Recombinant 2-enoyl-CoA hydratase derived from rat peroxisomal multifunctional enzyme 2: role of the hydratase reaction in bile acid synthesis

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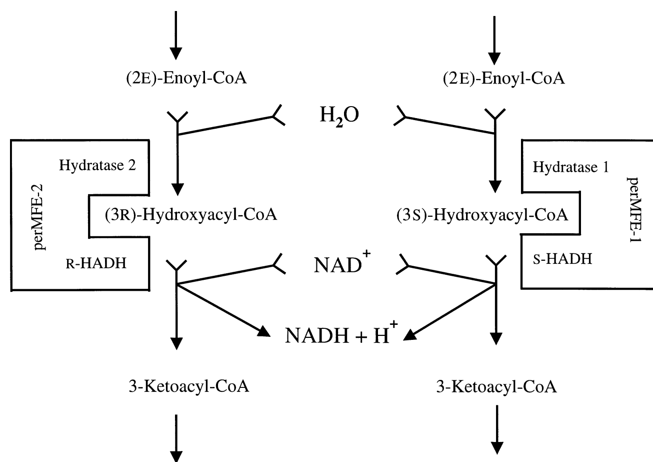
Rat liver peroxisomes contain two multifunctional enzymes: (1) perMFE-1 [2-enoyl-CoA hydratase 1/Δ^3,Δ^2-enoyl-CoA isomerase/(*S*)-3-hydroxyacyl-CoA dehydrogenase] and (2) perMFE-2 [2-enoyl-CoA hydratase 2/(*R*)-3-hydroxyacyl-CoA dehydrogenase]. To investigate the role of the hydratase activity of perMFE-2 in β-oxidation, a truncated version of perMFE-2 was expressed in *Escherichia coli* as a recombinant protein. The protein catalyses the hydration of straight-chain (2*E*)-enoyl-CoAs to (3*R*)-hydroxyacyl-CoAs, but it is devoid of hydratase 1 [(2*E*)-enoyl-CoA to (3*S*)-hydroxyacyl-CoA] and (3*R*)-hydroxyacyl-CoA dehydrogenase activities. The purified enzyme (46 kDa hydratase 2) can be stored as an active enzyme for at least half a year. The recombinant enzyme hydrates (24*E*)-3α,7α,12α-trihydroxy-5β-cholest-24-enoyl-CoA to (24*R*,25*R*)-3α,7α,12α,24-tetrahydroxy-5β-cholestanoyl-CoA, which has previously been characterized as a physiological intermediate in bile acid synthesis. The stereochemistry of the products indicates that the hydration reaction catalysed by the enzyme proceeds via a *syn* mechanism. A monofunctional 2-enoyl-CoA hydratase 2 has not been observed as a wild-type protein. The recombinant 46 kDa hydratase 2 described here survives in a purified form under storage, thus being the first protein of this type amenable to application as a tool in metabolic studies.

INTRODUCTION

Mammalian peroxisomal β-oxidation involves oxidative chain-shortening of a great variety of fatty acids of medium, long and very long chain lengths, dicarboxylic and branched-chain fatty acids, prostaglandins and other eicosanoids, the carboxy side chains of bile acid intermediates, di- and trihydroxycholestanoic acids and some xenobiotics [1]. Each of the four reactions of β-oxidation is catalysed by several enzymes, and recent reports have shown that mammalian peroxisomes contain two multifunctional enzymes Δ^3,Δ^2-enoyl-CoA isomerase/2-enoyl-CoA hydratase 1 (perMFE-1) and peroxisomal multifunctional 2-enoyl-CoA hydratase 2 (perMFE-2) involved in the second and third reactions [2–5]. Both perMFE-1 and perMFE-2 catalyse the hydration of (2*E*)-enoyl-CoA to 3-hydroxyacyl-CoA esters and, in the presence of NAD^+, their subsequent oxidation to 3-keto compounds. However, the course of the overall reaction catalysed by the two multifunctional enzymes proceeds via the formation of β-hydroxy intermediates of opposite chirality (Scheme 1).

The chain-shortening of C_{27} intermediates in the biosynthesis of cholic acid from 3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA) occurs via β-oxidation in liver peroxisomes [6]. The CoA ester of THCA is oxidized to (24*E*)-Δ^{24}-THCA-CoA in a reaction catalysed by THCA-CoA oxidase [7]. However, the enzyme(s) involved in the subsequent hydration reaction have not been characterized at present. Recently Xu and Cuebas [8] demonstrated that the hydratase component of perMFE-1 from rat liver efficiently catalyses the hydration of (24*E*)-Δ^{24}-THCA-CoA to a diastereomer of 24-OH-THCA-CoA that is either not acted on or only very poorly acted on by the dehydrogenase component of this enzyme. This finding suggests that perMFE-1 is not the multifunctional enzyme responsible for the hydration and dehydrogenation reactions involved in the



Scheme 1 Schematic representation of the β-oxidation reaction catalysed by mammalian peroxisomal multifunctional enzymes

perMFE-1 represents peroxisomal multifunctional Δ^3,Δ^2-enoyl-CoA isomerase/2-enoyl-CoA hydratase (hydratase 1) (*S*)-3-hydroxyacyl-CoA dehydrogenase (*S*-HADH) and perMFE-2 represents 2-enoyl-CoA hydratase (hydratase 2)/(3*R*)-hydroxyacyl-CoA dehydrogenase (*R*-HADH).

Abbreviations used: 24-OH-THCA, 3α,7α,12α,24ξ-tetrahydroxy-5β-cholestanoic acid; perMFE-1, peroxisomal multifunctional Δ^3,Δ^2-enoyl-CoA isomerase/2-enoyl-CoA hydratase 1/(*S*)-3-hydroxyacyl-CoA dehydrogenase; perMFE-2, peroxisomal multifunctional 2-enoyl-CoA hydratase 2/(*R*)-3-hydroxyacyl-CoA dehydrogenase; THCA, 3α,7α,12α-trihydroxy-5β-cholestanoic acid.

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peroxisomal metabolism of THCA-CoA and other α-methyl-branched acyl-CoAs.

In the present study we expressed amino acid residues 318–735 of rat perMFE-2 in *Escherichia coli* and purified the recombinant polypeptide (46 kDa hydratase 2). The results demonstrate that the 46 kDa hydratase 2 has (2*E*)-enoyl-CoA hydratase 2 activity and it catalyses the hydration of (24*E*)-Δ^{24}-THCA-CoA to (24*R*,25*R*)-24-OH-THCA-CoA, the physiological intermediate in cholic acid synthesis [9–12].

EXPERIMENTAL

Materials

[α-^{35}S]dATP was purchased from Amersham (Little Chalfont, Bucks., U.K.). The GeneClean II kit was from Bio101 (Vista, CA, U.S.A.) and Moloney murine leukaemia virus reverse transcriptase was from Gibco BRL (Gaithersburg, MD, U.S.A.). The Sure-Clone ligation kit, restriction enzymes, T7 sequencing kit, coenzyme A and DNase were obtained from Pharmacia Biotech (Uppsala, Sweden); the REX total RNA isolation kit was from USB (Cleveland, OH, U.S.A.), *Pfu* DNA polymerase was from Stratagene; lactate dehydrogenase (from rabbit muscle), (*S*)-3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) (from pig heart), lysozyme, RNase, BSA, NAD^+ and crotonyl-CoA were obtained from Sigma Chemicals (St. Louis, MO, U.S.A.). Dithioerythritol was from Boehringer-Mannheim (Steinheim, Germany), benzamidine hydrochloride was from Ega Chemie (Steinheim, Germany), PMSF was from Aldrich Chemie (Steinheim, Germany) and sodium pyruvate was from E. Merck (Darmstadt, Germany). The low-molecular-mass standard kit for SDS/PAGE was from Bio-Rad (Hercules, CA, U.S.A.). pET expression vector was purchased from Novagen (Milwaukee, WI, U.S.A.)

Expression of hydratase 2

Total RNA from the livers of Wistar rats (Laboratory Animal Centre of the University of Oulu, Oulu, Finland) was isolated by the use of a REX total RNA isolation kit in accordance with the manufacturer's instructions. The region of cDNA encoding residues 318–735 of rat perMFE-2 [4] was obtained from the isolated RNA (2 μg) by reverse transcription with Moloney murine leukaemia virus reverse transcriptase and amplification by PCR with rat perMFE-2-specific primers: 5′ primer, cacttccatATGGCAGATGCATCAGGATTTGCT and 3′ primer, catctggatccTCAGAGCTTGGCATAGTCTTTCA (with lower case sequences indicating mismatches to the rat perMFE-2 cDNA sequence). The embedded *Nde*I and *Bam*HI restriction endonuclease sites in the PCR primers allowed the release of a 1279 bp insert and subsequent cloning into pET-3a yielded the plasmid pET-Hydr2. The insert in the plasmid was sequenced and used for expression in *E. coli* BL21(DE3) plysS cells.

Production and purification of recombinant 2-enoyl-CoA hydratase-2

M9ZB medium supplemented with ampicillin (50 μg/ml) and chloramphenicol (34 μg/ml) was used for expression experiments. A 10 ml portion of an overnight culture of host cells containing the plasmid pET-Hydr2 was used to inoculate 1 litre of culture. The cells were grown at 37 °C under aerobic conditions until a D_{600} of 0.6 was reached. The culture was supplemented with additional ampicillin (50 μg/ml), and expression of the plasmid was induced by addition of isopropyl β-D-thiogalactoside to a final concentration of 0.4 mM. After 2 h of additional incubation at 35 °C, the cells were stored at -70 °C until used.

Bacterial cell pellet (4.2 g wet weight) was suspended in 42 ml of 100 mM potassium phosphate/0.5 mM PMSF/0.5 mM benzamidine hydrochloride/1 mM dithioerythritol (pH 8.0) (buffer A). After the cell wall had been digested with lysozyme (100 μg/ml) for 20 min at 22 °C, the viscosity of the cell lysate was reduced with DNase (25 μg/ml) and RNase (25 μg/ml) in the presence of 10 mM $MgCl_2$. Cell debris was sedimented by centrifugation at 40000 ***g*** for 45 min at 4 °C. The supernatant was applied to a 2.5 cm $\times$ 12.5 cm DEAE-Sephacel (Pharmacia) column equilibrated with buffer A. The non-bound protein fraction (40 ml) was concentrated in polyethylene glycol (20 kDa), dialysed against 20 mM Mes/80 mM NaCl (pH 6.0) and applied to a cation-exchange Resource S (1 ml) column equilibrated with the same buffer. The bound proteins were eluted in a NaCl gradient increasing linearly from 80 to 250 mM for 20 min at a flow rate of 1.0 ml/min; fractions of 1.0 ml were collected. Peak activity fractions were pooled, concentrated and applied to a Superdex® 200 HR 10/30 column equilibrated with 200 mM potassium phosphate, pH 7.4. Proteins were eluted at a flow rate of 0.5 ml/min.

Synthesis of substrates

A mixture of the four C-24,25 diastereomers of $3\alpha,7\alpha,12\alpha,24\xi$-tetrahydroxy-$5\beta$-cholestanoic acid (24-OH-THCA) was prepared with procedures previously described by condensing $3\alpha,7\alpha,12\alpha$-triformyloxy-5β-cholan-24-al with methyl-D,L-2-bromopropionate [13]. The Reformatsky condensation gave the ester, methyl $3\alpha,7\alpha,12\alpha,24\xi$-tetrahydroxy-$5\beta$-cholestanoate, and saponification with refluxing 5% (w/v) KOH in methanol, followed by acidification, gave 24-OH-THCA. A sample of the tetrahydroxy free acid was analysed by HPLC after derivatization to *p*-bromophenacyl esters and gave the four characteristic peaks of the C-24,25 diastereomers as has been reported previously [14]. (24*E*,*Z*)-$3\alpha,7\alpha,12\alpha$-Trihydroxy-5β-cholest-24-enoic acid was prepared from $3\alpha,7\alpha,12\alpha$-triformyloxy-5β-cholan-24-al by published procedures [14–16], except that the ylide, (carbethoxyethylidene) triphenyl phosphorane, was obtained from a commercial source and used as is. The ratio of *E* to *Z* isomers was estimated to be 9:1 by NMR (relative ratio of triplet signals due to *E* and *Z* protons on C-24) and by HPLC analysis of the *p*-bromophenacyl esters. CoA derivatives were prepared by the mixed anhydride method and purified by HPLC as previously described [17].

Enzyme assays and HPLC analysis

The activity of 2-enoyl-CoA hydratase 1 was measured in the direction of hydration of (2*E*)-enoyl-CoA to (3*S*)-hydroxyacyl-CoA esters; the activity of 2-enoyl-CoA hydratase 2 was measured in the direction of hydration of (2*E*)-enoyl-CoA to (3*R*)-hydroxyacyl-CoA. The incubation mixture consisted of 60 nmol of (2*E*)-enoyl-CoA in 50 mM Tris/HCl, pH 8.0, containing 50 μmol of KCl, 1 μmol of NAD^+ and, as auxiliary enzymes, 5 μg of pig (3*S*)-hydroxyacyl-CoA dehydrogenase or 5 μg of yeast recombinant (3*R*)-hydroxyacyl-CoA dehydrogenase (Y.-M. Qin, unpublished work) in a volume of 1 ml at 23 °C. The reaction was started by adding a sample to a cuvette and monitored by following NADH production at 340 nm. The reverse reaction catalysed by 2-enoyl-CoA hydratase 2 was measured as described by Hiltunen et al. [18].

Incubation mixtures for the reactions catalysed by the 46 kDa hydratase 2 contained either 45 μM (24*E*)-Δ^{24}-THCA-CoA or an 80 μM mixture of C-24,25 diastereomers of 24-OH-THCA-CoA

as substrate in 0.15 M potassium phosphate containing 0.025 M ammonium phosphate, pH 7.0. The reactions were initiated by the addition of 46 kDa hydratase 2 to a final concentration of 20 μg/ml. At intervals a 150 μl aliquot of the incubation mixture was withdrawn and the reaction was stopped by the addition of 25 μl of 2 M HCl; after 1 min it was neutralized with 25 μl of 2 M KOH. Experiments were performed with all CoA derivatives to demonstrate that the recovery of material on HPLC was virtually quantitative after the addition of 2 M HCl and 2 M KOH, although some spontaneous reactions did occur when the reactions were stopped with 6 M HCl and 6 M KOH. Aliquots of 150 μl were analysed by HPLC on a Waters dual-pump gradient system with a YMC-Pack ODS-A reverse-phase column (5 μm particle size; 0.6 cm × 15 cm) at 25 °C with an eluent of 0.05 M ammonium phosphate, pH 5.5, with a linear gradient of 60–80 % (v/v) methanol over 45 min at a flow rate of 2 ml/min; the absorbance of the effluent was monitored at 260 nm. Column re-equilibration was accomplished with a reversed gradient of 80–60 % (v/v) methanol over 5 min followed by isocratic elution at 60 % (v/v) methanol for an additional 15 min.

Other methods

Calculations for kinetic parameters (K_m and k_{cat}) were performed by using a Grafit 3.0 program.

Table 1 Summary of purification of recombinant 46 kDa hydratase 2

The cDNA fragment of perMFE-2 encoding the terminal portion was expressed in *E. coli* cells, and chromatographic purification of the protein was performed from cell lysates as described in the Experimental section.

Step	Activity (μmol/min)	Protein (mg)	Specific activity (μmol/min per mg)	Yield (%)
Soluble extract	1946	146	13.3	100
Anion exchanger (DE52)	1833	114	16.1	97.4
Cation exchanger (Resources S)	396	13	30.0	20.0
Size exclusion (Superdex 200 HR)	264	5.5	48.0	13.6

Table 2 Kinetic constants of recombinant 46 kDa hydratase 2

The activity of 2-enoyl-CoA hydratase 2 was measured in the direction of hydration of (2*E*)-enoyl-CoA to (3*R*)-hydroxyacyl-CoA in a mixture consisting of 60 nmol of (2*E*)-enoyl-CoA in 50 mM Tris/HCl, pH 8.0, 50 μmol of KCl and 1 μmol of NAD^+ in a volume of 1 ml at 23 °C. Kinetic parameters of the enzyme were measured with concentrations of substrates from 5 to 200 μM and calculated with the GraFit program and the Michaelis–Menten enzyme kinetics equation.

Substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)
Crotonyl-CoA	2.3	60	3.8×10^4
(2*E*)-Hexenoyl-CoA	22.8	8.7	2.6×10^6
(2*E*)-Decenoyl-CoA	26.0	4.6	5.7×10^6

RESULTS

Expression, purification and characterization of recombinant 46 kDa hydratase 2 derived from rat perMFE-2

When pET-Hydr2 was expressed in *E. coli*, the soluble extract of the cells hydrated (2*E*)-decenoyl-CoA to (3*R*)-hydroxydecanoyl-CoA at a rate of 33.4 μmol/min per mg of protein. The activity was below the detection limit of the assay system when using extracts from non-transformed cells or cells transformed with the vector only. The expressed recombinant protein, 46 kDa hydratase 2, was purified from the cell extract to apparent homogeneity by three chromatographic steps on anion-exchange, cation-exchange and size-exclusion columns (Table 1). By SDS/PAGE analysis, the molecular mass of the recombinant protein was 46 kDa (Figure 1A), which agrees with the 46.58 kDa estimate from the amino acid sequence. Size-exclusion chromatography on a Superdex 200 HR column gave a native molecular mass of 59 kDa, suggesting that the recombinant protein was monomeric. An antibody raised against 31.5 kDa rat liver hydratase 2 [19], which arose by partial proteolysis from perMFE-2 [4], recognized the 46 kDa hydratase 2 (Figure 1B). When the 46 kDa hydratase 2 was incubated with 30 nmol of crotonyl-CoA, the product of the hydration reaction served as a substrate for (3*R*)-hydroxyacyl-CoA dehydrogenase but not for the (3*S*)-hydroxy-specific enzyme.

Table 2 summarizes the kinetic constants of the 46 kDa

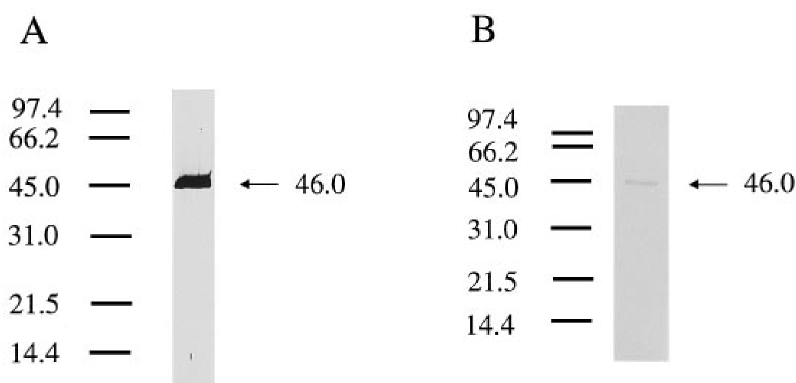


Figure 1 Purification of recombinant 46 kDa hydratase 2

Recombinant 46 kDa hydratase 2 was purified from *E. coli* cell extract. (**A**) Purified hydratase 2 (7.0 μg) was analysed by SDS/PAGE. (**B**) Polyclonal antibody raised against purified 31.5 kDa hydratase 2 [21] from rat liver was used as the primary antibody, and affinity-purified goat anti-(rabbit IgG) conjugated with horseradish peroxidase as the second antibody. The recognized epitopes were revealed with 4-chloro-1-naphthol.

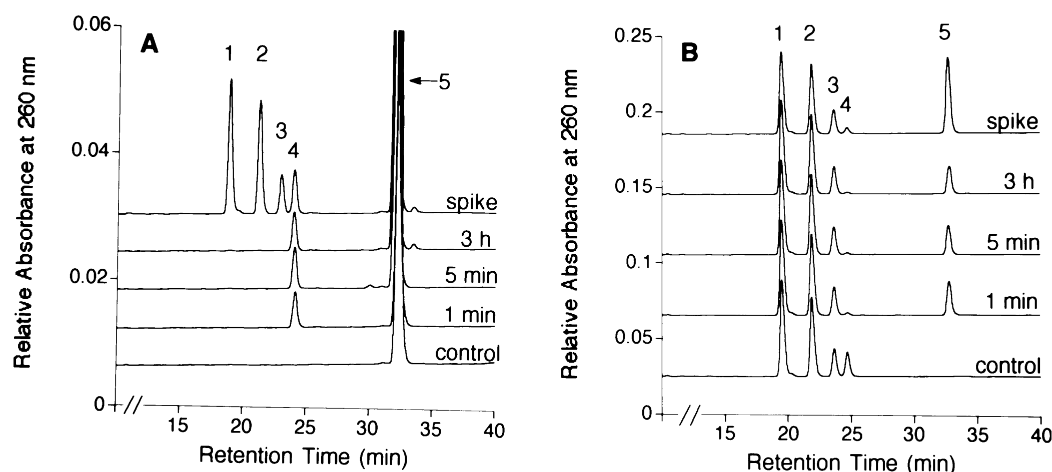


Figure 2 Metabolism of 24-OH-THCA-CoA by 46 kDa hydratase 2 at various intervals

(**A**) Hydration of (24*E*)-Δ^{24}-THCA-CoA. Traces: control, no 46 kDa hydratase 2 enzyme; spike, a small amount of a diastereomeric mixture of 24-OH-THCA-CoA was added to the 3 h incubation mixture before the reaction was stopped with acid. (**B**) Dehydration of the C-24,25 diastereomers of 24-OH-THCA-CoA. Traces: control, no 46 kDa hydratase enzyme; spike, a small amount of (24*E*)-Δ^{24}-THCA-CoA was added to the 3 h incubation mixture before the reaction was stopped with acid. Peak 1, (24*S*,25*R*)-24-OH-THCA-CoA; peak 2, (24*R*,25*S*)-24-OH-THCA-CoA; peak 3, (24*S*,25*S*)-24-OH-THCA-CoA; peak 4, (24*R*,25*R*)-24-OH-THCA-CoA; peak 5, (24*E*)-Δ^{24}-THCA-CoA. The small shoulder just after peak 1 is a small quantity of cholyl-CoA contaminating the diastereomeric 24-OH-THCA-CoA preparation.

hydratase 2 with (2*E*)-enoyl-CoA substrates. The K_m value for (2*E*)-decenoyl-CoA (C_{10}) was approx. one-tenth of that with crotonyl-CoA (C_4). The catalytic rate ratio with C_{10}/C_4 substrates was 11.3, which agreed well with a previously reported value (14.4) for rat liver hydratase 2 and 100 μM substrate [18]. The specificity constant (k_{cat}/K_m) of the 46 kDa hydratase 2 with crotonyl-CoA (Table 2) was approx. $1/10^4$ of that determined for mitochondrial short-chain 2-enoyl-CoA hydratase 1 (crotonase) [20,21]. The enzyme preparation retained more than 90 % of the original activity if it was stored as specified in the Experimental section for 6 months at +4 °C or frozen at −20 °C.

The metabolism of (24*E*)-Δ^{24}-THCA-CoA by recombinant 46 kDa hydratase 2

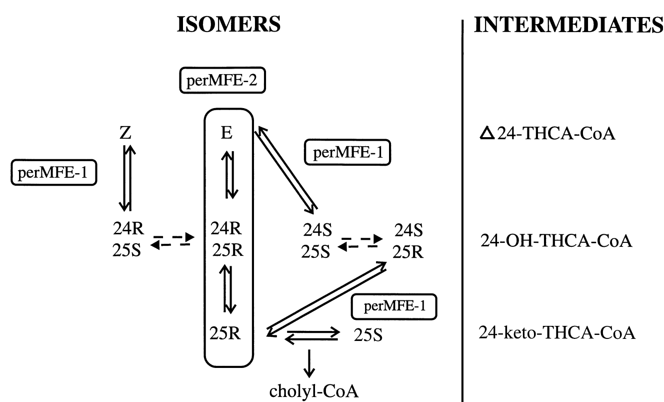
To evaluate the role of perMFE-2 in the β-oxidative side-chain modifications that occur in bile acid biosynthesis, the stereospecificity of the hydration reaction catalysed by the recombinant 46 kDa hydratase 2 was investigated by reverse-phase HPLC. Figure 2(A) shows the results obtained when the incubation medium contained 46 kDa hydratase 2 and (24*E*)-Δ^{24}-THCA-CoA. The protein rapidly converted (24*E*)-Δ^{24}-THCA-CoA to peak 4, corresponding to (24*R*,25*R*)-24-OH-THCA-CoA.

That peak 4 was formed from the 24*E* isomer of Δ^{24}-THCA-CoA is supported by the following: peak 4 has a retention time identical to that of the (24*R*,25*R*) diastereomer of 24-OH-THCA-CoA, and previous work has established that perMFE-2 hydrates (2*E*)-enoyl-CoAs to the (3*R*)-hydroxy stereoisomer [19]. In agreement with previous reports on the hydration of 2-methyl-(2*E*)-enoyl-CoAs [22] and on the hydration of (24*E*)-Δ^{24}-THCA-CoA by the inducible multifunctional enzyme (perMFE-1) [8], the equilibrium lies in the direction of dehydration. Detailed examination of the chromatogram obtained after 3 h of incubation revealed that the 46 kDa hydratase 2 gave only the (24*R*,25*R*) diastereomer of 24-OH-THCA-CoA when incubated with a sample of (24*E*)-Δ^{24}-THCA-CoA contaminated with a small amount of the (*Z*)-isomer. This observation contrasts with results obtained in similar experiments performed with perMFE-1 [8], where slow formation of the (24*R*,25*S*) diastereomer (peak 2) was also observed. The lack of enzymic formation of the (24*S*,25*R*) diastereomer of 24-OH-THCA-CoA from the small amount of contaminating (*Z*)-isomer accords with a previous report that the peroxisomal enzyme responsible for the hydration of (2*E*)-enoyl-CoAs to the (3*R*)-hydroxy stereoisomers is inactive towards (2*Z*)-enoyl-CoAs [23]. The identity of the small peak that is eluted just after (24*E*)-Δ^{24}-THCA-CoA (peak 5) after prolonged incubation is not known.

To investigate the stereospecificity of the reaction catalysed by the recombinant 46 kDa hydratase 2 in the direction of dehydration, a diastereomeric mixture of 24-OH-THCA-CoA was used as a substrate (Figure 2B). The rapid formation of a peak with the same retention time as synthetically prepared (24*E*)-Δ^{24}-THCA-CoA (peak 5) was observed with a simultaneous decrease in the peak corresponding to the (24*R*,25*R*) diastereomer of 24-OH-THCA-CoA (peak 4). The equilibrium ratio of the peak areas for (24*E*)-Δ^{24}-THCA-CoA (peak 5) and (24*R*,25*R*)-24-OH-THCA-CoA (peak 4) was 14:1 in both the hydration and dehydration experiments. Evidence that the 46 kDa hydratase 2 was still active after 3 h of incubation is supported by the results shown in the spiked chromatogram of Figure 2(B), where (24*E*)-Δ^{24}-THCA-CoA was added to the 3 h incubation mixture before the reaction was quenched with acid. The increase in (24*R*,25*R*)-24-OH-THCA-CoA (peak 4) was due to the rapid establishment of a new equilibrium on the addition of (24*E*)-Δ^{24}-THCA-CoA.

DISCUSSION

The oxidative cleavage of THCA, an intermediate in the biosynthetic pathway of cholic acid, is believed to proceed by a mechanism similar to the peroxisomal β-oxidation of fatty acids [6]. The first intermediate of this reaction sequence, Δ^{24}-THCA-CoA, has the (*E*)-configuration under physiological conditions [9,16,24,25]. In rat liver peroxisomes, this reaction is now known to be catalysed exclusively by a THCA-CoA oxidase distinct from fatty acyl-CoA oxidase and pristanoyl-CoA oxidase [7,26–28]. Using a rat liver mitochondrial fraction, some authors have



Scheme 2 Alternative pathways for the metabolism of (24*E*)-3α,7α,12α-trihydroxy-5β-cholest-24-enoyl-CoA (Δ^{24}-THCA-CoA) to 24-keto,3α,7α,-12α-trihydroxy-5β-cholest-24-enoyl-CoA (24-keto-THCA-CoA) in rat liver

The reaction catalysed by perMFE-2 is enclosed in the cartouche. The dotted double arrows indicate reactions that could hypothetically be catalysed by an α-methyl-acyl-CoA racemase.

shown that all four diastereomers of 24-OH-THCA are formed from (25*R*,*S*)-THCA [25] and that all four 24-OH-THCA diastereomers can be metabolized to cholic acid [10], whereas others have shown the exclusive formation of (24*R*,25*S*)-24-OH-THCA {revised assignment, (24*R*,25*R*) [11]} in both rat liver homogenates [12] and purified peroxisomes [24].

The dehydrogenase part of the recently characterized enzyme perMFE-2 catalyses the oxidation of (24*R*,25*R*)-24-OH-THCA-CoA [3]. {This isomer was previously referred to as the (24*R*,25*S*) isomer [3], as originally assigned by Une et al. [14], but recent work has resulted in the revised assignment of this isomer to (24*R*,25*R*) [11,29]}. Here we provide evidence that the hydratase component of perMFE-2 converts (24*E*)-Δ^{24}-THCA-CoA to (24*R*,25*R*)-24-OH-THCA-CoA, which can serve as a substrate for the dehydrogenase part of perMFE-2. Therefore it is apparent that, in rats, both hydratase and dehydrogenase portions of perMFE-2 are involved in the β-oxidation of the cholesterol side chain in bile acid synthesis and, potentially, α-methyl-branched substrates.

A summary of the stereospecific reactions in bile acid synthesis catalysed by hydratase and dehydrogenase parts of both perMFE-1 and perMFE-2 is shown in Scheme 2. The reactions catalysed by perMFE-2 provide a direct route for the formation of 24-keto-THCA-CoA from (24*E*)-Δ^{24}-THCA-CoA. However, the accumulation of THCA in patients with perMFE-1 deficiency [30] suggests that this enzyme can also participate in bile acid synthesis. This is puzzling in view of the results of experiments on the stereochemistry of reactions catalysed by perMFE-1 indicating that the product of the hydratase reaction cannot serve as a substrate for the dehydrogenase component of the enzyme during bile acid biosynthesis [8]. One possible alternative pathway for the formation of a 24-keto-THCA-CoA intermediate could involve the following sequential steps: (1) the hydration of (24*E*)-Δ^{24}-THCA-CoA to (24*S*,25*S*)-24-OH-THCA-CoA catalysed by perMFE-1, (2) the hypothetical racemization of the α-methyl group to (24*S*,25*R*)-24-OH-THCA-CoA by an α-methylacyl-CoA racemase, and (3) the dehydrogenation of (24*S*,25*R*)-24-OH-THCA-CoA to 24-keto-THCA-CoA catalysed by perMFE-1. Although an α-methylacyl-CoA racemase with activity towards THCA-CoA has been purified from rat [31] and human [32] liver, and α-methyl-THCA-CoA racemase activity has been demonstrated in purified peroxisomes [33], direct evidence that rat liver peroxisomes contain a racemase that is capable of racemizing the α-methyl group of 24-OH-THCA-CoA diastereomers is not available at present. Indirect evidence supporting the existence of such a racemase is provided by previous work demonstrating that a crude mitochondrial fraction from rat liver can catalyse the formation of all four C-24,25 diastereomers of 24-OH-THCA from (25*R*)-THCA, (25*S*)-THCA or (24*E*)-Δ^{24}-THCA [22], and that a crude mitochondrial fraction from rat liver can also catalyse the formation of cholic acid from all four C-24,25 diastereomers of 24-OH-THCA [10]. Further work is needed to determine the viability of an alternative pathway involving perMFE-1 and an α-methylacyl-CoA racemase in the synthesis of cholic acid.

Both experiments *in vitro* [34] and the structural architecture of the catalytic site of hydratase 1 (crotonase) [35] point to the occurrence of hydration/dehydration reactions of (2*E*)-enoyl-CoA esters following *syn* stereochemistry. In agreement with this stereochemistry, the end product of the hydration of (24*E*)-Δ^{24}-THCA-CoA by perMFE-1 has been found to be (24*S*,25*S*)-24-OH-THCA-CoA [8]. The end product of hydration of the same substrate by 46 kDa hydratase 2 was (24*R*,25*R*)-24-OH-THCA-CoA, indicating that proton and hydroxy group addition by hydratase 2 also occurs via *syn* stereochemistry, although such addition takes place on the opposite face of the double bond from the addition by hydratase 1.

The presence of different acyl-CoA oxidases for the first reaction of β-oxidation [7,26–28], two multifunctional enzymes for the second and third reactions [2–5] and three thiolases for the last reaction [36–38] raises the question of whether, instead of a common pathway, parallel pathways operate in mammalian peroxisomes, each showing a distinct preference towards their substrates. The treatment of rats with peroxisomal proliferators results in severalfold increases in the activities of straight-chain acyl-CoA oxidase, perMFE-1 and thiolase 1 with a simultaneous increase of peroxisomal straight-chain fatty acid oxidation capacity [1]. Chain shortening of THCA-CoA seems to be separate from the inducible oxidation pathway of straight-chain substrates. It is unclear whether perMFE-2, which is not induced by peroxisomal proliferators [2], participates in the β-oxidation of straight-chain fatty acyl-CoA *in vivo*. It is interesting to note that the second and third reactions of β-oxidation in yeast peroxisomes are catalysed by a multifunctional enzyme utilizing (3*R*)-hydroxyacyl-CoA intermediates [39]. Both genetic and biochemical experiments have demonstrated that the yeast multifunctional enzyme can be functionally complemented by rat perMFE-1, resulting in the introduction of a (3*S*)-hydroxyacyl-specific pathway in yeast [40].

A monofunctional protein catalysing the hydratase 1 reaction is commercially available (crotonase), and it is widely applied in metabolic studies. In contrast with hydratase 1, no monofunctional hydratase 2 has yet been found in Nature. In this work we describe a truncated version of rat perMFE-2 yielding a recombinant monofunctional hydratase 2, which can be stored as an active protein. Furthermore the usefulness of the product is demonstrated by applying the purified enzyme to studies of the hydratase reaction in bile acid synthesis.

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