Evidence that multifunctional protein 2, and not multifunctional protein 1, is involved in the peroxisomal β-oxidation of pristanic acid

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The second (enoyl-CoA hydratase) and third (3-hydroxyacyl-CoA dehydrogenase) steps of peroxisomal β-oxidation are catalysed by two separate multifunctional proteins (MFPs), MFP-1 being involved in the degradation of straight-chain fatty acids and MFP-2 in the β-oxidation of the side chain of cholesterol (bile acid synthesis). In the present study we determined which of the two MFPs is involved in the peroxisomal degradation of pristanic acid by using the synthetic analogue 2-methylpalmitic acid. The four stereoisomers of 3-hydroxy-2-methylpalmitoyl-CoA were separated by gas chromatography after hydrolysis, methylation and derivatization of the hydroxy group with (*S*)-2-phenylpropionic acid, and the stereoisomers were designated I–IV according to their order of elution from the column. Purified MFP-1 dehydrated stereoisomer IV but dehydrogenated stereoisomer III, so by itself MFP-1 is not capable of converting a branched enoyl-CoA into a 3-ketoacyl-CoA. In contrast, MFP-2 dehydrated and dehydrogenated the same stereoisomer (II), so it is highly probable that MFP-2 is involved in the peroxisomal degradation of branched fatty acids and that stereoisomer II is the physiological intermediate in branched fatty acid oxidation. By analogy with the results obtained with the four stereoisomers of the bile acid intermediate varanoyl-CoA, stereoisomer II can be assigned the 3*R*-hydroxy, 2*R*-methyl configuration.

INTRODUCTION

Peroxisomes are the subcellular site of the β-oxidation of a wide variety of lipophilic carboxylates including very-long-chain fatty acids, 2-methyl-branched fatty acids (e.g. the naturally occurring pristanic acid and the synthetic 2-methylpalmitic acid) and the bile acid precursors di- and tri-hydroxycoprostanic acids [1–3]. In rat liver the first step of peroxisomal β-oxidation is catalysed by three different acyl-CoA oxidases: palmitoyl-CoA oxidase [4,5], pristanoyl-CoA oxidase [6] and trihydroxycoprostanoyl-CoA oxidase [7], involved in the oxidation of the CoA esters of straight-chain fatty acids, 2-methyl-branched fatty acids and the bile acid precursors respectively [8]. Pristanoyl-CoA oxidase and trihydroxycoprostanoyl-CoA oxidase desaturate exclusively the 2*S*-methyl stereoisomers of their respective substrates (the 25-methyl group of di- and tri-hydroxycoprostanic acid corresponds to a 2-methyl group in their side chain) [9]. The second (enoyl-CoA hydratase) and third (3-hydroxyacyl-CoA dehydrogenase) reactions of peroxisomal β-oxidation are catalysed by two separate multifunctional proteins (MFPs) [10–12]. MFP-1 is the 'classical' multifunctional β-oxidation enzyme first identified and cloned by Hashimoto and co-workers [13]. As well as 2-enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities, it also displays Δ^3,Δ^2 enoyl-CoA isomerase activity required for the oxidation of unsaturated fatty acids [14]. The enzyme hydrates 2-*trans*-enoyl-CoA to L-3-hydroxyacyl-CoA and specifically dehydrogenates L-3-hydroxyacyl-CoAs. The hydroxy group of these CoA esters has a 3*S* configuration [15]. MFP-2 possesses 2-enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 17β-hydroxysteroid dehydrogenase activities [11]. The enzyme was described independently by Adamski and co-workers in pig [16] and in man [17] as a 17β-hydroxysteroid dehydrogenase type IV and by us and other groups in rat [11,12,18,19] and in man [20] as a second peroxisomal multifunctional β-oxidation enzyme. MFP-2 specifically forms and dehydrogenates D-3-hydroxyacyl-CoAs (3*R*-hydroxyacyl-CoAs) [11,12,20]. A portion of the enzyme is cleaved after import into the peroxisome, yielding an active homodimeric (subunit 34 kDa) D-3-hydroxyacyl-CoA dehydrogenase/17β-hydroxysteroid dehydrogenase (the N-terminal part of MFP-2) and a 45 kDa D-specific 2-enoyl-CoA hydratase (the C-terminal part of MFP-2) [11,12,21].

The naturally occurring 3-hydroxyacyl-CoA intermediate in the β-oxidation of straight-chain fatty acids has the L-3 configuration, implying that MFP-1 is involved in the β-oxidation of straight-chain fatty acids [22,23].

The physiological 3-hydroxyacyl-CoA intermediate in bile acid synthesis is 24*R*,25*R*-varanoyl-CoA (first erroneously described as 24*R*,25*S*-varanoyl-CoA) [24–28], in which the 24*R* hydroxy group corresponds to a D-3-hydroxy group in the side chain [24,25]. Because MFP-2 displays D-stereospecificity and because the enzyme specifically dehydrates and dehydrogenates 24*R*,25*R*-varanoyl-CoA [11,12], one can safely conclude that MFP-2 is involved in bile acid synthesis (and not in the degradation of straight-chain fatty acids). In contrast, MFP-1 forms 24*S*,25*S*-varanoyl-CoA and dehydrogenates 24*S*,25*R*-varanoyl-CoA [11,12,29], further supporting the notion that not MFP-1 but MFP-2 (and its cleavage products) is responsible for the hydration and dehydrogenation of the side chain of the bile acid intermediates.

The stereochemical configuration of the physiological 3-hydroxyacyl-CoA intermediate in the β-oxidation of 2-methyl-branched fatty acids remains unknown, so that it is currently impossible to predict which of the two MFPs is involved in the peroxisomal degradation of branched fatty acids. In this report we studied the stereospecificity of the two MFPs with 3-hydroxy-

Abbreviation used: MFP, multifunctional protein.

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2-methylpalmitoyl-CoA as the substrate, and provide evidence that MFP-2, but not MFP-1, has a role in the β-oxidative breakdown of 2-methyl-branched fatty acids.

MATERIALS AND METHODS

Materials

CoA, NAD^+ and Percoll were from Pharmacia Benelux. 2-Hydroxystearic acid was from Larodan AA. Stearoyl-CoA was from Sigma. C_{18} Sep-Pak cartridges (2 g) were from Waters. (*S*)-2-Phenylpropionic acid was from Fluka.

Substrate syntheses

2-Methylpalmitoyl-CoA [6] and 3-hydroxy-2-methylpalmitoyl-CoA [10] were prepared as described previously.

The CoA ester of 2-hydroxystearic acid was synthesized by transesterification of the corresponding thiophenyl ester, analogously to the synthesis of formyl-CoA [30] with some modifications. The thiophenyl ester was obtained as follows. To 180 μmol of 2-hydroxystearic acid, dissolved in 10 ml of anhydrous and peroxide-free tetrahydrofuran, thiophenol (1.2-fold molar excess) was added and the solution was stirred on ice for 20 min. After 360 μmol of dicyclohexylcarbodi-imide had been added, stirring was continued for 2 h at 0 °C. TLC analysis of the reaction mixture revealed a spot with an R_F of 0.58 [silica gel 60F254 0.2 mm; hexane/ether/acetic acid (60:40:1, v/v)]. After the addition of water (18 ml) containing sodium iodoacetate (65 mg), the thiophenyl ester formed was extracted twice into diethyl ether (25 ml each). The organic extract was washed with 0.1 M K_2HPO_4, dried over sodium sulphate, filtered and evaporated. The residue, taken up in tetrahydrofuran, was passed through a Blauband filter, dried and again dissolved in 6 ml of anhydrous and peroxide-free tetrahydrofuran. Half of this solution was transferred to a 25 ml reaction vessel containing 1 ml of ethanol, 3 ml of 0.1 M $NaHCO_3$, pH 8.5, and 60 μmol of CoA, after which the mixture was stirred overnight. After removal of the organic solvents by evaporation, the mixture was brought to pH 1 by the addition of $HClO_4$ and the precipitated CoA ester was recovered by centrifugation. The pellet was washed once with 0.8 % (v/v) $HClO_4$ and once with acetone. The CoA ester, dissolved in 10 ml of 0.05 M $NaHCO_3$, was applied to a C_{18} Sep-Pak cartridge and eluted with increasing methanol concentration. Methanol fractions were analysed by TLC and those containing CoA ester [R_F 0.49; n-butanol/acetic acid/water (5:2:3, v/v)] were combined, evaporated and dried under vacuum over P_2O_5. A yield of 66 % (related to the amount of CoA used) was obtained.

Incubation of peroxisomes and extraction of CoA esters

A light mitochondrial fraction of rat liver was prepared in 0.25 M sucrose containing 0.1 % (v/v) ethanol, 1 mM EDTA and 5 mM Mops, pH 7.2 [31], and subfractionated by centrifugation through an iso-osmotic self-generating Percoll gradient [32]. The gradient was collected in 15 fractions of equal volume, starting from the bottom; the fractions containing the peroxisomes were pooled and diluted approx. 10-fold with the sucrose medium. After centrifugation for 20 min at 16200 ***g***, the supernatant was removed and the peroxisomes were resuspended in 7 ml of sucrose medium. The purified peroxisomes (6 mg of protein, in a volume of 6 ml) were incubated for 1 h at 37 °C in a reaction medium containing 96.4 mM KCl, 1 mM KH_2PO_4, 20 mM $NaHCO_3$, 3.8 mM NaCl, 8 mM ATP, 1 mM CoA, 10 mM $MgCl_2$, 7.5 μM FAD, 0.05 % BSA and 100 μM 2-methylpalmitoyl-CoA in a total volume of 30 ml. NAD^+ was omitted, to suppress 3-hydroxyacyl-CoA dehydrogenase reaction and to bring about an accumulation of 3-hydroxy-2-methylpalmitoyl-CoA. Incubations were terminated by adding acetic acid [20 % (v/v) final concentration]. After acidification, stearoyl-CoA (600 nmol) was added as internal standard. Zero-time controls were performed by adding acetic acid before the addition of the substrate. The CoA esters were subsequently precipitated with $HClO_4$ [2.3 % (v/v) final concentration], extracted as described previously [33] and dissolved in 50 mM potassium phosphate, pH 5.5, before their injection on a C_{18} reverse-phase column.

Enzyme purification and enzyme incubations

MFP-1, MFP-2 and the cleaved-off 3-hydroxyacyl-CoA dehydrogenase domain of MFP-2 were purified from rat liver peroxisomes as described before [10]. 3-Hydroxyacyl-CoA dehydrogenase and dehydratase activities were measured as described previously [11], with 25 μM 3-hydroxy-2-methylpalmitoyl-CoA as the substrate. To enable a study of their stereospecificity, the enzymes were incubated in a total volume of 1 ml with racemic 3-hydroxypalmitoyl-CoA (substrate concentration 100 μM) or with the HPLC fractions 1 and 2 (substrate concentration 50 μM). At the end of the reactions acetic acid, 50 nmol of 2-hydroxystearoyl-CoA (internal standard) and $HClO_4$ were added successively and the CoA esters were then purified as described above.

Analytical procedures

HPLC analyses were performed on a Waters 600 system, equipped with a reverse-phase column (Econosphere C_{18}, 150 mm × 4.6 mm, 8 nm pore size, 5 μm particle size; Alltech). The 3-hydroxy-2-methylpalmitoyl-CoA, 2-methyl-2-hexadecenoyl-CoA and 2-methylpalmitoyl-CoA esters were separated by a gradient of acetonitrile in 50 mM potassium phosphate, pH 5.5 (percentage of acetonitrile: 0 to 2 min, 20 %; then linearly increasing to 40 % over 3 min, to 55 % over 18 min and to 65 % over 5 min). This method permits the separation of the racemic mixture of 3-hydroxy-2-methylpalmitoyl-CoA in two fractions, each containing two stereoisomers (see the Results section).

The semi-preparative separation of racemic 3-hydroxy-2-methylpalmitoyl-CoA into two fractions, containing stereoisomers I and III, and II and IV, respectively (see Figure 1), was performed under isocratic conditions [acetonitrile/50 mM potassium phosphate, pH 5.5 (37:63, v/v)].

GLC analyses were made on a Pye Unicam PU4550 Philips gas chromatograph equipped with an Econo Pac (EC-1) capillary column (30 m × 0.32 mm, 0.25 μm film thickness; Alltech) and a flame ionization detector. Before analysis, purified 3-hydroxy-2-methylpalmitoyl-CoA or other CoA ester fractions were hydrolysed, methylated and converted to (*S*)-2-phenylpropionyl derivatives by the method of Jin et al. [34] with some modifications. (*S*)-2-Phenylpropionyl chloride was prepared from (*S*)-2-phenylpropionic acid as described by Hammarstrom and Hamberg [35], dried under a stream of nitrogen to remove the acetyl chloride and stored at −20 °C. To achieve complete conversion to phenylpropionyl derivatives, the dried 3-hydroxy-2-methylpalmitic methyl esters were incubated for 3 h at 60 °C with 2 μl of (*S*)-2-phenylpropionyl chloride in 20 μl of pyridine. At the end of the reaction the samples were dried. The residues were dissolved in hexane and dried again to remove all volatile compounds. The final residues were dissolved in hexane and injected into the gas chromatograph. The purification on silica

cartridges (silica Bondelut cartridges; Varian), necessary to remove the remaining phenylpropionic acid [34], was omitted to avoid important losses of 2-methyl-2-hexadecenoic methyl ester. For GLC, the temperature was kept at 80 °C for 1 min, increased to 210 °C (10 °C per min), then to 250 °C (3 °C per min), kept at 250 °C for 10 min, and again increased to 300 °C (5 °C per min).

RESULTS

Separation of the four stereoisomers of 3-hydroxy-2-methylpalmitoyl-CoA

A prerequisite for the study of the substrate stereospecificity of the two MFPs towards branched-chain fatty acids is the development of a method capable of separating the four stereoisomers of 3-hydroxy-2-methylacyl-CoAs. However, by means of reverse-phase HPLC, racemic 3-hydroxy-2-methylpalmitoyl-CoA was resolved into only two peaks. The isomers of racemic mixtures of straight-chain 3-hydroxyacyl-CoAs have been separated by others as methyl esters by means of GLC after derivatization of the hydroxy group with chiral compounds such as α-methoxy-α-trifluoromethylphenyl acetate [36] and (*S*)-2-phenylpropionic acid [34,35]. For 3-hydroxy-2-methylpalmitoyl-CoA, only derivatization with (*S*)-2-phenylpropionic acid gave a satisfactory gas-chromatographic resolution of the four stereoisomers (Figure 1, top panel); derivatization with α-methoxy-A-trifluoromethylphenyl acetate resulted in only two broad peaks (results not shown). The (*S*)-2-phenylpropionic acid-derivatized stereoisomers were designated I–IV on the basis of their order of elution from the column. Analysis of the two peaks collected after HPLC (see above) showed that the first peak consisted of stereoisomers I and III (Figure 1, middle panel) and the second peak of stereoisomers II and IV (Figure 1, bottom panel).

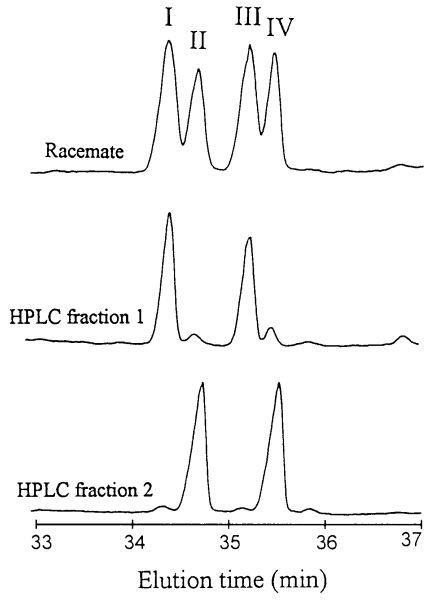


Figure 1 GLC analysis of racemic 3-hydroxy-2-methylpalmitoyl-CoA and of HPLC fractions 1 and 2

Top panel: GLC analysis of racemic 3-hydroxy-2-methylpalmitoyl-CoA after hydrolysis and derivatization to phenylpropionic methyl esters (see the Materials and methods section). The four stereoisomers of 3-hydroxy-2-methylpalmitoyl-CoA were resolved by HPLC into two fractions (1 and 2) and were analysed by GLC (middle and bottom panel).

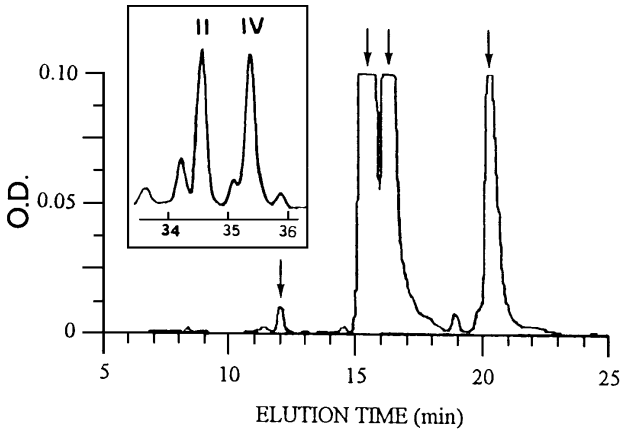


Figure 2 HPLC and GLC (inset) analysis of the 3-hydroxy-2-methylpalmitoyl-CoA stereoisomers formed after incubation of peroxisomes with 2-methylpalmitoyl-CoA

Purified peroxisomes (6 mg of protein) were incubated with 100 μM 2-methylpalmitoyl-CoA in a total volume of 30 ml (see the Materials and methods section). After 1 h of incubation the CoA esters were precipitated with $HClO_4$ and extracted. The partly purified CoA esters were analysed by HPLC. The vertical arrows mark the peaks of 3-hydroxy-2-methylpalmitoyl-CoA (12 min), 2-methyl-2-hexadecenoyl-CoA (15.4 min), 2-methylpalmitoyl-CoA (16.4 min) and stearoyl-CoA (20.5 min; internal standard). The fraction that was eluted between 11 and 13 min was collected, hydrolysed and derivatized with phenylpropionyl chloride and further analysed by GLC (inset). Stereoisomers of 3-hydroxy-2-methylpalmitic acid are indicated by roman numerals.

Stereochemical configuration of the 3-hydroxy-2-methylacyl-CoA intermediate in peroxisomal branched-chain fatty acid oxidation

To provoke an accumulation of 3-hydroxy-2-methylpalmitoyl-CoA, highly purified peroxisomes were incubated with racemic (*R*,*S*)-2-methylpalmitoyl-CoA in the absence of NAD^+. HPLC analysis of the extracted acyl-CoAs showed that after 1 h of incubation 63% of the substrate had been converted to 2-methyl-2-hexadecenoyl-CoA (Figure 2). Because pristanoyl-CoA oxidase desaturates the 2*S* isomer exclusively, this high percentage of conversion supports the presence of a 2-methylacyl-CoA racemase in rat liver peroxisomes [9]. Despite the absence of NAD^+, accumulating 3-hydroxy-2-methylpalmitoyl-CoA amounted to only 1% of the accumulated 2-methyl-2-hexadecenoyl-CoA. The detection of only one peak of 3-hydroxy-2-methylpalmitoyl-CoA by HPLC analysis suggested that only one or two stereoisomers were produced by the peroxisomes. Further analysis of the fraction that was eluted between 11 and 13 min by GLC demonstrated that stereoisomers II and IV were formed in substantial and equal amounts (Figure 2, inset). A small amount of the other stereoisomers was, however, also detected.

Hydratase and dehydrogenase stereospecificities of the MFPs

Both MFP-1 and MFP-2 display 3-hydroxyacyl-CoA dehydratase and 3-hydroxyacyl-CoA dehydrogenase activity when incubated with racemic 3-hydroxy-2-methylpalmitoyl-CoA [11,12]. The purified MFPs were therefore incubated with racemic 3-hydroxy-2-methylpalmitoyl-CoA in the absence and the presence of NAD^+, and the stereoisomer composition of the substrate remaining after 15 min of incubation was analysed by GLC

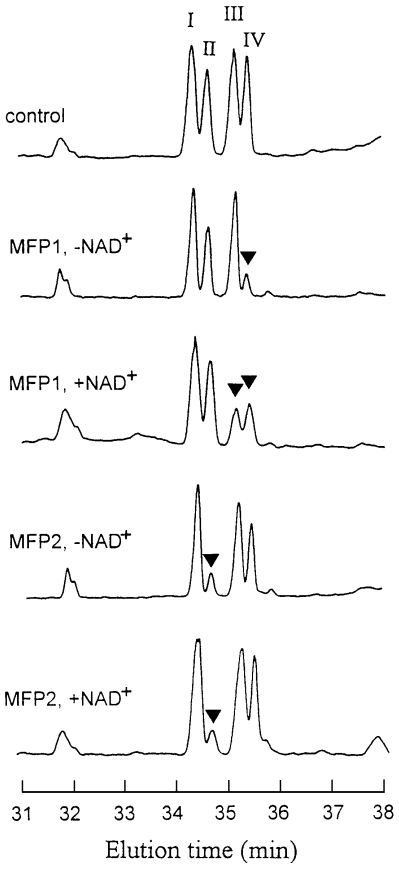


Figure 3 GLC analysis of the stereoisomers of 3-hydroxy-2-methylpalmitoyl-CoA after incubation of the racemic mixture with purified MFP1 and MFP2

Incubation of the purified MFPs with racemic 3-hydroxy-2-methylpalmitoyl-CoA and GLC analysis were performed as described in the Materials and methods section. The reaction mixture (1 ml) contained 5 μg or 50 μg of MFP-1 and 1 μg or 10 μg of MFP-2 when the incubations were performed in the absence of NAD^+ (dehydratase reaction) or in the presence of NAD^+ (dehydratase plus dehydrogenase reactions) respectively. The 2-hydroxystearoyl-CoA, added as internal standard, was resolved after derivatization in two peaks, being eluted at 41.4 and 41.7 min (results not shown). The substrate concentration was 100 μM. The arrowheads indicate stereoisomers that decreased significantly after 15 min of reaction. Stereoisomers are indicated by roman numerals.

(Figure 3). In the absence of NAD^+, when only the dehydration reaction is operative, MFP-1 metabolized stereoisomer IV exclusively, whereas MFP-2 converted stereoisomer II exclusively. Because the (de)hydration reaction is reversible, the results indicate that MFP-1 and MFP-2 also hydrate 2-methyl-2-hexadecenoyl-CoA to stereoisomers IV and II respectively and that the production of stereoisomers IV and II, as detected in purified peroxisomes incubated with racemic 2-methylpalmitoyl-CoA, is due to the action of MFP-1 and MFP-2 respectively.

In the presence of NAD^+, when both the dehydration and dehydrogenation reactions are operative, MFP-1 metabolized stereoisomers III and IV, whereas MFP-2 metabolized only stereoisomer II, as in the absence of NAD^+. These results lead us to conclude that (1) MFP-2 exclusively forms and dehydrogenates stereoisomer II, (2) MFP-1 exclusively forms stereoisomer IV, and (3) MFP-1 dehydrogenates stereoisomer III. Whether MFP-1 is capable of also dehydrogenating stereoisomer IV is not clear, however, because in the presence of NAD^+ the decrease in the area of peak IV might be due solely to the dehydratase activity or to the combined dehydratase and dehydrogenase activities.

Table 1 Specific activities of the hydratase and 3-hydroxyacyl-CoA dehydrogenase activities of the two MFPs

The two MFPs and the split-off dehydrogenase domain of MFP-2 (DH-2) occurring *in vivo* were purified as described previously [10]. Dehydratase and dehydrogenase activities were measured with the two HPLC fractions of 3-hydroxy-2-methylpalmitoyl-CoA as described in the Materials and methods section. Abbreviation: n.d., not detected.

	3-Hydroxy-2-methylpalmitoyl-CoA dehydratase activity (μmol/min per mg of protein)		3-Hydroxy-2-methylpalmitoyl-CoA dehydrogenase activity (μmol/min per mg of protein)	
Enzyme	Fraction 1	Fraction 2	Fraction 1	Fraction 2
MFP-1	n.d.	47.6	1.78	n.d.
MFP-2	n.d.	4.82	n.d.	0.09
DH-2	n.d.	n.d.	n.d.	1.11

In a subsequent experiment we therefore incubated the purified MFPs but also the purified cleaved-off 3-hydroxyacyl-CoA dehydrogenase domain of MFP-2 (see the Introduction section) with the two HPLC fractions containing isomers I and III (fraction 1, see above) and isomers II and IV (fraction 2) respectively, in the absence (dehydratase reaction) and the presence (dehydratase plus dehydrogenase reaction) of NAD^+. Dehydratase activities were measured spectrophotometrically as the appearance of a double bond at 263 nm; dehydrogenase activities were measured fluorimetrically as the reduction of NAD^+. As shown in Table 1, MFP-1 displayed dehydratase activity with fraction 2 but 3-hydroxyacyl-CoA dehydrogenase activity with fraction 1, indicating that isomer IV was used in the dehydratase reaction but not in the dehydrogenase reaction. MFP-2 showed dehydratase activity and 3-hydroxyacyl-CoA dehydrogenase activity with fraction 2 and not with fraction 1, in agreement with the evidence already presented above that MFP-2 forms and dehydrogenates stereoisomer II exclusively. In confirmation, the 3-hydroxyacyl-CoA dehydrogenase domain of MFP-2 displayed dehydrogenase activity with fraction 2 only. Interestingly, dehydrogenase rates were approx. 10-fold higher on a protein basis and 5-fold higher on a molar basis for the isolated dehydrogenase domain than for the parent enzyme. Although the reasons for this apparent discrepancy were not investigated further, one of the contributing factors might be the presence of the competing (and highly active) dehydratase activity in the parent molecule. In a similar experiment, the purified enzymes (the MFPs and the dehydrogenase domain) were incubated for 15 min with the two fractions of 3-hydroxy-2-methylpalmitoyl-CoA, with or without NAD^+; at the end of the reaction the acyl-CoA esters were extracted and analysed by GLC for the presence of the 3-hydroxy-2-methylpalmitoyl-CoA stereoisomers and 2-methyl-2-hexadecenoyl-CoA (Figure 4 and Table 2). 3-Keto derivatives (products of the dehydrogenase reaction) were not detected, probably because of degradation during the acidic extraction. In confirmation of the results described above, we observed the formation of enoyl-CoA concomitantly with a decrease in stereoisomer IV in the incubations with MFP-1 and in stereoisomer II in the incubations with MFP-2 (in the absence or presence of NAD^+). After incubation, approx. 75% of the 3-hydroxy-2-methylpalmitoyl-CoA was converted to 2-methyl-2-hexadecenoyl-CoA with MFP-1, confirming previous reports that the equilibrium of the hydration reaction lies in the direction of dehydration when 2-methyl-

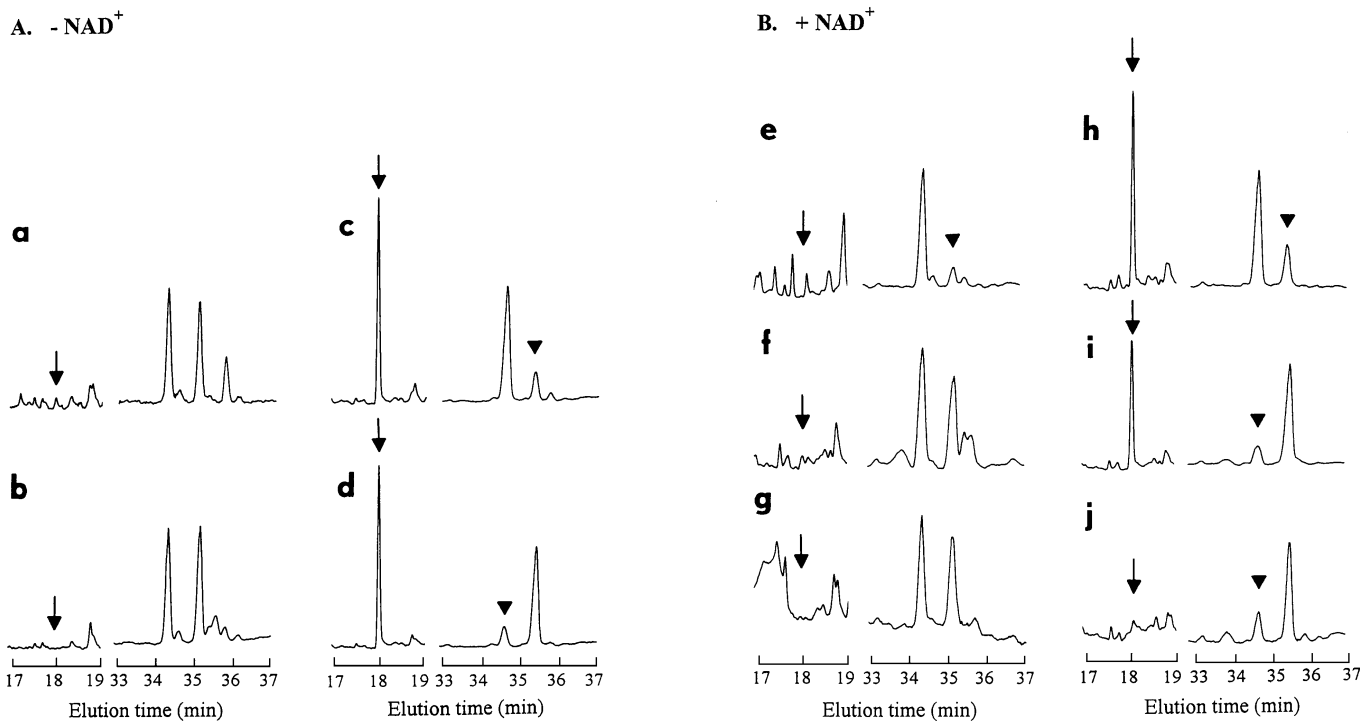


Figure 4 GLC analysis of the stereoisomers of 3-hydroxy-2-methylpalmitoyl-CoA after incubation of HPLC fractions 1 and 2 with purified MFP-1, MFP-2 and the 3-hydroxyacyl-CoA dehydrogenase domain of MFP-2 (DH-2)

(**A**) Purified MFP-1 (a,c) and MFP-2 (b,d) were incubated with the HPLC fractions 1 (a,b) and 2 (c,d) of 3-hydroxy-2-methylpalmitoyl-CoA in the absence of NAD^+. (**B**) Purified MFP-1 (e,h), MFP-2 (f,i) and DH-2 (g,j) were incubated with the HPLC fractions 1 (e–g) and 2 (h–j) in the presence of NAD^+. The incubation conditions were the same as for Figure 3, except that the substrate concentration was only 50 μM to obtain the same stereoisomer concentration. In the experiment with DH-2, 0.5 μg of protein was added. The arrows and arrowheads indicate respectively the position of the 2-methyl-2-hexadecenoyl-CoA formed in the dehydratase reaction and the stereoisomers that decreased significantly. The peaks associated with the derivatized isomers of the internal standard (2-hydroxystearoyl-CoA, elution time 41.4 and 41.7 min) are not shown.

Table 2 GLC analysis of the 3-hydroxy-2-methylpalmitoyl-CoA stereoisomers and 2-methyl-2-hexadecenoyl-CoA after incubation of purified MFP-1, MFP-2 and the dehydrogenase domain of MFP-2 (DH-2) with HPLC fractions 1 and 2

This was the same experiment as described in the legend to Figure 4. After 15 min of incubation the remaining amount of each stereoisomer and the amount of the 2-methyl-2-hexadecenoyl-CoA formed were estimated by GLC analysis. Results are expressed as percentages of the areas of peak I or II (corrected for recoveries by using the internal standard, 2-hydroxystearoyl-CoA), in HPLC fractions 1 or 2 respectively, in zero time controls. Abbreviation: n.d., not detected. Similar findings were obtained in two additional experiments.

			3-Hydroxy-2-methylpalmitoyl-CoA (%)				
			Isomers in fraction 1		Isomers in fraction 2		
Enzyme	Time (min)	NAD^+	I	III	II	IV	Enoyl-CoA (%)
Control	0	–	100	92			n.d.
					100	99	n.d.
MFP-1	15	Absent	100	92			n.d.
					96	21	71
MFP-2	15	Absent	93	93			n.d.
					18	93	71
MFP-1	15	Present	102	18			n.d.
					104	36	64
MFP-2	15	Present	111	98			n.d.
					20	108	44
DH-2	15	Present	100	95			n.d.
					19	103	n.d.

branched carboxylates {25(*E*)-trihydroxycoprostenoyl-CoA [29] or 2-methyl-2(*E*)-enoyl-CoAs [37]} are used as the substrates. Similar results were obtained with MFP-2, thus explaining the small amount of 3-hydroxy-2-methylpalmitoyl-CoA formed when peroxisomes were incubated with 2-methylpalmitoyl-CoA. In the presence of NAD^+, stereoisomer III was also metabolized

by MFP-1, presumably as a result of the dehydrogenase activity displayed towards this isomer by MFP-1 as already demonstrated above. The decrease in isomer II alone with the isolated dehydrogenase domain of MFP-2 confirms the above-described stereospecificity of the 3-hydroxyacyl-CoA dehydrogenase activity of MFP-2.

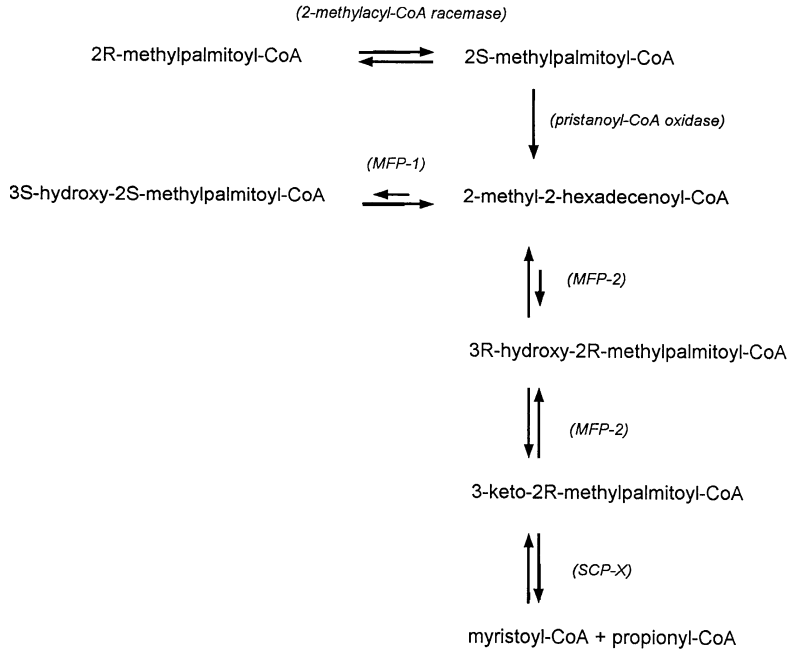
DISCUSSION

In the present study we attempted to determine which MFP is responsible for the second and third step in the peroxisomal β-oxidation of 2-methyl-branched fatty acids such as pristanic acid. Our results show that MFP-1 forms stereoisomer IV but dehydrogenates stereoisomer III of 3-hydroxy-2-methylpalmitoyl-CoA so that by itself MFP-1 is not capable of converting 2-methyl-2-hexadecenoyl-CoA into 3-keto-2-methylpalmitoyl-CoA. In contrast, MFP-2 forms and dehydrogenates stereoisomer II, indicating that MFP-2, and not MFP-1, is involved in the peroxisomal β-oxidation of 2-methyl-branched fatty acids (see Scheme 1). These results are reminiscent of the results obtained with the bile acid intermediate varanoyl-CoA. Whereas MFP-1 forms and dehydrogenates two different isomers (the 24*S*,25*S* and 24*S*,25*R* isomers respectively), MFP-2 forms and dehydrogenates the same (and physiological) 24*R*,25R isomer.

The results therefore also indicate that stereoisomer II is the naturally occurring intermediate in the peroxisomal β-oxidation of 2-methyl-branched fatty acids. Nevertheless, on incubation of purified peroxisomes with 2-methylpalmitoyl-CoA not only stereoisomer II but also stereoisomer IV of 3-hydroxy-2-methylpalmitoyl-CoA was found. Clearly, stereoisomers II and IV were formed by MFP-2 and MFP-1 respectively. One could reasonably doubt, however, whether stereoisomer IV is present *in vivo*. There is evidence that, at least in mitochondria, the β-oxidation enzymes are associated in multienzyme complexes, channelling intermediates from one enzyme to the other without releasing them into the surrounding matrix [38]. If this model holds for peroxisomes, pristanoyl-CoA oxidase (or branched-chain acyl-CoA oxidase in man) might be in close association with MFP-2, so that branched enoyl-CoAs might never have access to MFP-1. *In vitro*, association of the enzymes and intermediate channelling might be less tight, resulting in leakage and in the formation of stereoisomer IV. Alternatively, even if channelling is not occurring *in vivo*, the accumulation of stereoisomer IV would not be excessive because of the reversibility of the hydration reaction and the equilibrium of the reaction lying in the direction of dehydration (see Scheme 1).

According to our earlier results, MFP-2 exclusively forms and dehydrates D-3-hydroxyacyl-CoAs, which correspond to a 3*R* configuration in straight-chain acyl-CoAs and the 2-methyl-branched side chain of varanoyl-CoA. Thus the hydroxy group of stereoisomer II of 3-hydroxy-2-methylpalmitoyl-CoA most probably has the D- or *R*-configuration. MFP-1 is L-specific; the hydroxy groups of stereoisomers III and IV therefore most probably have the L- or *S*-configuration. Furthermore MFP-1 dehydrates 24*S*,25*S*-varanoyl-CoA and dehydrogenates 24*S*, 25*R*-varanoyl-CoA, whereas MFP-2 dehydrates and dehydrogenates 24*R*,25*R*-varanoyl-CoA. From the combination of these results we deduce that the physiological intermediate in branched fatty acid oxidation has the 3*R*,2*R* configuration (stereoisomer II), whereas the other stereoisomers have the 3*R*,2*S* (stereoisomer I), 3*S*,2*R* (stereoisomer III) and 3*S*,2*S* (stereoisomer IV) configurations.

Finally, our recent findings [11,12] and our present results that MFP-2, and not MFP-1, is involved in the synthesis of bile acids and in the degradation of 2-methyl-branched fatty acids will



Scheme 1 Reactions catalysing the peroxisomal β-oxidation of 2-methyl-branched fatty acids

SCP-X is sterol carrier protein X, shown by Seedorf et al. [39] to be a peroxisomal 3-oxoacyl-CoA thiolase. According to recent results from our laboratory ([40], and V. Antonenkov, G. P. Mannaerts and P. P. Van Veldhoven, unpublished work), SCP-X is the only peroxisomal thiolase that cleaves the 3-ketoacyl-CoA derivatives of 2-methyl-branched fatty acids and the bile acid intermediate trihydroxycoprostanic acid.

necessarily result in a reinterpretation of the molecular defects in a number of cases of human peroxisomal β-oxidation deficiencies.

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