]. Peroxisomes and subfractions were analysed for protein, hydratase activity and acetoacetyl-CoA reductase activity. The activities measured in peroxisomes, expressed in μmol/min per mg of protein, are means ($n = 2$) or means ± S.E.M. for $n \geqslant 3$ (shown in parentheses) independent determinations. Activities measured in the subfractions, expressed as percentages of the total recovered amount or activity, are means for two to six independent experiments. Recoveries were between 75% and 150%.

Fraction	Protein amount (% of total)	Crotonyl-CoA hydratase	Octenoyl-CoA hydratase	Hexadecenoyl-CoA hydratase	3-Hydroxy-2-methyl-palmitoyl-CoA dehydratase	24*R*,25*R*-Varanoyl-CoA dehydratase	24*S*,25*S*-Varanoyl-CoA dehydratase	Acetoacetyl-CoA reductase
		Activity (μmol/min per mg of protein)						
Peroxisomes		18.2 ± 2.3 (6)	16.4 ± 1.4 (6)	2.2 ± 0.3 (4)	2.1 (2)	1.2 ± 0.2 (4)	0.2 ± 0.03 (3)	0.5 ± 0.06 (5)
		Activity in subfraction (% of total)						
S1	36	12	10	7	9	9	7	17
S2	18	67	67	48	66	66	80	56
P2	46	21	23	45	25	25	13	27

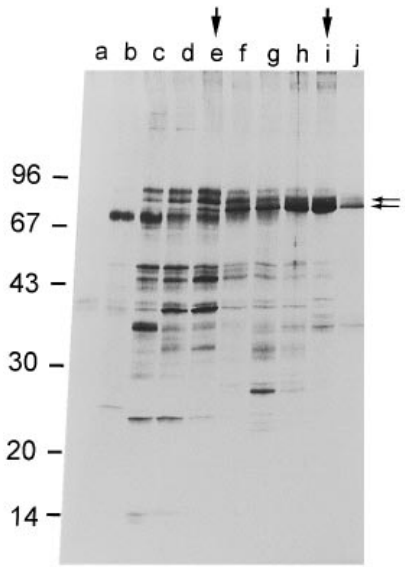

Figure 2 Analysis of the fractions from the sulphopropyl column by SDS/PAGE and silver staining

Lanes a–j correspond to proteins contained in 10 μl of fractions 13–22 from the sulphopropyl column described in Figure 1. The left-hand vertical arrow indicates the peak fraction containing MFP-2 (fraction 17) and the right-hand vertical arrow indicates the peak fraction containing MFP-1 (fraction 21). The horizontal arrows indicate the positions of MFP-2 (79 kDa) and MFP-1 (78 kDa). The positions of the protein markers, their molecular masses expressed in kDa, are shown at the left.

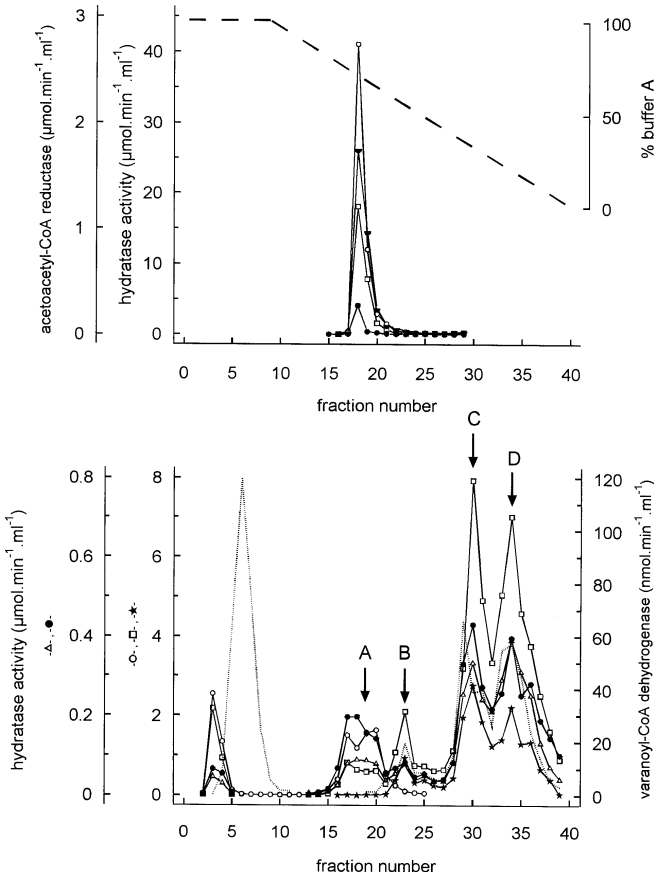

Figure 3 Hydrophobic chromatography of the 2-enoyl-CoA hydratases

Fractions 20–22 (upper panel) and fractions 11–19 (lower panel) from the sulphopropyl column (Figure 1) were separately pooled, concentrated, and dialysed overnight against 20 mM potassium phosphate buffer (pH 7.5)/0.6 M $(NH_4)_2SO_4$ and injected on the phenyl column. The bound proteins were eluted by means of a negative salt and a positive ethylene glycol gradient as described in the Experimental section (percentage of buffer A, broken line). Fractions of 1.6 ml were collected and assayed for enoyl-CoA hydratase activity with crotonyl-CoA (○), octenoyl-CoA (□), hexadecenoyl-CoA (●), 3-hydroxy-2-methylpalmitoyl-CoA (▲) and 24*R*,25*R*-varanoyl-CoA (★), and for 24*R*,25*R*-varanoyl-CoA dehydrogenase (dotted line) and acetoacetyl-CoA reductase (⬬). Upper panel: 1.1 ml of the concentrated fraction containing 221 units of crotonase (recovery 44%), 113 units of octenoyl-CoA hydratase (recovery 49%), 22 units of hexadecenoyl-CoA hydratase (recovery 39%), and 6.75 units of acetoacetyl-CoA reductase (recovery 79%) were injected. Lower panel: 1.7 ml of the concentrated fraction containing 35 units of crotonase (recovery 55%), 197 units of octenoyl-CoA hydratase (recovery 58%), 12 units of hexadecenoyl-CoA hydratase (recovery 54%), 12 units of 3-hydroxy-2-methylpalmitoyl-CoA dehydratase (recovery 44%), 52 units of 24*R*,25*R*-varanoyl-CoA dehydratase (recovery 54%) and 1.3 units of 24*R*,25*R*-varanoyl-CoA dehydrogenase (recovery 90%) were injected. Symbols are omitted where no activity was found in a fraction. Vertical arrows indicate the positions of the four peak fractions of hydratase activity (A to D).

designated A to D according to their order of elution (Figure 3, lower panel). SDS/PAGE and immunoblotting demonstrated that the activities contained in peak A were due to the presence of contaminating MFP-1 (results not shown), a finding that agrees with the substrate spectrum of peak A. Surprisingly, the three additional peaks, B (fractions 22–24), C (fractions 28–31) and D (fractions 33–36) showed similar substrate spectra, displaying a high activity towards octenoyl-CoA and only very low activity towards crotonyl-CoA. Activities were also recorded in all three peaks with hexadecenoyl-CoA (hydration) and with 3-hydroxy-2-methylhexadecanoyl-CoA and 24*R*,25*R*-varanoyl-CoA (dehydration) as the substrates. The column eluate was also analysed for 24*R*,25*R*-varanoyl-CoA dehydrogenase activity. Activity was found in fractions 6–8, corresponding to the presence of the N-terminal MFP-2 cleavage product (see the Introduction section; results not shown), and also in peaks B, C and D. Careful inspection of these peaks revealed that for peaks B and D the varanoyl-CoA dehydrogenase activity co-eluted with the hydratase activities, but that for peak C the varanoyl-CoA dehydrogenase activity was eluted one fraction earlier than the hydratase activities. Gel electrophoresis of the eluate fractions showed that the activities of peaks C and D (for peak B, see below) corresponded to the relative abundance of two polypeptides with molecular masses of 79 and 45 kDa (Figure 4), presumably being MFP-2 and the C-terminal hydratase/sterol carrier protein 2 domain of MFP-2 (see below) respectively. In support of this contention, both polypeptides reacted with the antiserum raised against MFP-2 (results not shown). In peak D the relative abundances of the 79 and 45 kDa polypeptides were superimposable, whereas in peak C the 79 kDa polypeptide was most abundant in fraction 29 and the 45 kDa polypeptide in fraction 30 (Figure 4), probably explaining the shift by one fraction between the peak varanoyl-CoA dehydrogenase and the hydratase activities.

Fractions 28–39 (peaks C and D) were pooled and injected on different columns (cation-exchange and anion-exchange columns) to separate the 45 kDa polypeptide from the 79 kDa polypeptide. During further chromatographic steps, complete separation of the two polypeptides was not achieved (results not shown), suggesting that MFP-2 and its C-terminal cleavage product are rather tightly associated. The (inhomogeneous) association between MFP-2 and its C-terminal cleavage product may explain why the hydratase activities were eluted in two peaks (C and D) from the phenyl column.

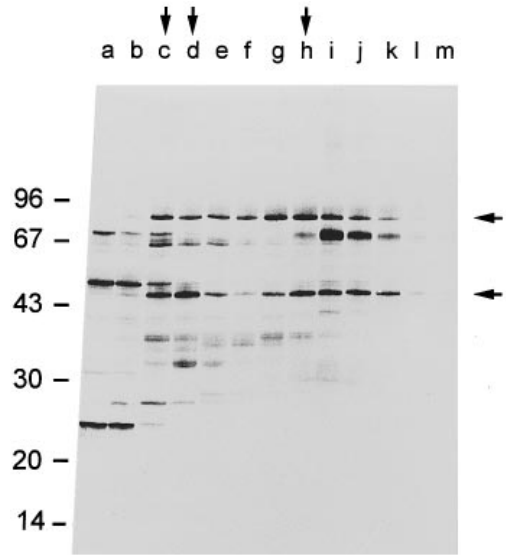

Figure 4 Analysis of fractions eluted from the phenyl column by SDS/PAGE and silver staining

Fractions from the column described in Figure 3 (lower panel) were analysed by SDS/PAGE. Lanes a–m correspond to proteins contained in 40 μl of fractions 27–39 from the phenyl column. The horizontal arrows indicate the position of the MFP-2 (79 kDa) and the 45 kDa polypeptide, and vertical arrows indicate fractions corresponding to peaks of 2-enoyl-CoA hydratase activity (fractions 29 and 34) and/or 3-hydroxyacyl-CoA dehydrogenase activity (fractions 30 and 34) on the phenyl column. The positions of the protein markers, their molecular masses expressed in kDa, are shown at the left.

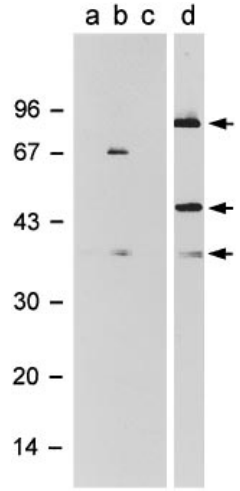

Figure 5 Immunoblot analysis of fractions eluted from the phenyl column

After separation by SDS/PAGE, proteins contained in fractions 22–24 (lanes a–c) and 34 (lane d) from the phenyl column (Figure 3, lower panel) were transferred to nitrocellulose and the blot was incubated with antibodies raised against MFP-2, followed by detection with alkaline phosphatase-labelled anti-rabbit IgG. The horizontal arrows indicate the position of the MFP-2 (79 kDa) and its two cleavage products. The positions of the protein markers, their molecular masses expressed in kDa, are shown at the left.

Fraction 30 from the phenyl column also contained a 33 kDa polypeptide (Figure 4) that might correspond to the 33.5 kDa D-dehydratase described by Malila et al. [24]. However, separation of peroxisomal proteins from clofibrate-treated rats indicated that the 33 kDa polypeptide does not display hydratase activity, as detailed below.

Finally, electrophoresis and immunoblot analysis of fractions 22–24 from the phenyl column (peak B) demonstrated the presence of a 67 kDa polypeptide cross-reacting with antibodies raised against MFP-2 (Figure 5), suggesting that the hydratase activities of peak B were due to the presence of a proteolytic degradation product of MFP-2. The 67 kDa polypeptide was not purified further to confirm this possibility.

The 45 kDa polypeptide is the C-terminal hydratase/sterol carrier protein 2 domain of MFP-2

Previous data from our laboratory demonstrated that a portion of MFP-2 is cleaved *in vivo*, giving rise to a separate enzyme that comprises the N-terminal domain of MFP-2, containing the 17β-hydroxysteroid and D-3-hydroxyacyl-CoA dehydrogenase activities [10]. Those experiments and the present study also revealed the occurrence of a 45 kDa polypeptide cross-reacting with antibodies raised against MFP-2. These results suggested that the 45 kDa polypeptide might represent the C-terminal hydratase and sterol carrier protein 2 domains of MFP-2. N-terminal sequencing of the 45 kDa polypeptide revealed a major sequence Ser-Ala-Asp-Ala-Ser-Gly-Phe-Ala-Gly and a minor sequence Gly-Gln-Val-Ala-Ser-Ala-Asp-Ala-Ser, which start at amino acids 317 and 313 of MFP-2 respectively. Additionally, ligand-blotting analysis (M. Fransen, G. P. Mannaerts and P. P. Van Veldhoven, unpublished work) demonstrated that MFP-2 and the 45 kDa polypeptide are recognized by the bacterially expressed peroxisome targeting signal 1 receptor [29], indicating that the 45 kDa protein still carries the C-terminal peroxisomal targeting signal (Ala-Lys-Leu) of MFP-2 [10]. Overall, our earlier and the present results show that a portion of MFP-2 is cleaved into a 17β-hydroxysteroid/D-3-hydroxyacyl-CoA dehydrogenase and a 2-enoyl-CoA hydratase/sterol carrier protein 2. The molecular masses of the dehydrogenase and hydratase/sterol carrier domains, calculated from the deduced amino acid sequence [10], are 33753 and 45525 Da and 34108 and 45170 Da when the protein is cleaved after amino acid residues 312 and 316 respectively.

MFP-2 displays D-hydratase activity

Previous results from our laboratory showed that MFP-2 specifically dehydrogenates D-3-hydroxyacyl-CoAs including the physiological 24*R*,25*R*-varanoyl-CoA stereoisomer [10]. We also demonstrated that MFP-2 specifically dehydrates 24*R*,25*R*-varanoyl-CoA, but the stereospecificity of the hydration reaction in the forward direction was not investigated [10]. Therefore purified MFP-2 was incubated with 2-*trans*-octenoyl-CoA in the absence of NAD^+, leading to the partial conversion of the enoyl-CoA to the 3-hydroxyacyl-CoA as measured at 263 nm. When a plateau was reached, 1 mM NAD^+ and mitochondrial L-3-hydroxyacyl-CoA dehydrogenase (40 μg/ml) were added, and the reduction of NAD^+ was followed by measuring the absorbamce at 340 nm. No 3-hydroxyacyl-CoA dehydrogenase activity was detected. However, when 2-*trans*-octenoyl-CoA was incubated with crotonase (instead of MFP-2), which specifically produces L-3-hydroxyoctanoyl-CoA, the 3-hydroxyacyl-CoA was rapidly dehydrogenated after addition of NAD^+ and the mitochondrial 3-hydroxyacyl-CoA dehydrogenase (results not shown). These results demonstrate that MFP-2 catalyses the hydration of a 2-*trans*-enoyl-CoA to a D-3-hydroxyacyl-CoA. The high ratio of hydratase activity towards 3-hydroxyacyl-CoA dehydrogenase activity observed for MFP-2 [10] explains the

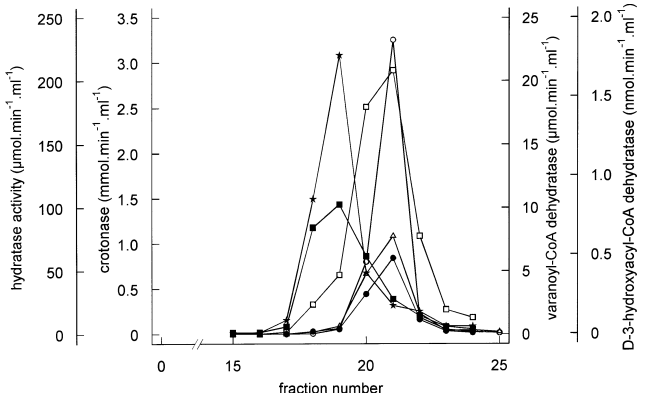

Figure 6 Separation of hepatic peroxisomal 2-enoyl-CoA hydratases from clofibrate-treated rats by cation exchange on a sulphopropyl column

A 23.5 ml portion of the peripheral-membrane protein fraction (S2), derived from 57 g of clofibrate-treated rat liver, was concentrated approx. 4-fold and dialysed overnight against buffer A. After removal of particulate material by centrifugation, the protein solution (5.5 ml) contained 33 673 units of crotonase, 4029 units of octenoyl-CoA hydratase, 983 units of hexadecenoyl-CoA hydratase, 1395 units of 3-hydroxy-2-methylpalmitoyl-CoA dehydratase, 30 units of 24*R*,25*R*-varanoyl-CoA dehydratase and 164 units of D-3-hydroxyoctanoyl-CoA dehydratase. A sample of 1.7 ml was injected on the sulphopropyl column equilibrated with buffer A. The bound proteins were eluted by means of a linear phosphate gradient (20–250 mM). Fractions of 1.6 ml were collected and 2-enoyl-CoA hydratase activities were assayed with crotonyl-CoA (○, recovery 65%), octenoyl-CoA (□, recovery 73%), hexadecenoyl-CoA (●, recovery 58%), 3-hydroxy-2-methylpalmitoyl-CoA (▲, recovery 59%), 24*R*,25*R*-varanoyl-CoA (★, recovery 73%) and D-3-hydroxyoctanoyl-CoA (■, recovery 68%). Fractions 16–20 from three runs were pooled separately and used for further purification. Symbols are omitted where no activity was found in a fraction.

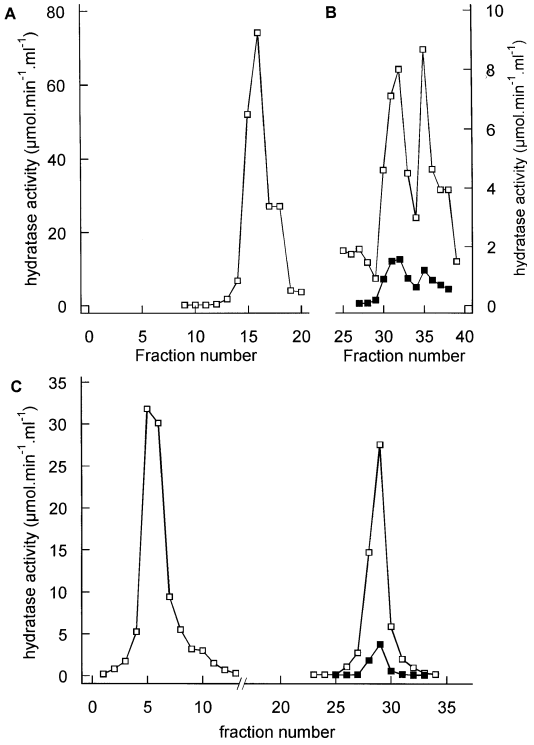

Figure 7 Hydrophobic chromatography on a phenyl column (A, B) and on an alkyl column (C) of the 2-enoyl-CoA hydratases contained in fractions 16–20 from the sulphopropyl column

Fractions 16–20 from the sulphopropyl column (Figure 6) were pooled, concentrated to 2 ml and dialysed overnight against 20 mM potassium phosphate buffer (pH 7.5)/0.6 M $(NH_4)_2SO_4$. Enoyl-CoA hydratase activities were measured with octenoyl-CoA (□) and D-3-hydroxyoctanoyl-CoA (■). (**A**, **B**) a 0.7 ml portion of the concentrated fraction containing 540 units of octenoyl-CoA hydratase (recovery 55%) and 16 units of D-3-hydroxyoctanoyl-CoA dehydratase (recovery 60%) was injected on the phenyl column equilibrated with the same buffer. After 10 min the bound proteins were eluted as described in the Experimental section, at a flow rate of 0.9 ml/min. Fractions of 1.8 ml [(**A**) fractions 1–21; (**B**) fractions 24–40] were collected. (**C**) A 0.6 ml portion of the concentrated fraction described above was brought to the composition of buffer A by adding 0.3 ml of 20 mM potassium phosphate buffer (pH 7.5)/2.4 M $(NH_4)_2SO_4$. After the removal of particulate material by centrifugation, 0.8 ml of this fraction, containing 400 units of octenoyl-CoA hydratase (recovery 35%) and 12 units of D-3-hydroxyoctanoyl-CoA dehydratase (recovery 55%), was injected on the alkyl column equilibrated with the same buffer. After 10 min the bound proteins were eluted by means of a negative salt and a positive ethylene glycol gradient [100–0% buffer A; 0–100% buffer B containing 20 mM potassium phosphate, pH 7.5 and 30% (w/v) ethylene glycol] over 50 min at a flow rate of 0.5 ml/min. Fractions of 1 ml were collected. Symbols are omitted where no activity was found in a fraction.

absence of visible D-3-hydroxyacyl-CoA dehydrogenase activity after the addition of NAD^+.

Separation of the peroxisomal 2-enoyl-CoA hydratases from livers of clofibrate-treated rats

Malila et al. [24] purified a D-dehydratase consisting of a homodimer with a subunit molecular mass of 33.5 kDa from livers of clofibrate-treated rats. The enzyme was active essentially towards medium-chain fatty acids. Because we did not find any clear indication for the presence of this enzyme in livers from untreated rats, we suspected that the enzyme might be inducible by clofibrate treatment. We therefore purified peroxisomes from livers of clofibrate-treated rats and subfractionated the organelles into matrix, peripheral membrane and integral membrane proteins. D-3-Hydroxyoctanoyl-CoA was used to measure the D-dehydratase activity specifically. As in control rats, 70–80% of the hydratase and dehydratase activities were associated with the peripheral-membrane protein fraction for all substrates used (results not shown). The peripheral-membrane protein fraction was applied to a sulphopropyl column as described for control rats. One large peak of hydratase and dehydratase activities (fractions 20–22) was observed with crotonyl-CoA, octenoyl-CoA hexadecenoyl-CoA and with 3-hydroxy-2-methyl-hexadecanoyl-CoA respectively (Figure 6), owing to the presence of the inducible MFP-1 (results not shown). The use of 24*R*,25*R*-varanoyl-CoA and D-3-hydroxyoctanoyl-CoA allowed the detection of a second peak of dehydratase activity that was eluted in fractions 17–21. Fractions 16–20 were combined and, after concentration, an aliquot was injected on a phenyl column (Figures 7A and 7B). The elution profile of the dehydratase activities was similar to that observed for a control liver: a first peak corresponding to the presence of MFP-1 (confirmed by gel electrophoresis and immunoblotting; data not shown) inactive towards 24*R*,25*R*-varanoyl-CoA and D-3-hydroxyoctanoyl-CoA, and two additional peaks (fractions 29–33 and 34–39) that specifically dehydrated 24*R*,25*R*-varanoyl-CoA and D-3-hydroxyoctanoyl-CoA. Gel electrophoresis and immunoblot analysis with antibodies against MFP-2 revealed the presence of MFP-2 and its C-terminal 45 kDa cleavage product in these two peaks (results not shown). As with control liver, a prominent band of 33 kDa was present in fractions 31–34. In a second experiment, an aliquot of the pooled fractions 16–20 from the sulphopropyl column was injected on an alkyl column (Figure 7C). MFP-1 was not retained on the column and only one peak of D-3-hydroxyoctanoyl-CoA and 24*R*,25*R*-varanoyl-CoA dehydratase activity was eluted from the column in fractions 26–32. Gel electrophoresis of the eluate revealed the presence of MFP-2 and its cleaved 45 kDa polypeptide in these fractions. In

contrast, the 33 kDa protein was not retained on the column and was eluted in fractions 3 and 4 (results not shown), which did not show D-dehydratase activity (Figure 7).

DISCUSSION

Each step of peroxisomal β-oxidation seems to be catalysed by several enzymes with distinct substrate specificities. In the present study we attempted to account for the 2-enoyl-CoA hydratases present in peroxisomes. Our results indicate that rat liver peroxisomes contain two hydratase activities: (1) an L-specific hydratase activity associated with MFP-1, and (2) a D-specific hydratase activity associated with MFP-2 and its 45 kDa C-terminal cleavage product.

The L- and D-stereospecificity of MFP-1 and MFP-2 respectively imply that MFP-1 is involved in the degradation of straight-chain fatty acids and that MFP-2 is responsible for the β-oxidation of the side chain of cholesterol. In support of this contention are our observations that MFP-2 specifically dehydrates and dehydrogenates the physiological 24*R*,25*R*-varanoyl-CoA stereoisomer ([10], and present study). MFP-1 dehydrates and dehydrogenates the 24*S*,25*S* and 24*S*,25*R* stereoisomers of varanoyl-CoA respectively, so that the dehydratase and dehydrogenase activities of MFP-1 are not active on the same 25-stereoisomers. On the basis of similar findings, Xu and Cuebas [30] also concluded recently that MFP-1 is most probably not involved in bile acid synthesis.

MFP-2 consists of an N-terminal short-chain alcohol dehydrogenase/3-hydroxyacyl-CoA dehydrogenase domain, a central hydratase domain and a C-terminal domain that shows homology with sterol carrier protein 2. A previous report from our laboratory [10] and the present studies demonstrate that a portion of MFP-2 is cleaved into an N-terminal part of 34 kDa that shows 17β-hydroxysteroid and D-3-hydroxyacyl-CoA dehydrogenase activities and a 45 kDa C-terminal part that shows 2-enoyl-CoA hydratase activity. N-terminal sequencing of the 45 kDa polypeptide gave two sequences that started at residues 313 and 317 respectively. It remains to be investigated whether the cleavage site between residues 312 and 313 is the primary cleavage site and the polypeptide is then further cleaved between residues 316 and 317, or whether both sites function as primary cleavage sites. The fact that on gel electrophoresis the N-terminal 17β-hydroxysteroid/3-hydroxyacyl-CoA dehydrogenase part forms a hardly discernible (because so closely apposed) doublet [9] suggests that the latter possibility should be considered.

The cleavage of MFP-2 occurs most probably *in vivo* because the 34 and 45 kDa polypeptides are present in tissue homogenates freshly prepared in the presence of proteinase inhibitors [10]. The observation that MFP-2 and the 45 kDa polypeptide but not the 34 kDa polypeptide bind to the human peroxisome targeting signal 1 receptor [10] suggests that MFP-2 is cleaved after import into the peroxisome. Leenders et al. [14], who originally described MFP-2 as a pig 17β-oestradiol dehydrogenase IV (see the Introduction section), also found that a portion of the protein is cleaved. The exact contribution of MFP-2 on the one hand, and its cleavage products on the other hand, to peroxisomal bile acid and steroid metabolism requires further investigation.

Three other laboratories have reported the presence in peroxisomes of D-specific hydratases. Li et al. purified an enzyme with a (subunit) molecular mass of 44 kDa [23], whereas the enzyme purified by Cook et al. consisted of polypeptides of 78, 71 and 47 kDa [25]. The 78 kDa polypeptide might be identical with MFP-2, whereas the 44 kDa and 47 kDa polypeptides might represent the C-terminal cleavage product. The 71 kDa polypeptide might be a proteolytic degradation product of MFP-2, formed during purification, because we also found a polypeptide of similar size that displayed hydratase activity and cross-reacted with antibodies raised against MFP-2. We did not find this polypeptide, however, in freshly prepared tissue homogenates. Because antibodies against MFP-2 are now available and because the amino acid sequence of MFP-2 is known, it should be possible to verify how closely the enzymes purified by Li et al. [23] and Cook et al. [25] are related to MFP-2. In contrast, we did not find any indication of the presence of a 33.5 kDa D-hydratase as reported by Hiltunen and colleagues [24]. One possibility might be that it is a degradation product of MFP-2 or its C-terminal cleavage product.

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