Peroxisomal multifunctional enzyme of β -oxidation metabolizing D-3-hydroxyacyl-CoA esters in rat liver: molecular cloning, expression and characterization

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In the present study we have cloned and characterized a novel rat peroxisomal multifunctional enzyme (MFE) named perMFE-II. The purified 2-enoyl-CoA hydratase 2 with an M_r of 31 500 from rat liver [Malila, Siivari, Mäkelä, Jalonen, Latipää, Kunau and Hiltunen (1993) J. Biol. Chem. **268**, 21578–21585] was subjected to tryptic fragmentation and the resulting peptides were isolated and sequenced. Surprisingly, the full-length cDNA, amplified by PCR, had an open reading frame of 2205 bp encoding a polypeptide with a predicted M_r of 79 331 and contained a potential peroxisomal targeting signal in the C-terminus (Ala-Lys-Leu). The sequenced peptide fragments of hydratase 2 gave a full match in the middle portion of the cDNA-derived amino acid sequence. The predicted amino acid sequence showed a high

degree of similarity with pig 17 β -hydroxysteroid dehydrogenase type IV and MFE of yeast peroxisomal β -oxidation. Recombinant perMFE-II (produced in *Pichia pastoris*) had 2-enoyl-CoA hydratase 2 and D-specific 3-hydroxyacyl-CoA dehydrogenase activities and was catalytically active with several straight-chain *trans*-2-enoyl-CoA, 2-methyltetradecenoyl-CoA and pristenoyl-CoA esters. The results showed that in addition to an earlier described multifunctional isomerase-hydratase-dehydrogenase enzyme from rat liver peroxisomes (perMFE-I), another MFE exists in rat liver peroxisomes. They both catalyse sequential hydratase and dehydrogenase reactions of β -oxidation but through reciprocal stereochemical courses.

INTRODUCTION

Fatty acid degradation in living organisms occurs mainly via the β -oxidation pathway, which in eukaryotes can take place in mitochondria and/or peroxisomes [1–5]. In both cases it consists of a spiral of four sequential reactions utilizing the same set of substrates in one turn, namely acyl-, *trans*-2-enoyl-, 3-hydroxyacyl- or 3-ketoacyl-CoA esters. In addition to straight-chain saturated acyl-CoA esters, a great variety of unsaturated and branched-chain fatty acids can undergo chain shortening by β -oxidation in mammalian peroxisomes [6–9]. This assumes the participation of auxiliary enzyme activities [10]. Recent research into eukaryotic β -oxidation and related reactions has indeed revealed the existence of a 2-methyl branch acyl-CoA racemase [11], operation of the $\Delta^3,5, \Delta^{2,4}$ -dienoyl-CoA isomerase-dependent pathway [12–14], the occurrence of several enzymes with partly overlapping chain-length specificities for each step in the β -oxidation reaction [15], the fact that β -oxidation in yeast peroxisomes proceeds via D-3-hydroxyacyl-CoA intermediates [16], and the epimerization of 3-hydroxyacyl-CoA esters via a dehydration-hydration mechanism [17,18] catalysed by stereospecific hydratases: 2-enoyl-CoA hydratase 2 and 2-enoyl-CoA hydratase 1 (crotonase).

Hydratase 2 catalyses the hydration of *trans*-2-enoyl-CoA to D-3-hydroxyacyl-CoA esters in a reversible manner [17]. At least three hydratase 2 species with different M_r values have been purified from rat liver. The isoforms with subunit M_r values of

33 500 and 44 000 have been suggested as being located in peroxisomes [19,20], and the isoform with a subunit M_r of 31 500 to be located in microsomes [19]. In fungal peroxisomes, hydratase 2 is an integral part of the peroxisomal multifunctional enzyme (MFE) and it participates in the β -oxidation of fatty acyl-CoA esters [16]. The aim of the present study was the characterization of 2-enoyl-CoA hydratase 2 from rat liver; the results provide evidence that hydratase 2 can be either an integral part and/or a fragmentation product of a multifunctional β -oxidation protein.

EXPERIMENTAL

Materials

The SureClone ligation kit, T7 sequencing kit and restriction enzymes were purchased from Pharmacia Biotech (Uppsala, Sweden), the GeneClean II kit from Bio 101 (Vista, CA, U.S.A.), Moloney murine leukaemia virus (MMLV) reverse transcriptase from Gibco BRL (Gaithersburg, MD, U.S.A.), the Dynazyme DNA polymerase from Finnzymes (Espoo, Finland), Dynabeads mRNA DIRECT kit from Dynal (Oslo, Norway), the enhanced chemiluminescence detection kit and [α - 32 P]dCTP from Amersham International (Little Chalfont, Bucks., U.K.), the Marathon[®] cDNA Amplification Kit from Clontech (Palo Alto, CA, U.S.A.), goat anti-rabbit IgG conjugated with horse-

Abbreviations used: 17 β -HSD, 17 β -hydroxysteroid dehydrogenase; cpMFE-II, coding region of perMFE-II; MMLV, Moloney murine leukaemia virus; MFE, multifunctional enzyme; perMFE-I, peroxisomal multifunctional Δ^3, Δ^2 -enoyl-CoA isomerase, 2-enoyl-CoA hydratase 1, L-specific 3-hydroxyacyl-CoA dehydrogenase; perMFE-II, peroxisomal multifunctional 2-enoyl-CoA hydratase 2, D-specific 3-hydroxyacyl-CoA dehydrogenase.

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The nucleotide sequence reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number U37485.

Table 1 Oligonucleotide probes used for the isolation of rat liver peroxisomal MFE II cDNA clones

Key: R = A + G, Y = C + T, N = A + C + G + T.

Name	Nucleotide sequence	Basis for the oligonucleotide
Oligonucleotide A	5'-GCNGCNGTNGCNGTRCCGAG-3'	Amino acid sequence AAVAVPD
Oligonucleotide B	5'-ACRTCRTTRTCNGCRAACTGCTG-3'	Amino acid sequence QQFADNDV
Oligonucleotide C	5'-GGGTTTCTCAAACCGGCAATGCT-3'	Rat perMFE-II cDNA bp 1561–1584
Oligonucleotide D	5'-CCATCCTAATACGACTCACTATAGGGC-3'	Adapter primer AP1
Oligonucleotide E	5'-TACCGCCTCAGTGGAGACTCGAA-3'	Rat perMFE-II cDNA bp 1510–1532
Oligonucleotide F	5'-CACTTCAATTGATGGCTTCGCCTGAGGTTTCA-3'	Rat perMFE-II cDNA bp 1–23 tailed with <i>Mun</i> I restriction site
Oligonucleotide G	5'-CATCGCAATTGTCAGAGCTTGGCATAGTCTTCA-3'	Rat perMFE-II cDNA bp 2184–2208 tailed with <i>Mun</i> I restriction site

radish peroxidase from Bio-Rad Laboratories (Richmond, CA, U.S.A.), 4-chloro-1-naphthol and CoA (lithium salt) from Sigma Chemicals (St. Louis, MO, U.S.A.), and the Pichia Expression Kit from Invitrogen (San Diego, CA, U.S.A.). Toluene sulphonic acid, 2,3-dihydropyran, LiAlH_4 , (1-ethoxycarbonyl ethylidene)-triphenylphosphorane, farnesol, palladium on charcoal, di-isobutylaluminium hydride and trifluoroacetic acid anhydride were from Aldrich Chemie (Steinheim, Germany), silica gel 60, ethyl chloroformate and lauraldehyde from Merck (Darmstadt, Germany), tetrahydrofuran and triethylamine from Fluka Chemica-Biochemica (Buchs, Switzerland), dichloromethane and ethyl acetate from Riedel-de Haen (Seelze, Germany), modified trypsin (sequencing grade) from Promega (Madison, WI, U.S.A.), and reverse-phase silica gel (RP-18) from ICN Chemicals (Costa Mesa, CA, U.S.A.). All other chemicals were of analytical grade or of the highest purity available.

Tryptic peptides for 2-enoyl-CoA hydratase 2 from rat liver

For the purification of hydratase 2 proposed to be associated with microsomes, the homogenization of rat liver and extraction and chromatographic purification of the enzyme were performed as described by Malila et al. [19]. SDS/PAGE of the fractions from a Mono S[®] column enabled the 31 500- M_r band of hydratase 2 to be identified. Fragmentation of the protein band was performed in the gel slice as previously described [21]. The resulting fragments were separated by reverse-phase liquid chromatography using a μ RPC C₂/C₁₈ SC 2.1/10 column connected to a SMART micropurification system (Pharmacia Biotech). The peptides were eluted in a linear gradient of acetonitrile in 0.05% (v/v) trifluoroacetic acid. Fractions containing non-homogeneous peptides, as judged from the UV pattern, were re-chromatographed on a Sephasil C₈ SC 2.1/10 column (Pharmacia Biotech). Six peptides were selected and subjected to automatic Edman degradation in an Applied Biosystems model 470A sequencer.

Cloning of 2-enoyl-CoA hydratase 2 and DNA sequencing

mRNA from the livers of Wistar rats obtained from the Laboratory Animal Centre of the University of Oulu, Finland, was isolated through the use of Dynabeads oligo(dT)₂₅ in accordance with the manufacturer's instructions. Subsequently the mRNA (1 μ g) was transcribed to the first cDNA strand with MMLV reverse transcriptase [22]. For the first PCR amplification of the resultant cDNA, an aliquot of 5 μ l of the reaction mixture was used as a template and oligo A and oligo B as the primers (Table 1). The amplification for 30 cycles was performed with Dynazyme DNA polymerase. The amplified PCR fragment (Figure 1) was subcloned into pUC 18 plasmids with a SureClone

Figure 1 Isolation of cDNA for rat perMFE-II

Clone PCR 1 was isolated by amplification with oligonucleotide primers A and B, derived from degenerated amino acid sequences of isolated tryptic fragments of hydratase 2. Clones PCR 2, PCR 3 and PCR 4 were obtained by reverse transcriptase-PCR (see the Experimental section). The direction of transcription is from left to right, and rat liver cDNA was used as a template. The arrows represent the direction and extent of the sequence determinations. Encircled E, H and S under the isolated perMFE-II indicate the restriction sites for *Eco*RI, *Hind*III and *Sa*I respectively.

ligation kit and used to transform *Escherichia coli* DH 5 α competent cells. Both strands of the subcloned fragments were sequenced by the dideoxynucleotide sequencing method.

To obtain full-length cDNA, the adaptor primer PCR method was employed, with a Marathon[®] cDNA amplification kit. mRNA isolated from rat liver was taken as a template and first-cDNA-strand synthesis was performed with the Marathon cDNA synthesis primer using MMLV reverse transcriptase. Second-cDNA-strand synthesis was performed in accordance with the method of Gubler and Hoffmann [23] with a cocktail of *E. coli* DNA polymerase I, RNase H and *E. coli* DNA ligase. After the creation of blunt ends with T4 DNA polymerase, the double-stranded cDNA was ligated to the Marathon cDNA adaptor, and the adaptor-ligated cDNA was taken as the template for PCR amplification, with specific oligonucleotide C as the anti-sense primer and the adaptor primer oligonucleotide D as the sense primer to reach the 5' end, and specific oligonucleotide E as the sense primer and the adaptor primer oligonucleotide D as the anti-sense primer to reach the 3' end. The amplified PCR

fragments were subcloned into pUC 18 plasmids and subsequently sequenced.

Construction of a plasmid for integration into the *Pichia pastoris* genome

pHILD2 vector derived from the *P. pastoris* expression kit was digested with *EcoRI* and dephosphorylated. The coding region of cloned cDNA was generated by PCR amplification with oligonucleotide F as the sense primer and oligonucleotide G as the anti-sense primer with a tailing *MunI* restriction site at the 5' ends of both primers; the adaptor-ligated cDNA was taken as the template. The amplified PCR fragment (cpMFE-II) was isolated using a GeneClean II kit, digested with *MunI* and ligated into the pHILD2 vector. The resulting construct (pHILD2-perMFE-II) was used to transform the *E. coli* strain Top10 F'. The plasmid was isolated and used to transform *Pichia* strain GS115 by the electroporation method (Gene Pulser®; Bio-Rad Laboratories), yielding the yeast strain PP-MFE-II. Yeast cells transformed with pHILD2 were used as a control (PP-HILD).

Northern blot analysis

mRNA species (10 µg) were fractionated in 1 % (w/v) agarose gel in the presence of formaldehyde, transferred to a nylon membrane by capillary blotting overnight and fixed by UV cross-linking. The membrane was prehybridized at 42 °C for 4 h and hybridized with ³²P-labelled cpMFE-II. The membrane was exposed to X-ray film with intensifying screens at -70 °C.

Immunoblot analysis

Yeast cells were disrupted by agitation with glass beads (0.5 mm) in a Bead-Beater® (Biospec Products, Bartlesville, OK, U.S.A.). Samples of the yeast cell lysates, rat liver homogenate or isolated peroxisomes from rat liver were subjected to SDS/PAGE analysis followed by electroblotting on a nitrocellulose membrane. For immunodetection, polyclonal rabbit antibody to rat 2-enoyl-CoA hydratase 2 was used as the first antibody and affinity-purified goat anti-rabbit IgG conjugated with horseradish peroxidase as the second antibody. The recognized epitopes were detected by either 4-chloro-1-naphthol or an enhanced chemiluminescence detection system. Peroxisomes were isolated from a sample of the light mitochondrial fraction from rat liver [24] with a continuous Nycodenz density gradient (15–40 %, w/w) as described [19,25].

Enzyme activity assays

Cells of *P. pastoris* (20 g wet weight) grown on methanol were suspended in 50 ml of 50 mM potassium phosphate, pH 8.6, containing 1 mM EDTA, 1 mM EGTA, 0.1 mM PMSF and 10 % (v/v) glycerol, then disrupted. The cell homogenate was centrifuged at 27000 g for 15 min and the resulting supernatant recentrifuged at 100000 g for 1 h. The proteins precipitated with (NH₄)₂SO₄ at 35–60 % saturation were dissolved in 20 mM potassium phosphate (pH 7.0)/0.5 mM EDTA/0.5 mM EGTA/0.5 mM benzamidine hydrochloride/0.5 mM dithiothreitol containing 0.5 M (NH₄)₂SO₄ (buffer A), and applied to a phenyl-Sepharose hydrophobic interaction column (6.5 cm × 2.6 cm) equilibrated with buffer A. The bound proteins were eluted in a linear gradient (0–40 %, v/v) of ethylene glycol in 150 ml of buffer A without (NH₄)₂SO₄.

The activities of 2-enoyl-CoA hydratase 2, 2-enoyl-CoA hydratase 1, D- and L-specific 3-hydroxyacyl-CoA dehydrogenases, 3-hydroxyacyl-CoA epimerase, Δ³,Δ²-enoyl-CoA iso-

merase and combined activities (metabolism of *trans*-2-enoyl-CoA esters to 3-ketoacyl-CoA esters) were measured as described previously [17,26,27]. The metabolism of 2-pristenoyl-CoA and 2-methyltetradec-2-enoyl-CoA esters (hydration and dehydrogenation reactions) was assayed spectrophotometrically by monitoring the production of NADH in a mixture consisting of 30 nmol of substrate, 1 µmol of NAD⁺, 50 µmol of Tris/HCl, pH 9.0, 50 µg of BSA, 50 µmol of KCl and the sample in a total volume of 1 ml at 23 °C. Activity measurements of 17β-hydroxysteroid dehydrogenase (17β-HSD) were performed *in vitro* as previously described [28].

Synthesis of 2-pristenic acid

Farnesol (9 mmol) was hydrogenated to 3,7,11-trimethyl-1-dodecanol by stirring with 20 mg of palladium on charcoal in a hydrogen atmosphere for 2 days. The product (7.9 mmol) was purified by TLC over silica gel with hexane/ethyl acetate (9:1, v/v) as solvent. A sample of 1 mmol of the alcohol was treated with 1.2 mmol of trifluoroacetic acid anhydride and evaporated under argon. The residue was resuspended in 2 ml of tetrahydrofuran/DMSO (1:1, v/v) containing 4 mmol of NaCN and heated at 90 °C for 2 h. The resulting 4,8,12-trimethyltridecanonitrile (0.64 mmol) was purified by TLC over silica gel with hexane/ethyl acetate (4:1, v/v) as solvent. The nitrile (0.422 mmol) was dissolved in 1 ml of hexane containing 1 M diisobutylaluminium hydride and stirred for 1 h. The product (0.4 mmol of 4,8,12-trimethyltridecanal) was purified by TLC over silica gel with hexane/ethyl acetate (9:1, v/v) as solvent. A sample of the aldehyde (0.25 mmol) was dissolved in 3 ml of acetonitrile containing 0.83 mmol of (1-ethoxycarbonyl-ethylidene)triphenylphosphorane and stirred for 1 h at 85 °C. The resulting 2-pristenic acid ethyl ester (0.175 mmol) was purified by TLC over silica gel with hexane/ethyl acetate (9:1, v/v) as solvent. The hydrolysis of ethyl ester to obtain pristenic acid was performed as described earlier [15]. 2-Methyltetradec-2-enoic acid was synthesized essentially by the same method, starting from lauraldehyde. CoA esters were prepared from the corresponding carboxylic acids by a mixed-anhydride method as modified by Rasmussen et al. [29].

Other methods

Multiple comparison of the amino acid sequences of the polypeptides was performed by applying a multiple alignment program [30] with default parameters. The proposal for this research project was approved by the University of Oulu committee on animal experimentation.

RESULTS

cDNA encoding hydratase 2

The cloning of hydratase 2 from rat liver was performed by applying PCR-based techniques. When degenerated oligonucleotides based on the amino acid sequence of the tryptic peptide of hydratase 2 were used as primers, PCR amplification yielded a cDNA fragment of 228 bp in length (PCR 1) (Figure 1). With the subsequent PCRs with rapid amplification of cDNA ends, two overlapping PCR products of 1660 bp (PCR 2) and 1008 bp (PCR 3) were obtained. Full-length cDNA was also amplified (PCR 4) and used for production of recombinant protein. PCR 2 contained 49 nucleotides before the putative ATG initiation codon, whereas PCR 3 contained TGA as a stop codon followed by 250 non-coding 3'-flanking nucleotides that

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-49      M A S P L R F D G R V V L V T G A G G G L G R A Y A L A F A
1      ATGGCTTCGCCTTCAGGATTCGACGGCGTGTGGTCTGGTCACCGCGCGGGGAGGTTGGCGAGACTTATGCCCTGGCTTTTGA
60      E R G A L V V V N D L G G D F K G V G K G S S A A D K V V E
91      GAAGGAGGAGCATTAGTGTGTGAATGACTTAGAGGGGACTTCAAGGGCGTTGGGAAAGGCTCTTCTGCGCAGACAAAGTGCTGGAA
E T R F R G C K A V A N L V D S V E R G E K L V K T A L D T F
181      GAATTAAGAGAGAGAGCGGAAAGCGGTGGCCATTCAGTTCAGAGCACTCGACATCTTGAGAGCACTGGACACATTC
G R I D V V V N N A G I L R D R S F S R I S D E D W D I I Q
271      GGCAGAAATAGATGTGTGGTGAACAATGCTGGGACTCTGAGGACCGTTCCTTCTCTAGGATAAGTGATGAAGACTGGGATATAATTCAA
R V H L R G S F Q V T R A A W D H M K K Q N Y G R I I M T A
361      AGAGTTCATTTCGGGGCTCTCTTCAAGTGAACCGGGCAGCATGGGATCATATGAAGAAGCAGAAATATGGAAGAAATCATATGACGGCC
S A S G I Y G N F G Q A N Y S A A K L G L L G L A N T L V I
451      TCAGCTTCGGAATATACGGCACTTTGGCCAGGCAATATAGTGTGCAAGCTGGGCCCTCTGGGCTTCGCCAATCTCTCGTGATT
E G R K N N I H C N T I A P N A G S R M T E T V M P E D L V
541      GAAGCAGGAGAGACACATTCATTGTACACCATTCGCCCAACGCTGGGTACGGATGACAGAGACGCTGATGCCAGAAGACCTGGTT
E A L F P E Y V A P L V L V L H E S C E N G G L F E V G
631      GAAGCCTGAAGCAGAGTATGTGGCACCGCTGGTCTTTGGCTTTGCCATGAGAGCTGTGAGGAAATGGTGGCTTGTGTGAGGTGGA
A G W I G K L R W E R T L G A I V R K R N Q P M T P E A V R
721      CGAAGTGGATTGGAATATTCGCTGGGAGAGGACCTGGGAGCCATTGTCAAGGAAGCGGAATCAGCCATGACTCCGAGGCACTGAGG
D N W V K I C D F S N A S K P K S I Q E S T G G I I E V L H
811      GACAACCTGGGTGAAGATCTGTGACTTCAGCAATGCCAAGCCGAAGACATTCAGAGTCCACAGGTGGTATAATCGAAGTTTACAT
K I D S E G I S Q N H T G Q V A S A D A S G F A G V V G H K
901      AAAATAGATTTCAGAGGAATCTCACAATAACACCGGTCAAGTGGCATCTGCAGATGCATCAGGATTGTCTGGCGTCTGGCCACAA
L P L F S G S Y T E L Q C I M Y A L I G V G A S V K N P K D L
991      CTTCCTTCACTTCTCTCATATACGGAATCGAGTGCATTATGATGCCCTCGGAGTAGAGCTTCAGTCAAAAATCCAAAGCACTTG
K P V V Y E G S A D F S C L P T F G V I V A Q K S L M S G G L
1081      AAGTTTGTATGAAGGAGTGTGCTCTCTCTGTTTGGCTACATTTGGAGTCAATGTGCTCAGAAGTCTTGTATGAGTGGAGGCTTA
A E V P G L S I N F A K V L H G E Q Y L E L Y K P L P R S G
1171      CGCAGAGTTCCTGGGCTGTCAATCACTTTGCAAGGTTCTTCAATGGGAGCAGTACTTGGAGTTGTATAAGCCACTTCCCGATCAGG
E L K C E A V I A D I L D K G S G I V I V M D V Y S Y S G K
1261      GAATTAATAATGAAGCAGTATTCGTCACATCTGGATAAAGGCTCTGGCATAGTGATTTGTATGGACGCTTATTTCTATTCTGGCAAG
E L I C Y N Q P S V F V V G S G G F G V K R T S E K L K A A
1351      GAATCTATATGCTATATCACTCTCTGCTCTGTGCTTGGCTCTGGAGGCTTTGGTGAAGACGACATCAAGAAACTCAAGACGCT
V A V P S R P P D A V L R D T T S L N Q A A L Y R L S G D S
1441      GTAGCCGTACCGAGTCCGCTTCAGATGCTGTACTGAGAGATACCACTTCACTGAATCAGGCGCTCTGTACCGCTCAGTGGAGACTCG
N P L H I D P S F A S I A G F E K P I L H G L C T F G F S A
1531      AATCTTTACACATTGACCCGAGCTTTGCGAGCATTCGCGGTTTGGAGAAACCATATACACGGATTATGTACTTTTGGGTTTTCGCA
R H V L Q Q F A D N D V S R F K A I K V R F A K P V Y P G Q
1621      AGGCATGTTTACAGCAGTTTGGGATAATGATGTCAAGATTCAAGCCATTAAAGTTCTGTTTGGCAAAACAGTGTATCCAGGACAA
T L Q T E M W R E G N R I H F O T K V Q E T G D I V I S N A
1711      ACTCTACAACTGAGATGTCAGAGAGAGAGACAGAACTATTCAAACAGCTCCAGAGAGACTGGAGACATTGTCATTTCCAAATGCA
Y V D L V S T P G V S A Q T P S E G G A L V F G E I
1801      TATGTGGATCTTGTCTTCACTACCTGGAGTTCCGCTCAGACACCTTCTGAGGGTGGAGCACTGCAGAGTGTCTTGTATTTGGGAAATA
G R R L K D V G R E V V K K V N A V F E W H I T K N G N V A
1891      GGTTCAGCGCTCAAGGATGTTGGAGCTGAGGTGGTAAAGAAATGCTGTATTTGAAATGGCATACAGAAATATGGGAATGTGCA
A K W T I D L K N G S G E V Y Q G P A K G S A D T T I T S
1981      GCCAAGTGACCATTGACCTGAAGAAGCGCTCTGGAGAGGTTTACCAAGGCCCTGCCAAAGGCTCTGTGACACGACCATCAAAATTTCT
D E D F M E V V L G K L N P Q N A F F S G R L K A R G N I M
2071      GTAGAGATTTCAATGGAGTGGTCTGGGCAAGCTTAAACCCACAGAAATGCTTCTTCAAGTGGCAGCTGAAGGCCCGAAGAACATCATG
L S Q K L Q M I L K D Y A K L
2161      CTGAGCCAGAAGCTACAGATGATCTGAAAGACTATGCCAAGCTCTGAAGGACCCACTGCGTCTTAAATAAAACAGAAATCATTAGTT
2251      CTGCTACGCACTGCTGCTCCAGCTTCTTTGAAACGATCCACGGTATGTGCAGCAGAAATCGCTTAACATTTTCAGATTCAGATAACT
2341      TCTCAGATTTTCATTTCTACTAATTTTTCACATATTTATTTATAAGGAACGTGAATCTAGCTAGCAATAATTTGTCTGTCTCATAGATC
2431      TGTATCTTAAATAAAAGTCAACCGAAAAAAGAAAAAAGAAAAAAGAAAAAAGAAAAAAGAAAAAAGAAAAAAGAAAAAAGAAAAAAG

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Figure 2 Nucleotide sequence and deduced amino acid sequence of the rat perMFE-II

A 2535 bp cDNA was generated by reverse transcriptase-PCR of mRNA isolated from rat liver as described in the Experimental section and shown schematically in Figure 1. The numbering of the nucleotides, given on the left, begins with the putative start codon, the nucleotides before the open reading frame being denoted by a negative sign. The numbering of the amino acids is indicated on the right. The presumed polyadenylation signal is indicated with a double underline. Amino acid sequences were determined by Edman degradation of trypsin fragments of the 31 500- M_r isoform of 2-enoyl-CoA hydratase 2 and are singly underlined.

had an AATAAA polyadenylation signal 20 bp upstream of the poly(A)⁺ tract (Figure 2). The coding region of the isolated cDNA translated to a 735-residue polypeptide predicting an M_r of 79 331, a value 2.5-fold that estimated for purified hydratase 2 (M_r 31 500). The cDNA-derived amino acid sequence between residues 318 and 588 was accommodated with full matching of the amino acid sequences obtained for the isolated cleaved peptides by protein sequencing. The C-terminal amino acid residues of the perMFE-II polypeptide chain were Ala-Lys-Leu, a triplet known to act as a peroxisomal targeting signal [31].

Amino acid sequence comparisons of perMFE-II with sequence-related proteins

A search for proteins related to perMFE-II revealed that its amino acid sequence has 81.3 % identity with pig 17 β -HSD type IV [32] (Figure 3). The high degree of identity, which stretched over the whole length of the polypeptides, suggested that perMFE-II could represent a 17 β -HSD type IV counterpart in the rat. Like the pig 17 β -HSD [32,33], the N-terminal part of perMFE-II (residues 1–292) also showed a high degree of identity with both the N-terminal parts (residues approx. 1–290) and the middle parts (residues approx. 315–600) of the peroxisomal MFEs from both *Candida tropicalis* [34] and *Saccharomyces cerevisiae* [16]. Residues approx. 337–606 were similar to those in the C-terminal third of the yeast MFE. The extreme C-terminus (residues approx. 612–731) was similar to the sterol carrier protein 2 of the rat [35].

Enzyme activities assigned to perMFE-II

The enzyme activities catalysed by perMFE-II were studied with a recombinant enzyme obtained via expression of cpMFE-II in *Pichia* cells. To confirm expression of the cDNA integrated into the genome, Northern blot analysis was performed and a 2.9 kb signal was detected in the cells transformed with pHILD2–perMFE-II and grown on methanol (Figure 4A). A 79 000- M_r signal and two smaller signals were detected in immunoblotting of the soluble protein extract from the transformed cells with antibody to purified wild-type hydratase 2 (Figure 4B). The metabolism of *trans*-2-decenoyl-CoA ester to 3-ketodecenoyl-CoA ester in the presence of NAD⁺ (combined activity) was 0.043 μ mol/min per mg of protein in the soluble extract of PP-MFE-II grown on methanol. The activity in the control PP-HILD cells was below the detection limits of the assay method used. (NH₄)₂SO₄ at 35–60 % saturation precipitated 92 % of the activity from the extract. The redissolved precipitate was subjected to chromatography on a phenyl-Sepharose column; the fractions containing the activity were pooled and used for substrate specificity studies (Table 2). The perMFE-II catalysed hydratase 2 and D-specific 3-hydroxyacyl-CoA dehydrogenase reactions with both the C₁₀ and C₄ substrates, the C₁₀/C₄ activity ratios being 6.6 and 1.4 for hydration and dehydrogenation respectively. Interestingly, in the combined-activity test, the 2-methyl-branched substrates pristanoyl-CoA and 2-methyltetradecenoyl-CoA esters were also metabolized, the rates being approx. 0.007 and 0.010 of that observed for *trans*-2-decenoyl-

Figure 3 Alignments in rat perMFE-II, pig 17 β -HSD type IV, yeast peroxisomal MFE and sterol carrier protein 2 amino acid sequences

The amino acid sequences of the rat perMFE-II and pig 17β -HSD type IV showed an overall identity of 81.3%. The N-terminal part of perMFE-II (residues 1–292) had 50.0% [53.1%] identity with the N-terminal third of the peroxisomal MFE from *C. tropicalis* (residues 1–291; CTMFEa) [*S. cerevisiae* (residues 1–290; SCMFEa)] and 43.7% [41.6%] identity with the middle third of the peroxisomal MFE from *C. tropicalis* (residues 315–594; CTMFEbc) [*S. cerevisiae* (residues 314–602; SCMFEbc)]. Residues 337–606 of perMFE-II had 37.8% [41.6] identity with residues 635–900 [625–894] of the MFE from *C. tropicalis* [*S. cerevisiae*], and residues 612–735 of perMFE-II an identity of 41.7% with residues 19–144 of the sterol carrier protein 2 (SCP2). Bold letters indicate identity and underlined letters indicate similarity.

Figure 4 Expression of cDNA for hydratase 2 in *P. pastoris* cells

(A) Total RNA (15 μ g per lane) was fractionated on 1% (w/v) agarose gel in the presence of 0.8% (v/v) formaldehyde, transferred to a nitrocellulose filter and hybridized with the amplified PCR 4 fragment. The labels 25 S and 18 S indicate the positions of yeast ribosomal RNA. (B) The cellular lysate of *P. pastoris* grown on methanol for 7 days was separated by SDS/PAGE and blotted on a nitrocellulose filter. Polyclonal antibody raised against purified rat liver hydratase 2 was used as the primary antibody, and the signals were detected with an enhanced chemiluminescence detection kit. Lane 1, *P. pastoris* transformed with pHLD2; lane 2, the yeast transformed with pHLD2-perMFE II. The positions of molecular mass standards are indicated ($10^{-3}M_r$) at the left of (B); deduced $10^{-3}M_r$ values are shown at the right of both (A) and (B).

Table 2 Enzyme activities detected for the peroxisomal MFEs

Rat perMFE-I was purified as described in [26], and partly purified rat perMFE-II was obtained as a recombinant protein from PP-MFE-II cells grown on methanol. The extract of soluble proteins was prepared as described in the Experimental section, followed by $(NH_4)_2SO_4$ precipitation and chromatography on a phenyl-Sepharose column. The fractions containing 2-enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities were pooled and other activities were measured. The values are means $\pm$ S.E.M. for three determinations. The activities below the detection limits of the assay are given as n.d. (not detectable).

	Activity (μ mol/min per mg of protein)	
	perMFE-I	perMFE-II
L-specific 3-hydroxyacyl-CoA dehydrogenase		
3-Hydroxybutyryl-CoA	4.22 $\pm$ 0.09	n.d.
3-Hydroxydecanoyl-CoA	4.17 $\pm$ 0.33	n.d.
D-specific 3-hydroxyacyl-CoA dehydrogenase		
3-hydroxybutyryl-CoA	n.d.	0.43 $\pm$ 0.025
3-hydroxydecanoyl-CoA	n.d.	0.58 $\pm$ 0.065
2-Enoyl-CoA hydratase 1		
Crotonoyl-CoA	95.9 $\pm$ 3.5	n.d.
trans-2-Decenoyl-CoA	72.7 $\pm$ 3.6	n.d.
2-Enoyl-CoA hydratase 2		
Crotonoyl-CoA	n.d.	0.94 $\pm$ 0.11
trans-2-Decenoyl-CoA	n.d.	6.22 $\pm$ 1.06
Combined activity		
Crotonoyl-CoA	1.99 $\pm$ 0.22	0.007 $\pm$ 0.001
trans-2-Decenoyl-CoA	3.82 $\pm$ 0.54	0.56 $\pm$ 0.053
Pristenoyl-CoA	0.009 $\pm$ 0.005	0.0041 $\pm$ 0.0016
2-Methyltetradecenoyl-CoA	0.021 $\pm$ 0.005	0.0058 $\pm$ 0.0019
Δ^3, Δ^2 -Enoyl-CoA isomerase		
trans-3-Hexenoyl-CoA	0.84 $\pm$ 0.043	n.d.
trans-3-Decenoyl-CoA	0.35 $\pm$ 0.025	n.d.
3-Hydroxyacyl-CoA epimerase	n.d.	n.d.
17- β -Hydroxysteroid dehydrogenase	n.d.	(1.58 $\pm$ 0.11) $\times 10^{-6}$

CoA. perMFE-II did not have detectable activities of 2-enoyl-CoA hydratase 1, L-specific dehydrogenase, Δ^3, Δ^2 -enoyl-CoA isomerase or 3-hydroxyacyl-CoA epimerase with the substrates tested. As the amino acid sequence of perMFE-II was very

Figure 5 Immunoblotting of tissue extracts from livers of control rats and clofibrate-treated rats

Each lane contained 15 μ g of proteins that were separated by SDS/PAGE, electrotransferred to a nitrocellulose filter and detected with the polyclonal antibody to purified hydratase 2 (31 500 M_r subunit). (A) Experiment with soluble extract from livers quickly frozen with liquid nitrogen and detected with an enhanced chemiluminescence detection kit. Lane 1, clofibrate-treated male rat; lane 2, control male rat; lane 3, clofibrate-treated female rat; lane 4, control female rat. (B) Blot obtained with peroxisomes from light mitochondrial fractions of clofibrate-treated rat liver purified on a Nycodenz density gradient. The revealed bands were detected with anti-(2-enoyl-CoA hydratase 2) antisera as the primary antibody, goat anti-rabbit IgG conjugated with horseradish peroxidase as the second antibody and 4-chloro-1-naphthol as the stain. The positions of molecular-mass standards ($10^{-3}M_r$) are indicated on the left.

similar to that of pig 17 β -HSD, the 17 β -HSD activity was measured, and a very low rate of oxidation of oestradiol to oestrone was observed. For comparison, the same activities were measured with rat liver perMFE-I: in agreement with earlier observations, hydratase 1, L-specific dehydrogenase [36] and isomerase [26] activities were found.

Immunoblotting of tissue extract with antibody to hydratase 2

To elucidate the apparent discrepancy between the M_r of purified hydratase 2 (31 500) and that predicted for perMFE-II (79 331), immunoblotting of rat liver extract was performed with polyclonal antibody to purified hydratase 2. In agreement with an earlier observation [19], immunoblotting revealed two major bands with M_r values of 46 000 and 31 500, and a weaker 79 000- M_r band (Figure 5A). A weak 66 000- M_r signal was also observed. When immunodetection by chemiluminescence and densitometric quantification was performed there were no significant changes in the bands in either male or female rat livers after feeding the animals with a clofibrate-containing diet. As perMFE-II had the potential peroxisomal targeting signal -Ala-Lys-Leu at its C-terminus, highly purified peroxisomes were immunoblotted (Figure 5B). The relative staining intensity of the 79 000- M_r band was higher in the isolated peroxisomes; the 46 000- M_r band was visible and the 66 000 band also appeared. However, the 31 500- M_r band was barely detectable. Interestingly, the expression of cpMFE-II in *Pichia* for 7 days yielded a 79 000- M_r band as well as two small fragmentation products recognized by the anti-hydratase 2 antibody (Figure 4B). COS cells expressing cpMFE-II demonstrated hydratase 2 activity (0.2 μ mol/min per mg of protein, using C_{10} substrate) not seen in the control. Immunoblotting of transformed cells detected the 79 000- M_r band. Northern blot analysis of the levels of mRNA for perMFE-II gave a single signal of 2.9 kb with variable intensity in several tissues of both female and male rats, indicating that the perMFE-II gene is widely expressed. perMFE-II mRNA was most abundant in the kidney and liver in both sexes (results not shown).

DISCUSSION

Although hydratase 2 activity is found in living organisms from bacteria to mammals [37], its physiological function in mammals is largely unknown. To investigate its role in mammalian metabolism, the 31 500- M_r isoform of hydratase 2 from rat liver was characterized in the present study. A cDNA clone encoding the protein was isolated, expressed in yeast, and the biochemical and catalytic properties of the recombinant protein were analysed.

The authenticity of the cDNA clone characterized here was established in several ways: (i) in the purification of hydratase 2 the dehydration of D-3-hydroxydecanoyl-CoA ester to *trans*-2-decenoyl-CoA ester was followed, and the amino acid sequence of the isolated peptides matched the amino acid sequence deduced from the nucleotide sequence; (ii) expression of the isolated clone in yeast and in COS cells resulted in a recombinant 79000- M_r protein that was recognized by an antibody raised against wild-type hydratase 2; (iii) the nucleotide and amino acid sequences of the protein cloned here show a pronounced similarity to the peroxisomal MFE from *S. cerevisiae*, which is known to have both hydratase 2 and D-specific 3-hydroxyacyl-CoA dehydrogenase activities; and (iv) when measured *in vitro*, the purified recombinant protein catalysed both hydratase 2 and D-specific 3-hydroxyacyl-CoA dehydrogenase reactions.

Much evidence exists to suggest that the N-terminal part of perMFE-II is responsible for the 3-hydroxyacyl-CoA dehydrogenase activity. The N-terminus of rat perMFE-II showed sequence similarity to the duplicated N-terminus of yeast peroxisomal MFEs, which have been shown biochemically and genetically to catalyse the D-specific 3-hydroxyacyl-CoA dehydrogenase reaction [16]. Baker [38] has shown the duplicated regions of *C. tropicalis* MFE to be homologous with the members of the secondary-alcohol dehydrogenase protein superfamily, which is composed of NAD(P)⁺-dependent dehydrogenases catalysing the reversible oxidation of hydroxy groups in a variety of substrates. In addition to the apparent nucleotide-binding site [39] in the N-terminus (residues 12–28), the highly conserved motif -Tyr-Xaa-Xaa-Xaa-Lys-, which interacts with the hydroxy group in the catalytic site of the substrate [40,41], emerges at residues 164–168 in perMFE-II. Furthermore the N-terminal fragment of 17 β -HSD isolated from pig endometrium is biochemically active with 17 β -hydroxysteroid substrates *in vitro* [42], and recombinant rat perMFE-II has the same activity, even though to a very low degree.

Truncation of the C-terminal part of yeast MFE resulted in a loss of hydratase 2 activity, whereas the D-specific dehydrogenase activity survived [16]. The C-terminal third of yeast MFE also showed a similarity to residues 337–606 of rat perMFE-II, suggesting that this could be the region involved in hydration reactions in the rat enzyme. This is also strongly supported by the fact that the amino acid sequences of tryptic peptides from the 31 500- M_r isoform of hydratase 2 were accommodated within this region. In connection with the hydration property, the purified wild-type 31 500- M_r hydratase 2 showed a higher catalytic rate with C₁₀ than with C₄ substrates [19]. The behaviour of recombinant perMFE-II was similar to this, whereas to a slight degree the opposite was true of perMFE-I.

In agreement with these proposals, Leenders et al. recently published a report indicating that in pig 17 β -HSD type IV, the N-terminal peptide catalyses the dehydrogenation of steroids at the C-17 position and also 3-hydroxyacyl-CoA esters, and the central part catalyses 2-enoyl-CoA hydratase reactions, but they did not provide evidence about the stereochemistry of the reactions [43].

Seedorf et al. [44] have recently characterized a peroxisomal sterol carrier protein X from rat liver. The protein consists of an N-terminal domain of 404 amino acids with intrinsic 3-ketoacyl-CoA thiolase activity and a 143-residue C-terminal domain with lipid-transferring activity. Similarly, perMFE-II is a peroxisomal protein with β -oxidation activities at the N-terminal part and a sterol carrier protein 2-like domain at the C-terminus. Whether perMFE-II is capable of metabolizing steroids other than 17 β -hydroxysteroids remains to be studied. Of special interest is the question of whether this enzyme participates in the chain shortening of cholesterol during the synthesis of primary bile acids.

Leenders et al. [32,33] have provided evidence that the 79000- M_r polypeptide of pig 17 β -HSD type IV is processed *in situ* somewhere between residues 314 and 328. This cleavage region can be aligned to residues 312–326 in rat perMFE-II. The extreme N-terminal fragment (Ala-Asp-Ala-Ser-Gly-Phe-Ala-) of the hydratase 2 isolated here chromatographically from rat liver is identical with the sequence that begins at residue 318 in the perMFE-II. Furthermore residue 317 was Ser; because Ser is not a cleavage site for trypsin, the observation is in line with the notion that this peptide represents the N-terminus of the 31 500- M_r hydratase 2. However, the appearance of peptide fragments of different sizes shows that the proteolytic processing of perMFE-II is incomplete. The 79000- M_r band that was detectable in immunoblotting of liver extracts and isolated peroxisomes with anti-hydratase 2 antibody represents unprocessed rat perMFE-II. Starting at residue 318 of rat perMFE-II (the putative N-terminus for the hydratase 2 domain), the whole C-terminal fragment has a predicted M_r of 46 580. This value is very close to that of one of the major bands identified in immunoblotting experiments, namely the 46000- M_r band also detected in liver extracts and isolated peroxisomes (Figure 5). Earlier immunological studies of the subcellular fractionation *in vitro* indicated that the 31 500- M_r isoform of hydratase 2 was associated with microsomes [19]. However, in view of the present results the mechanism yielding this isoform remains undetermined.

The results presented here, together with earlier results, indicate that there are two parallel pathways for the second and third reactions of β -oxidation in rat liver peroxisomes but that the reactions follow reciprocal stereochemistry. In this context it is noteworthy that recent experiments on the stereochemistry of β -oxidation have shown that the endogenous yeast peroxisomal MFE (metabolizing D-3-hydroxy intermediates) can be complemented with rat peroxisomal MFE-I (metabolizing L-3-hydroxy intermediates), and both stereochemical alternatives allow yeast cells to grow on oleic acid [27]. When studying the pathophysiology of patients with inborn errors of peroxisomal β -oxidation, it has been proposed that only one peroxisomal MFE could be responsible for the metabolism of all 2-enoyl-CoA esters [45]. This proposal might be an oversimplification, however, in view of the findings presented here and the results published by Novikov et al. [46] demonstrating the existence of several 3-dehydroxyacyl-CoA dehydrogenases in rat liver peroxisomes.

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