The role of α-methylacyl-CoA racemase in bile acid synthesis

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According to current views, the second peroxisomal β-oxidation pathway is responsible for the degradation of the side chain of bile acid intermediates. Peroxisomal multifunctional enzyme type 2 [peroxisomal multifunctional 2-enoyl-CoA hydratase/(*R*)-3-hydroxyacyl-CoA dehydrogenase; MFE-2] catalyses the second (hydration) and third (dehydrogenation) reactions of the pathway. Deficiency of MFE-2 leads to accumulation of very-long-chain fatty acids, 2-methyl-branched fatty acids and C_{27} bile acid intermediates in plasma, but bile acid synthesis is not blocked completely. In this study we describe an alternative pathway, which allows MFE-2 deficiency to be overcome. The alternative pathway consists of α-methylacyl-CoA racemase and peroxisomal multifunctional enzyme type 1 [peroxisomal multifunctional 2-enoyl-CoA hydratase/(*S*)-3-hydroxyacyl-CoA dehydrogenase; MFE-1]. (24*E*)-3α,7α,12α-Trihydroxy-5β-cholest-24-enoyl-CoA, the presumed physiological isomer, is hydrated by MFE-1 with the formation of (24*S*,25*S*)-3α,7α,12α,24-tetrahydroxy-5β-cholestanoyl-CoA [(24*S*,25*S*)-24-OH-THCA-CoA], which after conversion by a α-methylacyl-CoA racemase into the (24*S*,25*R*) isomer can again be dehydrogenated by MFE-1 to 24-keto-3α,7α,12α-trihydroxycholestanoyl-CoA, a physiological intermediate in cholic acid synthesis. The discovery of the alternative pathway of cholesterol side-chain oxidation will improve diagnosis of peroxisomal deficiencies by identification of serum 24-OH-THCA-CoA diastereomer profiles.

Key words: cholesterol side chain, β-oxidation, peroxisome.

INTRODUCTION

Chain-shortening of C_{27} intermediates in the biosynthesis of cholic acid from 3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA) occurs through a β-oxidative sequence of reactions located exclusively in liver peroxisomes [1,2]. Mammalian peroxisomes contain two distinct sets of β-oxidation enzymes [3]; the inducible enzymes that function in the oxidation of straight-chain acyl-CoAs, and a more recently characterized set of fibrate-uninducible enzymes that appear necessary for the β-oxidation of α-methyl-branched acyl-CoAs, such as 3α,7α,12α-trihydroxy-5β-cholestanoyl-CoA (THCA-CoA) and pristanoyl-CoA (Scheme 1). The oxidative cleavage of THCA is believed to proceed by a mechanism similar to the peroxisomal β-oxidation of fatty acids (Scheme 1). The first intermediate of this reaction sequence is (24*E*)-Δ^{24}-THCA-CoA. In rat peroxisomes, this reaction is catalysed by THCA-CoA oxidase [4]. However, the mitochondrial hydroxylation at C-26 of the cholesterol side chain affords specifically the (25*R*) diastereomer of THCA [5], whereas THCA-CoA oxidase is highly stereospecific for (25*S*)-THCA-CoA [6]. It has been shown that α-methylacyl-CoA racemase efficiently racemizes (25*R*)-THCA-CoA to the (25*S*) isomer [7].

The two subsequent reactions are catalysed by MFE-2 [peroxisomal multifunctional 2-enoyl-CoA hydratase/(*R*)-3-hydroxyacyl-CoA dehydrogenase; Scheme 1]. Its hydratase component converts (24*E*)-Δ^{24}-THCA-CoA into (24*R*,25*R*)-3α,7α,12α,24-tetrahydroxy-5β-cholestanoyl-CoA (24-OH-THCA-CoA) [8], which is converted into 24-keto-THCA-CoA by the dehydrogenase part of the same enzyme [9,10]. Then sterol carrier protein-x cleaves the 24-keto-THCA-CoA to choloyl-CoA and propionyl-CoA [11].

A number of patients with MFE-2 deficiency have been identified and, although these patients accumulate bile acid intermediates, they still synthesize mature bile acids [12–14], implying the existence of an alternative route to bile acids. In the present article, we demonstrate that rat α-methylacyl-CoA racemase can racemize the C-25 carbon of 24-OH-THCA-CoAs and, together with MFE-1 [peroxisomal multifunctional 2-enoyl-CoA hydratase/(*S*)-3-hydroxyacyl-CoA dehydrogenase], can provide a route for bile acid biosynthesis (and presumably pristanic acid degradation) that does not require MFE-2.

EXPERIMENTAL

Materials

Sigma was the source of CoA and NAD^+. Cholic acid, triethylamine, ethyl chloroformate, methyl-D,L-2-bromopropionate, (carbethoxyethylidene)triphenylphosphorane, Adam's catalyst and all other reagents were obtained from Aldrich. α-Methylacyl-CoA racemase was purified from rat liver as described previously [7]. Recombinant hydratase domain of rat liver MFE-2 was prepared as reported previously [8]. MFE-1 was purified from di(2-ethylhexyl)phthalate-treated rats according to published procedures [15].

General procedures

HPLC analyses were performed at 25 °C on a Waters dual-pump gradient system using a YMC-Pack ODS-A reversed-phase column (5 μm particle size; 0.6 cm × 15 cm). CoA derivatives

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

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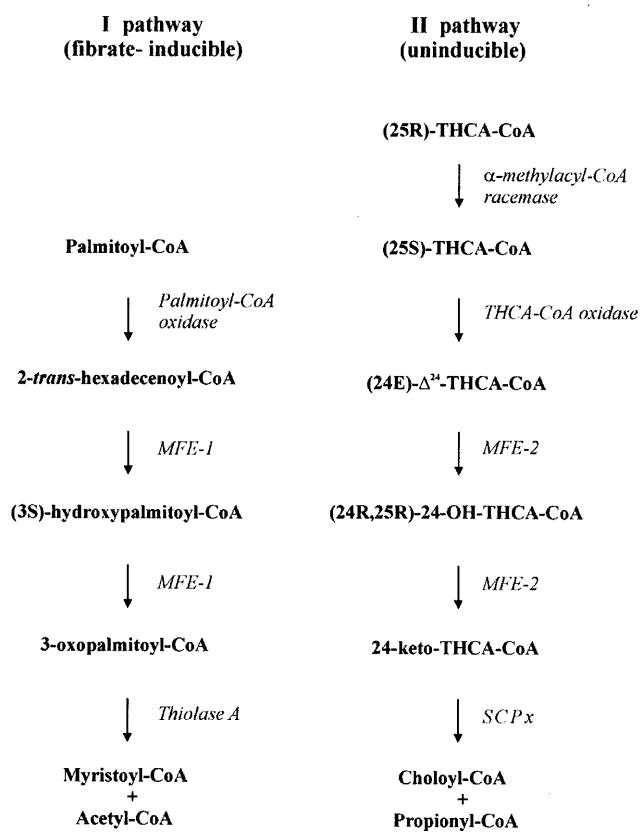


Scheme 1 Organization of the β-oxidation of straight-chain fatty acids and of bile acid intermediates in rat liver peroxisomes

SCPx, sterol carrier protein-x.

were prepared by the mixed anhydride method as described in [16] and were purified by HPLC. *p*-Bromophenacyl esters were prepared for analysis by HPLC using a published procedure [17] and chromatographed using methanol/water (80:20) as a solvent. Protein concentration was determined by the Coomassie dye-binding method of Sedmak and Grossberg [18] using BSA as a standard.

Synthetic procedures

A mixture of the four C-24,25 diastereomers of 3α,7α,12α,24ξ-tetrahydroxy-5β-cholestanoic acid (24-OH-THCA) was prepared by a modification of the procedures described previously for the Reformatsky condensation of 3α,7α,12α-triformyloxy-5β-cholan-24-al with 2-bromopropionate [19]. (24*E*,*Z*)-Δ^{24}-THCA was prepared from 3α,7α,12α-triformyloxy-5β-cholan-24-al according to published procedures [20–22]. The relative ratio of *E*:*Z* isomers was approx. 9:1 as estimated by NMR and HPLC analyses of the *p*-bromophenacyl esters. A mixture of the (25*S*,*R*) diastereomers of THCA was synthesized by catalytic hydrogenation of (24*E*,*Z*)-Δ^{24}-THCA with Adam's catalyst in methanol at atmospheric pressure and room temperature. The progress of the reaction was monitored by HPLC analysis of the reaction mixture following derivatization to *p*-bromophenacyl esters.

Enzyme assays

Enzyme activities were analysed by HPLC using an eluent of 0.05 M ammonium phosphate at pH 5.5 with a linear gradient of 60–80 % methanol over 45 min at a flow rate of 2.0 ml/min, except for the racemization of THCA-CoA, where the linear gradient was 20–60 % acetonitrile over 65 min. The absorbance of the effluent was monitored at 260 nm. Unless stated otherwise, all enzyme assays contained 15 mM potassium phosphate and 25 mM ammonium phosphate, and aliquots of the reaction mixture were quenched with a one-sixth vol. of 2 M HCl and neutralized with 2 M KOH. Incubation mixtures for the racemization of THCA-CoA contained 34 μM (24*R*)-THCA-CoA and 0.125 μg/ml α-methylacyl-CoA racemase at pH 7.0. Aliquots of 90 μl were analysed by HPLC. Incubation mixtures for the racemization of 24-OH-THCA-CoA diastereomers catalysed by α-methylacyl-CoA racemase contained 50 μM substrate and 5.5 μg/ml α-methylacyl-CoA racemase at pH 7.0, and aliquots of 60 μl were analysed by HPLC. Incubation mixtures for the metabolism of 24-ene-THCA-CoA to 24-keto-THCA-CoA contained 70 μM substrate, 20 μg/ml MFE-1, 1 mM NAD^+, 0.1 M Tris/HCl, 50 mM ammonium phosphate and 7.72 μg/ml α-methylacyl-CoA racemase at pH 9. Aliquots of 130 μl were analysed by HPLC. Incubation mixtures for the dehydration of (24*S*,25*R*)-24-OH-THCA-CoA or (24*R*,25*S*)-24-OH-THCA-CoA contained 36 μM substrate and 20 μg/ml MFE-2 or MFE-1, respectively, at pH 7.0. Aliquots of 135 μl were analysed by HPLC.

RESULTS

The racemization of (25*R*)-THCA-CoA to (25*S*)-THCA-CoA by α-methylacyl-CoA racemase

Previous work has shown that rat and human liver α-methylacyl-CoA racemase can efficiently racemize the α-carbon of various α-methylacyl-CoA derivatives [7,23]. Figure 1 shows the results obtained by HPLC analysis of the time-dependent racemization of (25*R*)-THCA-CoA (Figure 1, peak 2) to (25*S*)-THCA-CoA

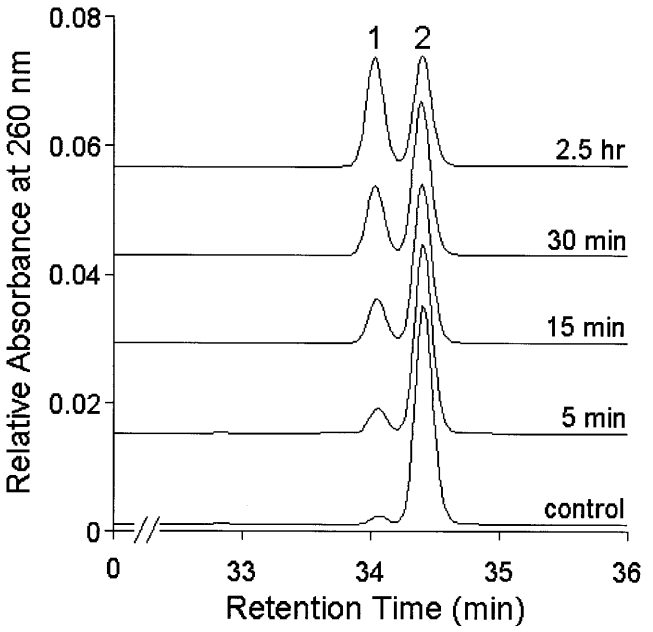


Figure 1 HPLC analysis of the time-dependent racemization of (25*R*)-THCA-CoA to (25*S*)-THCA-CoA by α-methylacyl-CoA racemase purified from rat liver

Peak 1, (25*S*)-THCA-CoA; peak 2, (25*R*)-THCA-CoA.

(Figure 1, peak 1) by α-methylacyl-CoA racemase purified from rat liver. After 2.5 h of incubation, the reaction mixture contained virtually identical concentrations of both THCA-CoA isomers. This result was unchanged upon the addition of more α-methylacyl-CoA racemase to the 2.5 h reaction mixture, indicating that equilibrium had in fact been reached (results not shown). The initial rate of conversion corresponds to a specific activity of 4.0 μmol/min per mg of protein for this substrate. This result is close to the value of 10 μmol/min per mg of protein determined previously for this enzyme from the release of tritium from diastereomeric [24,25-^{3}H]THCA-CoA [7]. The slightly lower specific activity determined by the HPLC assay may be a result of slight contamination of the (25*S*) isomer in our (25*R*)-THCA-CoA preparation, thereby inhibiting the forward reaction. Alternatively, the radiometric assay may overestimate the true specific activity because the ^{3}H exchange may be more rapid than the net racemization rate, as in the case of racemases with a single-base mechanism.

The racemization of C-24,25 diastereomers of 24-OH-THCA-CoA by α-methylacyl-CoA racemase

Although α-methylacyl-CoA racemase can racemize the methyl group of various α-methylacyl-CoA derivatives, it is not known what effect the presence of a functional group on the β-carbon would have on racemization of the α-carbon. Figure 2 shows the results obtained by HPLC analysis of the time-dependent racemization at C-25 of purified diastereomers of 24-OH-THCA-CoA catalysed by α-methylacyl-CoA racemase. The incubations were performed at pH 7, which was shown previously to be the pH maximum for this enzyme using THCA-CoA as a substrate [7]. Figure 2(A) shows the results obtained when the incubation medium contained α-methylacyl-CoA racemase and (24*S*,25*R*)-24-OH-THCA-CoA (Figure 2A, peak 1). The results show a time-dependent decrease in the area of peak 1 and the concomitant appearance of a peak that co-migrates with synthetic (24*S*,25*S*)-24-OH-THCA-CoA (Figure 2A, peak 3). That the reaction reached equilibrium at 24 h was shown by the fact that the addition of more α-methylacyl-CoA racemase did not change the proportions of peaks 1 and 3 (results not shown). The (24*S*,25*R*) isomer is slightly thermodynamically favoured over the (24*S*,25*S*) isomer. The inability to detect peaks corresponding to the other two diastereomers at C-24,25 following prolonged incubation provides strong evidence that the α-methylacyl-CoA racemase is absolutely specific for racemization at the α-carbon and has no ability to affect the stereochemistry of the hydroxy groups at the β-carbon. Assuming equal absorption coefficients for the two diastereomers, the specific activity of the α-methylacyl-CoA racemase for the conversion of the (24*S*,25*R*) isomer into the (24*S*,25*S*) isomer was estimated to be 8.8 nmol/min per mg of protein.

Figure 2(B) shows the results obtained under the same conditions as Figure 2(A) except that α-methylacyl-CoA racemase was incubated for various time intervals with the (24*R*,25*S*) diastereomer of 24-OH-THCA-CoA. The results show the exclusive formation of a peak whose retention time is identical with (24*R*,25*R*)-24-OH-THCA-CoA. These are the results to be expected if the racemization reaction catalysed by the α-methylacyl-CoA racemase is restricted to the α-carbon. In contrast with the results shown in Figure 2(A), it appears that the equilibrium was obtained after an incubation period of approx. 10 h. Assuming that the extinction coefficients of the two diastereomers are equal, the specific activity of the α-methylacyl-CoA racemase for the conversion of the (24*R*,25*S*) isomer into the (24*R*,25*R*) isomer was estimated to be 10 nmol/min per mg of protein.

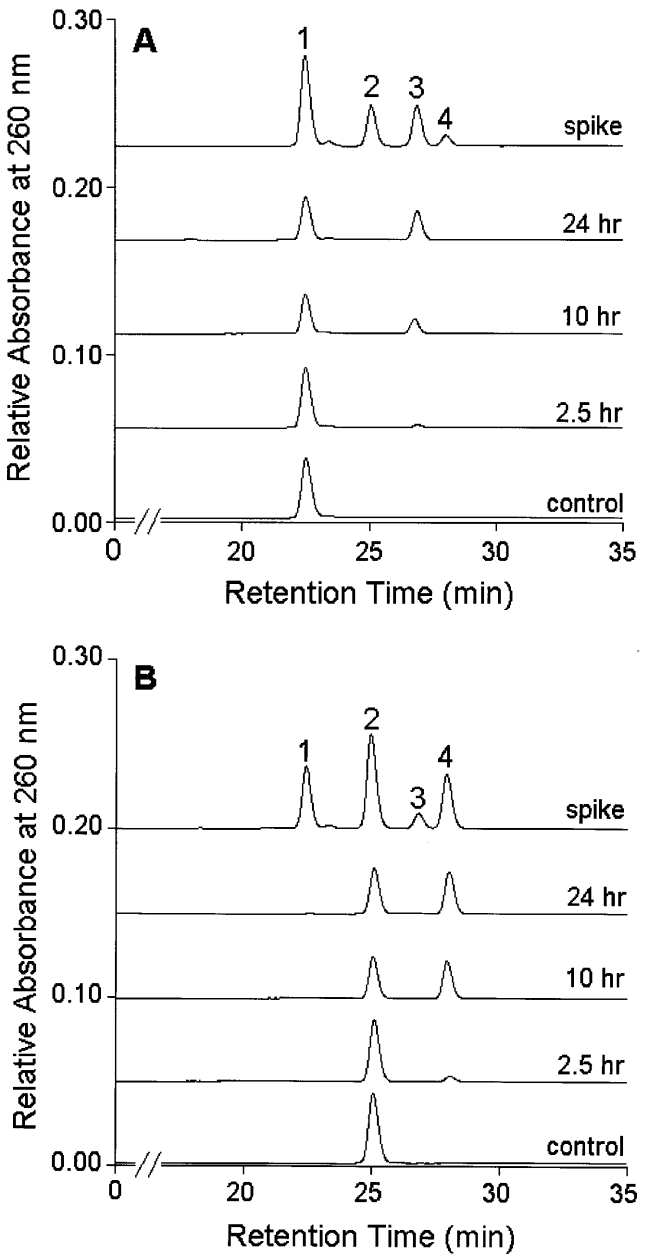


Figure 2 HPLC analysis of the time-dependent racemization of the C-25 carbon of 24-OH-THCA-CoA diastereomers by α-methylacyl-CoA racemase purified from rat liver

(**A**) The racemization of (24*S*,25*R*)-24-OH-THCA-CoA to the (24*S*,25*S*) isomer; (**B**) the racemization of (24*R*,25*S*)-24-OH-THCA-CoA to the (24*R*,25*R*) isomer. Spike indicates the addition of a small amount of a diastereomeric mixture of 24-OH-THCA-CoA to the 24 h incubation mixture before workup. Peak 1, (24*S*,25*R*)-24-OH-THCA-CoA; peak 2, (24*R*,25*S*); peak 3, (24*S*,25*S*); peak 4, (24*R*,25*R*). The small peak that elutes just after peak 1 in the spiked solution is a small quantity of cholyl-CoA contaminating the diastereomeric THCA-CoA preparation. α-Methylacyl-CoA racemase also converts the (24*S*,25*S*) into the (24*S*,25*R*) isomer and the (24*R*,25*R*) into the (24*R*,25*S*) isomer (results not shown).

The physiologically relevant direction of racemization for the newly proposed pathway is from the (24*S*,25*S*) isomer to the (24*S*,25*R*) isomer, which is the reverse of the reaction shown in

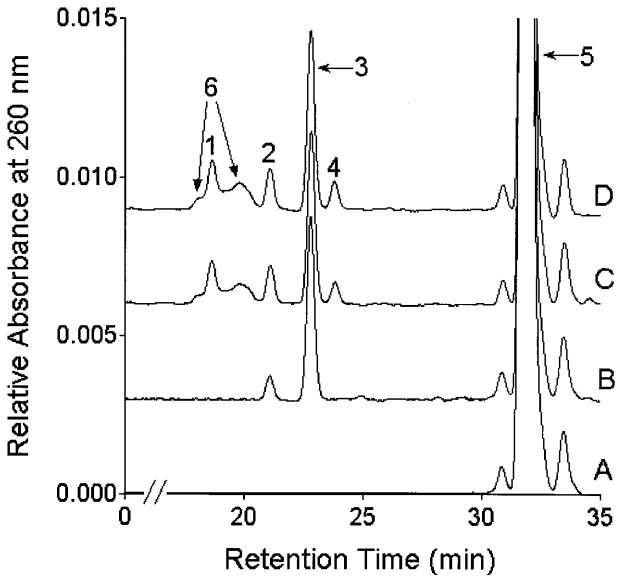


Figure 3 HPLC analysis of the time-dependent conversion of (24*E*)-Δ^{24}-THCA-CoA into 24-keto-THCA-CoA by the combined actions of MFE-1 and α-methylacyl-CoA racemase

(**A**) Control incubation without enzymes; (**B**) results obtained after 30 min incubation with MFE-1; (**C**) results obtained after the addition of α-methylacyl-CoA racemase to the reaction mixture in (**B**) followed by a 2 h incubation prior to workup; (**D**) as (**C**) except the incubation time with α-methylacyl-CoA racemase was 3 h before workup. Assignments for peaks 1–4 are the same as shown in the legend to Figure 2; peak 5, (24*E*,*Z*)-Δ^{24}-THCA-CoA; peak 6, 24-keto-THCA-CoA.

Figure 2(A). The racemization of purified (24*S*,25*S*) isomer was also studied under the same conditions as those above for the other isomers (results not shown), and the specific activity of the α-methylacyl-CoA racemase for the conversion of the (24*S*,25*S*) isomer into the (24*S*,25*R*) isomer was found to be 94 nmol/min per mg of protein. Interestingly, this value is only 2.4% of the value obtained above for racemization of THCA-CoA and approximately one order of magnitude higher than the reverse reaction. It is clear that there is a strong steric effect exerted by the presence of the β-hydroxy group, and that the enzyme has different kinetic parameters towards the (24*S*,25*S*) and (24*S*,25*R*) isomers.

Formation of 24-keto-THCA-CoA from (24*E*)-Δ^{24}-THCA-CoA in the presence of α-methylacyl-CoA racemase and MFE-1

Figure 3 shows the results obtained by HPLC analysis of the metabolism of Δ^{24}-THCA-CoA by the combined actions of α-methylacyl-CoA racemase and the hydratase and dehydrogenase activities of MFE-1. Chromatogram A shows the results when the incubation medium contained Δ^{24}-THCA-CoA (Figure 3, peak 5) and NAD^+ but no enzymes. The result obtained after 30 min of incubation with MFE-1 is shown in chromatogram B, and shows the expected appearance of the syn-addition product (24*S*,25*S*)-24-OH-THCA-CoA (Figure 3, peak 3) and a small amount of the (24*R*,25*S*) diastereomer (Figure 3, peak 2), which forms by syn-addition to the small amount of *Z* isomer contaminating the (24*E*)-Δ^{24}-THCA-CoA preparation. The observation that the (24*R*,25*R*) isomer (Figure 3, peak 4) is not present in this chromatogram provides strong evidence that our MFE-1 preparation did not have any measurable contamination with MFE-2. Although NAD^+ is present and a pH of 9.0 was used to shift the oxidation equilibrium to the formation of ketoacyl-CoA, the formation of 24-keto-THCA-CoA was not observed, as has been reported previously [24,25]. The results obtained after the subsequent addition of α-methylacyl-CoA racemase with 2 and 3 h incubations are shown in chromatograms C and D, respectively. The formation of (24*S*,25*R*)-24-OH-THCA-CoA (Figure 3, peak 1) is clearly evident and results from the action of the α-methylacyl-CoA racemase on (24*S*,25*S*)-24-OH-THCA-CoA (Figure 3, peak 3). Subsequent oxidation of (24*S*,25*R*)-24-OH-THCA-CoA (Figure 3, peak 1) by the dehydrogenase domain of the MFE-1 would produce 24-keto-THCA-CoA, as evidenced by the broad peak (Figure 3, peak 6) that appears on either side of peak 1. That (24*S*,25*R*)-24-OH-THCA-CoA (Figure 3, peak 1) is the only 24-OH-THCA-CoA diastereomer that the dehydrogenase domain of MFE-1 acts upon has been shown previously [24]. The observation that the easily enolizable 24-keto-THCA-CoA intermediate exhibits a broad double peak has also been confirmed previously [24]. The formation of (24*R*,25*R*)-24-OH-THCA-CoA (Figure 3, peak 4) can be explained by the action of the α-methylacyl-CoA racemase on the (24*R*,25*S*) isomer (Figure 3, peak 2).

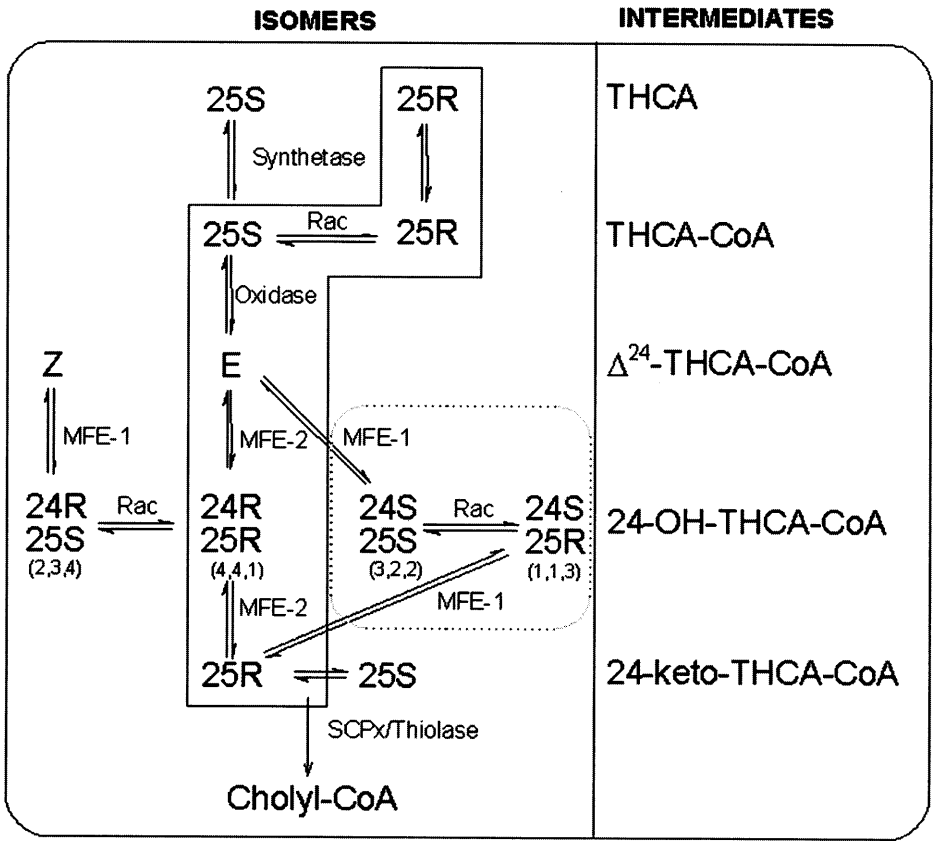
Formation of (24*Z*)-Δ^{24}-THCA-CoA from (24*R*,25*S*)- and (24*S*,25*R*)-24-OH-THCA-CoA catalysed by the hydratase domains of MFE-1 and MFE-2

The following experiments were performed to clarify the interconversions that are possible between the various geometric isomers of 24-ene-THCA-CoA and diastereomers of 24-OH-THCA-CoA. Our present and previous work [24] suggests that MFE-1 is capable of interconverting (24*Z*)-Δ^{24}-THCA-CoA and (24*R*,25*S*)-24-OH-THCA-CoA. In order to investigate this further, purified (24*R*,25*S*)-24-OH-THCA-CoA was incubated with MFE-1 and the formation of a peak that co-migrated with (*E*,*Z*)-24-ene-THCA-CoA was observed (results not shown). Our HPLC solvent system does not resolve the *E* and *Z* isomers of Δ^{24}-THCA-CoA, but the established syn-addition mechanism of the MFE-1 would predict that it was the *Z* isomer that formed. The initial rate was estimated to be 2 nmol/min per mg of protein and at equilibrium the reaction favoured the hydration product by a factor of 5. This result was not unexpected considering the thermodynamic instability of the Z-isomer, as has been shown previously for the hydration reaction's equilibrium for unbranched α,β-*cis*(*Z*)-enoyl-CoAs, where the hydration product is favoured by a factor of 137 [26].

In a separate experiment, (24*S*,25*R*)-24-OH-THCA-CoA was incubated with recombinant hydratase domain of MFE-2. In this case, the formation of a peak that co-migrated with (*E*,*Z*)-24-ene-THCA-CoA was not observed, even after an extended incubation time (results not shown). This result is in accordance with previous work where the reverse reaction was also not detectable [8], and in accord with a report where the peroxisomal hydratase responsible for the hydration of (2*E*)-enoyl-CoAs to the (3*R*)-hydroxy isomer is inactive towards (2*Z*)-enoyl-CoAs [26].

DISCUSSION

It is currently believed that α-methylacyl-CoA racemase catalyses an essential reaction during bile acid biosynthesis. Mitochondrial sterol 27-hydroxylase affords the (25*R*) isomer [5,27] of THCA, whereas its dehydrogenation, measured with either purified peroxisomal oxidases [6] or peroxisomes after immuno-



Scheme 2 The enzymes involved in the β-oxidative biosynthesis of cholyl-CoA from THCA

The steps enclosed by the solid line are the generally accepted intermediates and enzymes in the major pathway that uses the D(*R*)-specific MFE-2. The steps enclosed by the dotted line represent the alternative pathway involving the L(*S*)-specific MFE-1 and α-methylacyl-CoA racemase (Rac) demonstrated in this paper. The numbers in parentheses below each 24-OH-THCA-CoA isomer represent the relative elution order of these diastereomers during reversed-phase HPLC as CoA derivatives [24]. SCPx, sterol carrier protein-x.

precipitation of α-methylacyl-CoA racemase [28], displays strict specificity for the (25*S*) isomer. A recent report described how α-methylacyl-CoA racemase deficiency leads to 3α,7α-dihydroxy-5β-cholestanoic acid (DHCA) and THCA accumulation in plasma, and LC-MS detected an accumulation of (25*R*) isomers only [29].

The results of our study show that α-methylacyl-CoA racemase can be involved in an alternative oxidative pathway of the cholesterol side chain in peroxisomes. The current view that MFE-2 is the major multifunctional enzyme involved in oxidation of α-methyl-branched acyl-CoA is most strongly supported by the recent clarification that most, if not all, patients whose condition has been diagnosed as MFE-1 deficiency are, in fact, MFE-2-deficient [12]. In general, these patients show increased plasma concentrations of very-long-chain fatty acids, pristanic and phytanic acids, and bile acid intermediates, as well as depressed peroxisomal β-oxidation activity towards both cerotic and pristanic acid in fibroblasts. Another group has found that 2,3-pristenic and 3-hydroxypristanic acids are elevated in plasma during MFE-2 deficiency, although 3-ketopristanic acid was within the normal range [30]. Taken together, these results imply that although MFE-2 appears necessary to β-oxidize bile acid intermediates and pristanic acid at rates that are sufficient to prevent their accumulation, there is an alternative pathway that operates independently of MFE-2 for the formation of mature bile acids and, presumably, for pristanic acid degradation.

Table 1 The C-24,25 diastereomers of 24-OH-THCA-CoA predicted to accumulate in various peroxisomal β-oxidation disorders

The Table shows bile acid intermediates that may accumulate in a case of MFE-2 or sterol carrier protein-x (SCP_x)/thiolase deficiency.

	24-OH-THCA-CoA diastereomer			
Defect	24*R*,25*S*	24*R*,25*R*	24*S*,25*S*	24*S*,25*R*
MFE-2 hydratase domain	Trace?	Trace?	+	–
MFE-2 dehydrogenase domain*	+	+	+	–
MFE-2 complete*	–	–	+	–
SCP_x/thiolase	+	+	+	+

* The predicted increases in unconjugated bile acids correspond to the profiles observed from plasma of patients with deficiencies of the dehydrogenase domain of or complete MFE-2 [13].

Scheme 2 shows a summary of the stereospecific reactions catalysed by enzymes in rat liver for the degradation of the steroid side chain during bile acid biosynthesis. The reactions surrounded by the dotted line represent the alternative pathway involving MFE-1 and α-methylacyl-CoA racemase demonstrated in this report. It is now possible to predict the 24-OH-THCA-CoA diastereomers that accumulate in various peroxisomal β-

oxidation deficiencies and these are shown in Table 1. These predictions assume that the α-methylacyl-CoA racemase step in the alternative pathway is rate-limiting and that therefore the (24*S*,25*R*) isomer should not accumulate in MFE-2 deficiency, since this is the only diastereomer being metabolized further. The validity of the proposed alternative pathway is supported by recent work that determined the stereochemistry of the 24-OH-THCA-CoA isomers that accumulated in plasma of a patient with an isolated defect in the dehydrogenase activity of MFE-2 as well as a patient with complete MFE-2 deficiency [14], and these results are in complete accord with our predictions. In light of the alternative pathway that we have proposed, it would appear puzzling that pristanic acid oxidation was not measurable in fibroblasts from the patient with an isolated defect in the dehydrogenase activity of MFE-2 [31]. This result is most probably explained by the fact that the α-methylacyl-CoA racemase activity towards [2-^{3}H]pristanoyl-CoA in human fibroblasts is approx. one-eighth of that found in human liver [23]. We would therefore predict that pristanic acid oxidation should be measurable, although lower than normal, from liver tissue of this patient, and that the alternative pathway is not operative in tissues that express relatively low levels of α-methylacyl-CoA racemase.

The involvement of MFE-1 in cholesterol side-chain oxidation is supported by data on (24*S*)-hydroxycholesterol oxidation. It has been reported that excess cholesterol in brain is converted into (24*S*)-hydroxycholesterol, which may be secreted across the blood–brain barrier into the circulation and eliminated via the liver [32,33]. It was suggested that (24*S*)-hydroxycholesterol is metabolized to (24*S*,25*R*)-24-OH-THCA-CoA, which is the correct stereoisomer for further dehydrogenation by MFE-1. The total input of cholesterol utilization via (24*S*)-hydroxycholesterol is a matter for further investigation.

Further work is necessary to clarify the role of α-methylacyl-CoA racemase in branched-chain acyl-CoA metabolism, and whether serum 24-OH-THCA-CoA diastereomer profiles are generally applicable for the diagnosis of peroxisomal β-oxidation disorders.

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