Stereochemistry of the α-oxidation of 3-methyl-branched fatty acids in rat liver

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Abstract The stereochemistry of the α-oxidation of 3-methyl-branched fatty acids was studied in rat liver. *R*- and *S*-3-methylhexadecanoic acid were equally well α-oxidized in intact hepatocytes and homogenates. Subcellular fractionation studies showed that α-oxidation of both isomers is confined to peroxisomes. Dehydrogenation of 2-methylpentadecanal, the end-product of the peroxisomal α-oxidation of 3-methylhexadecanoic acid, to 2-methylpentadecanoic acid, followed by derivatization with *R*-1-phenylethylamine and subsequent separation of the stereoisomers by gas chromatography, revealed that the configuration of the methyl-branch is preserved throughout the whole α-oxidation process. Metabolism and formation of the 2-hydroxy-3-methylhexadecanoyl-CoA intermediate were also investigated. Separation of the methyl esters of the four isomers of 2-hydroxy-3-methylhexadecanoic acid was achieved by gas chromatography after derivatization of the hydroxy group with *R*-2-methoxy-2-trifluoromethylphenylacetic acid chloride and the absolute configuration of the four isomers was determined. Although purified peroxisomes are capable of metabolizing all four isomers of 2-hydroxy-3-methylhexadecanoyl-CoA, they can only form the (2*S*,3*R*) and the (2*R*,3*S*) isomers. Our experiments exclude the racemization of the 3-methyl branch during the α-oxidation process. The configuration of the 3-methyl branch does not influence the rate of α-oxidation, but determines the side of the 2-hydroxylation, hence the configuration of the 2-hydroxy-3-methylacyl-CoA intermediates formed during the process.—Croes, K., M. Casteels, M. Dieuaide-Noubhani, G. P. Mannaerts, and P. P. Van Veldhoven. **Stereochemistry of the α-oxidation of 3-methyl-branched fatty acids in rat liver. *J. Lipid Res.* 1999.** 40: **601–609.**

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3-Methyl-branched fatty acids such as the naturally occurring phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) or the synthetic 3-methylhexadecanoic acid cannot undergo β-oxidation due to the presence of a methyl-group in the 3-position. They are first shortened by one carbon atom via α-oxidation, which for a long time has been the subject of controversy as to its subcellular localization, the intermediates involved, and the cofactors required. It is now clearly established that α-oxidation in the rat is a peroxisomal process during which the fatty acid is first activated, requiring ATP, Mg^{2+}, and CoA, and then 2-hydroxylated, requiring Fe^{2+}, 2-oxoglutarate, and ascorbate in the presence of O_2 (1–4). The resulting 2-hydroxy-3-methylacyl-CoA intermediate is cleaved into formyl-CoA (5) and a 2-methyl fatty aldehyde (6, 7). Formyl-CoA is hydrolyzed to formate, which is then converted to CO_2 (5). In an NAD^+-dependent process, the 2-methyl fatty aldehyde is converted to the corresponding 2-methyl fatty acid (6, 7), which is then further degraded mainly by peroxisomal β-oxidation (8–12). In the human, the conversion of the 2-methyl fatty aldehyde to the 2-methyl fatty acid may take place in the endoplasmic reticulum (13).

Naturally occurring phytanic acid is racemic at position 3 while its α-oxidation product pristanic acid (2, 6, 10, 14-tetramethylpentadecanoic acid) is racemic at position 2 (14, 15), suggesting that both stereoisomers of phytanic acid can undergo α-oxidation without a preceding racemization of the initial 3-methyl branch. Studies (16, 17) carried out with 3-methyl-branched fatty acids tritium-labeled in position 3 show that the label at C-3 is retained, which also supports this contention. This would, however, contrast with the peroxisomal β-oxidation of 2-methyl-branched compounds such as pristanic acid and trihydroxycoprostanic acid, where only the *S*-isomer can be degraded (18, 19). The *R*-isomer is therefore racemized to the *S*-isomer and this occurs at the level of the CoA-ester by a 2-methylacyl-CoA racemase (20).

If no racemization of the 3-methyl branch occurs, this would imply that, due to the introduction of an additional asymmetric centre during 2-hydroxylation, theoretically

Abbreviations: GC, gas chromatography; HPLC, high pressure liquid chromatography; MS, mass spectrometry; MTPA, *R*-2-methoxy-2-trifluoromethylphenylacetic acid chloride; Tris, tris(hydroxymethyl) aminomethane.

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four isomers of the 2-hydroxy-3-methylacyl-CoA intermediate can occur. However, a complete lack of stereospecificity seems unlikely for a biochemical process. The stereospecificity of the α-oxidation process has already been investigated by Tsai et al. (21) in heavy mitochondria. Using the amount of CO_2 produced as a measure for α-oxidation, they concluded that both isomeric forms of phytanic acid are α-oxidized. They also demonstrated that the introduction of the hydroxyl-group in the 2-position is highly stereospecific, but did not establish the absolute configurations of the 2-hydroxy intermediates formed. However, recently it has been clearly shown in rat liver that α-oxidation is a peroxisomal and not a mitochondrial process (1, 2), and that the overall process should be assessed by the measurement of both formate and CO_2 (2). In addition, the set of cofactors used by Tsai et al. (21) did not include all the cofactors recently shown to be required for α-oxidation (1, 2).

We therefore decided to reinvestigate the stereospecificity of the α-oxidation of 3-methyl-branched fatty acids, using synthetic *rac*, *R*-, and *S*-3-methylhexadecanoic acid as the substrates.

MATERIALS AND METHODS

Materials

2-Hydroxyhexadecanoic and 2-hydroxyoctadecanoic acids were from Larodan AA. *L*-, *D*-, *D-allo*-2-hydroxy-3-methylvaleric acid, and diethyl cyanophosphonate were from Sigma. *R*-2-methoxy-2-trifluoromethylphenylacetic acid chloride (MTPA), *R*-1-phenylethylamine, and triethylamine were from Fluka, Aldrich, and Pierce, respectively.

Synthesis of substrates

The *R*- and *S*-isomers of 2-methylpentadecanoic acid and of unlabeled and 1-^{14}C-labeled 3-methylhexadecanoic acid were synthesized as described previously by Van Veldhoven et al. (18) and Croes et al. (2), respectively. Unlabeled and 1-^{14}C-labeled 2-hydroxy-3-methylhexadecanoic acid and 2-hydroxy-3-methylhexadecanoyl-CoA were synthesized as described before (6).

Rac 3-methylheptadecanoic acid (22) and 2-hydroxyoctadecanoyl-CoA (23), used as internal standards, were prepared as described previously.

Animals

The experiments were performed with overnight-fasted male Wistar rats weighing between 120 and 200 g.

Incubation and extraction procedures

α-Oxidation of 3-methylhexadecanoic acid. Intact hepatocytes were prepared (24) and incubated (25) as described previously. Final substrate concentrations were 0.2 mm *R*- or *S*-3-methyl[1-^{14}C] hexadecanoic acid (specific radioactivity 0.9 Ci/mol), except for the substrate concentration curve.

Liver homogenates and subcellular fractions were prepared as described before (2). Incubations (37°C) were started by adding 100 μl of homogenate, corresponding to 12.5 mg of tissue, or appropriately diluted subcellular fractions to 400 μl of reaction medium containing final concentrations of 100 mm KCl, 50 mm tris (hydroxymethyl)aminomethane (Tris) (pH 7.5), 0.025 mm defatted albumin, 4 mm ATP, 2.4 mm $MgCl_2$, 0.2 mm CoA, 0.1 mm $FeCl_2$, 3 mm 2-oxoglutarate, and 10 mm l-ascorbate, referred to in the text as standard α-oxidation conditions. Final substrate concentrations were 0.05 mm *R*- or *S*-3-methyl[1-^{14}C]hexadecanoic acid (specific radioactivity 0.9 and 6.8 Ci/mol for incubation with homogenates and subcellular fractions, respectively).

All reactions were terminated after 10 min with 250 μl of 6% (w/v) perchloric acid, and CO_2 and formate were determined as described before (25).

Determination of isomers of 2-hydroxy-3-methylhexadecanoyl-CoA formed during α-oxidation of 3-methylhexadecanoic acid. Purified peroxisomes were prepared and incubated (300 to 800 μg protein/assay) as described before (5). Substrate concentrations were 0.05 mm *rac*, *R*- or *S*-3-methyl[1-^{14}C]hexadecanoic acid (specific radioactivity 6.8 Ci/mol) or 0.05 mm 2-hydroxy-3-methylhexadecanoyl-CoA. Reactions were terminated after 10 min with 100 μl of glacial acetic acid and, after addition of [^{3}H]hexadecanoyl-CoA as an internal standard, the CoA esters were extracted and analyzed by high pressure liquid chromatography (HPLC) as described previously (2).

To duplicate samples, used for gas chromatography (GC) analysis of the isomers of 2-hydroxy-3-methylhexadecanoyl-CoA formed from 3-methylhexadecanoic acid, no internal standard was added. The 2-hydroxy-3-methylhexadecanoyl-CoA peak was isolated from the acyl-CoA fraction by HPLC (2) and subjected to alkaline hydrolysis. After acid extraction with diethylether, the fatty acids were derivatized and analyzed as described below for 2-hydroxy fatty acids.

Determination of the configuration of 2-methylpentadecanal formed during α-oxidation of 3-methylhexadecanoic acid. Purified peroxisomes were incubated with *rac*, *R*- or *S*-3-methylhexadecanoic acid as described in the previous paragraph, except that the concentration of defatted albumin was 0.010 mm, reaction volumes were doubled, and incubations were terminated after 15 min in order to obtain a higher amount of 2-methylpentadecanal. The reaction was terminated with 1 ml of methanol containing 2% (v/v) acetic acid, and 2-methylpentadecanal was extracted with 3 ml of hexane. After evaporation of the solvent (under N_2 and on ice), the 2-methylpentadecanal was redissolved in 20 μl of tetrahydrofuran and 880 μl of reaction medium containing final concentrations of 100 mm KCl, 50 mm Tris (pH 7.5), 0.005 mm defatted albumin, and 2 mm NAD^+ was added. The 2-methylpentadecanal present was converted to 2-methylpentadecanoic acid by incubation (37°C) with 100 μl of purified peroxisomes for 15 min. After termination of the reaction with 100 μl of glacial acetic acid and addition of *rac* 3-methylheptadecanoic acid (10 nmol) as an internal standard, the fatty acids were extracted, derivatized, and analyzed as described below for 2- and 3-methyl-branched fatty acids.

Cleavage of 2-hydroxy-3-methylhexadecanoyl-CoA. Incubations (37°C) were started by addition of 100 μl of purified peroxisomes (100 μg of protein) to 400 μl of reaction medium containing final concentrations of 100 mm KCl, 50 mm Tris (pH 7.5), 0.010 mm defatted albumin, and 0.05 mm *rac* 2-hydroxy-3-methyl[1-^{14}C] hexadecanoyl-CoA. Reactions were terminated after 10 min with 250 μl of 6% (w/v) perchloric acid for the measurement of formate production (25).

For the determination of the isomers of the residual 2-hydroxy-3-methyl[1-^{14}C]hexadecanoyl-CoA, duplicate samples were acidified with 100 μl of glacial acetic acid instead of perchloric acid and *rac* 2-hydroxyoctadecanoyl-CoA (12.5 nmol) was added as internal standard. After alkaline hydrolysis and acid extraction, the fatty acids were derivatized and analyzed as described below for 2-hydroxy fatty acids.

Measurement of L-2-hydroxy acid oxidase activity. Rat kidney cortex homogenates were prepared in 0.25 m sucrose containing 0.1% (v/v) ethanol and 5 mm 3-(N-morpholino)propanesulphonic acid (pH 7.2), by five strokes up and down of a loose and tight-fitting pestle in a Dounce homogenizer. *L*-2-hydroxy acid

oxidase activity was measured essentially as described before (26) with some minor modifications. Final assay concentrations were 1 mm NaN_3, 1 μm FMN, 50 mm pyrophosphate (pH 8.0), 8 mm tribromohydroxybenzoate (sodium salt), 2 mm 4-aminoantipyrine, 16 U/ml horseradish peroxidase, and 0.2% (w/v) Triton X-100. Substrates were *D*-, *D-allo*-, *L*-2-hydroxy-3-methylvaleric acid (sodium salt, final concentration 1 mm), *rac* 2-hydroxyhexadecanoic acid and *rac* 2-hydroxy-3-methylhexadecanoic acid (final concentrations 0.025 to 0.5 mm for both).

Stock solutions (5 mm) of 2-hydroxyhexadecanoic acid and 2-hydroxy-3-methylhexadecanoic acid were prepared in 2% (w/v) Thesit. A final concentration of 0.2% (w/v) Thesit did not influence the oxidase activity as tested with *L*-2-hydroxy-3-methylvaleric acid as the substrate.

Determination of the L-isomer(s) of 2-hydroxy fatty acids. Incubations (37°C) were started by addition of 40 μl of rat kidney cortex homogenate to 200 μl of reaction medium containing final concentrations of 50 mm pyrophosphate (pH 8.0), 20 μm FAD, 0.2% (w/v) Triton X-100, and 0.5 mm *rac* 2-hydroxy fatty acid (2-hydroxyhexadecanoic acid or 2-hydroxy-3-methylhexadecanoic acid, solubilized in 0.5% (w/v) Tween 20 as Thesit interfered with the GC analysis). Reactions were terminated after 20 min with 50 μl of glacial acetic acid. After addition of *rac* 2-hydroxyoctadecanoic acid (125 nmol) as an internal standard, the fatty acids were extracted, derivatized, and analyzed as described below for 2-hydroxy fatty acids.

Analytical procedures

Derivatization and separation of the stereoisomers of 2- and 3-methyl-branched fatty acids. Standards or dried residues of extracts containing 2- and 3-methyl-branched fatty acids were dissolved in 60 μl of dimethylformamide to which 60 μl of 2 mm *R*-1-phenylethylamine (20), 60 μl of 2 mm diethyl cyanophosphonate (27), and 60 μl of 2 mm triethylamine were added subsequently. After incubation overnight at room temperature, 2 ml of chloroform was added to the mixture. Subsequently, the chloroform solution was extracted with 1 ml of 10 mm NaOH, 1 ml of 10 mm HCl (twice), and 1 ml of H_2O, and then dried under nitrogen. The residue was reconstituted in hexane, an aliquot of which was analyzed by gas chromatography–mass spectrometry (GC–MS) (GCQ Finnigan; capillary column: 25 m × 0.25 mm i.d. CP-sil 5 CB-MS, film thickness 0.25 μm (Chrompack); column temperature: 80°C for 0.5 min; 80–248°C at a rate of 20°C/min, 248°C for 10 min, 248–280°C at a rate of 5°C/min; injection port and GC–MS interface at 310 and 300°C, respectively; ionization energy: 70 eV; source temperature: 175°C). The mass spectra were recorded in full scan mode, but for interpretation single ion mode was used at *m/z* 359, 373, and 387, the masses of the molecular ions of the phenylethylamide derivatives of 2-methylpentadecanoic, 3-methylhexadecanoic, and 3-methylheptadecanoic acid, respectively.

Derivatization and separation of the stereoisomers of 2-hydroxy fatty acids. Standards or dried residues of extracts containing 2-hydroxy fatty acids were methylated with acidic methanol and then converted to their *R*-2-methoxy-2-fluoromethylphenylacetyl derivative (28) as follows. The dried 2-hydroxyacyl methyl esters were incubated for 3 h in a heating block at 60°C with 1 μl of MTPA in 20 μl of pyridine. After the reaction the samples were dried, dissolved in hexane, and dried again. The residues were then redissolved in hexane, an aliquot of which was subjected to GC analysis for separation and detection of the stereoisomers of the 2-hydroxy acid derivatives (Pye Unicam PU 4550 gas chromatograph; on-column injection; capillary column: 30 m × 0.32 mm Econocap EC-1 (SE-30), film thickness 0.25 μm (Alltech); column temperature: 80–200°C at a rate of 10°C/min, 200–300°C at a rate of 2°C/min; flame ionization detection, detector temperature: 300°C).

RESULTS

R- and *S*-3-methylhexadecanoic acid are equally well α-oxidized in isolated rat hepatocytes, rat liver homogenates, and subcellular fractions

α-Oxidation rates (CO_2 + formate) obtained in intact rat hepatocytes were 23.3 and 25.1 nmol·(10^8 cells)$^{-1}$·min^{-1} with 0.2 mm *R*- and *S*-3-methylhexadecanoate as the substrates, respectively (**Fig. 1**). Figure 1 also shows that in intact cells for both isomers the same time and substrate concentration dependency is observed.

As in intact rat hepatocytes, comparable α-oxidation rates (CO_2 + formate) were obtained for both isomers in rat liver homogenates incubated under standard conditions (23.2 versus 27.5 nmol·(g liver)$^{-1}$·min^{-1} for *R*- and *S*-3-methylhexadecanoate, respectively).

In subcellular fractions obtained by differential centrifugation and incubated with *R*- or *S*-3-methylhexadecanoate under standard conditions, production of formate + CO_2 showed the same distribution as the peroxisomal marker enzyme catalase (**Fig. 2**), demonstrating that the α-oxidation of each isomer is confined to peroxisomes.

No racemization of the 3-methyl branch during peroxisomal α-oxidation

In order to investigate whether a racemase exists that acts on free or activated 3-methyl-branched fatty acids, as is the case for activated 2-methyl-branched fatty acids

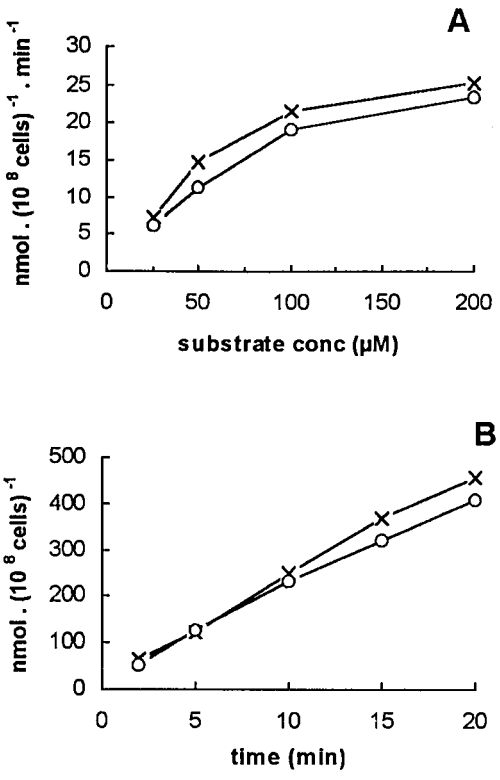

Fig. 1. α-Oxidation of *R*- and *S*-3-methylhexadecanoic acid in intact hepatocytes. Rates of α-oxidation (CO_2 + formate) were measured in isolated intact hepatocytes incubated with 0.2 mm (panel B) or a varying concentration (panel A) of *R*- (○) or *S*- (×) 3-methylhexadecanoic acid. Reactions were stopped after 10 min (panel A).

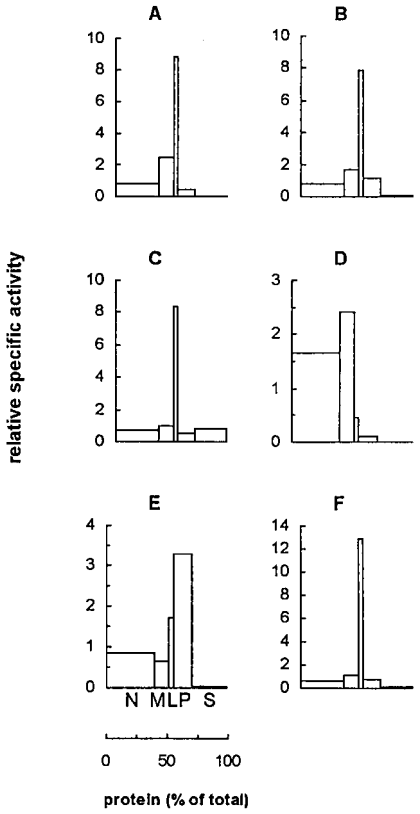

Fig. 2. Subcellular distribution of the production of CO_2 plus formate in rat liver incubated with *R*- or *S*-3-methylhexadecanoic acid. A fresh rat liver homogenate was fractionated by differential centrifugation into a nuclear (N), a heavy mitochondrial (M), a light mitochondrial (L), a microsomal (P), and a cytosolic (S) fraction. The fractions were incubated in the presence of ATP (4 mm), Mg^{2+} (2.4 mm), CoA (0.2 mm), Fe^{2+} (0.1 mm), 2-oxoglutarate (3 mm), and sodium ascorbate (10 mm) with 0.05 mm *R*- or *S*-3-methylhexadecanoic acid. Incubations were stopped after 10 min and the production of CO_2 and formate was measured. (A) and (B) show the sum of CO_2 and formate measured after incubation with *R*- and *S*-3-methylhexadecanoic acid, respectively. Protein and marker enzymes were determined in each fraction: (C) catalase (peroxisomal matrix); (D) glutamate dehydrogenase (mitochondria); (E) glucose-6-phosphatase (endoplasmic reticulum), and (F) acid phosphatase (lysosomes). Results are expressed as relative specific activity versus cumulative percentage of protein. Relative specific activity is defined as the percentage of recovered activity in a particular fraction, divided by the percentage of recovered protein in that fraction. Recoveries for the production of CO_2 plus formate were 42 and 47% for *R*- and *S*-3-methylhexadecanoic acid, respectively. Recoveries for protein and marker enzymes varied between 85 and 105%.

(20), purified peroxisomes were incubated with *R*- or *S*-3-methylhexadecanoic acid in the absence and the presence of ATP, CoA, and Mg^{2+}. After alkaline hydrolysis, the fatty acids were extracted and derivatized with *R*-1-phenylethylamine as described above. GC analysis of the derivatized fatty acids (data not shown, but see Fig. 3) showed that there was no conversion of one isomer to the other, suggesting that there is no peroxisomal racemase that acts on 3-methyl branched fatty acids or their CoA-esters.

To exclude the involvement of a 3-methyl racemase unequivocally, we determined the stereochemical configuration of the 2-methyl-branched fatty aldehyde resulting from the α-oxidation process, after dehydrogenation to the corresponding acid. This experiment was done in two steps, because addition of NAD^+, required for the conversion of the 2-methyl-branched aldehyde to its corresponding acid, to the initial standard incubation mixture containing ATP, Mg^{2+}, and CoA could lead to the formation of 2-methylacyl-CoA. As rat liver peroxisomes contain a 2-methylacyl-CoA racemase (26), a one-step experiment could have led to erroneous results. Therefore, purified peroxisomes were incubated under standard conditions with *rac*, *R*-, or *S*-3-methylhexadecanoic acid. The resulting 2-methylpentadecanal was extracted, dissolved in tetrahydrofuran, and reincubated with purified peroxisomes in the presence of NAD^+ in order to convert 2-methylpentadecanal to 2-methylpentadecanoic acid. After addition of *rac* 3-methylheptadecanoic acid as an internal standard, the resulting 2-methylpentadecanoic acid was extracted, derivatized with *R*-1-phenylethylamine, and then subjected to GC analysis. **Figure 3** shows that dehydrogenation of the 2-methylpentadecanal formed in purified peroxisomes incubated with *rac* 3-methylhexadecanoic acid resulted in the formation of two peaks, one eluting at about 14.1 min and the other at 15.0 min. The peaks coeluted with a standard of derivatized *rac* 2-methylpentadecanoic acid and showed a peak in the mass spectrum at *m*/*z* 359, the molecular ion of 2-methylpentadecanoylphenylethylamide. When purified peroxisomes were incubated with *R*- or *S*-3-methylhexadecanoic acid, two peaks were found at approximately 14.1 and 15.0 min, respectively, and they coeluted with a standard of *R*-2-methylpentadecanoylphenylethylamide and *S*-2-methylpentadecanoylphenylethylamide, respectively (Fig. 3). These results unequivocally prove that no racemization of the 3-methyl branch occurs during peroxisomal α-oxidation.

Separation of the four isomers of 2-hydroxy-3-methylhexadecanoyl-CoA

As no racemization of the 3-methyl branch occurs, theoretically four isomers of 2-hydroxy-3-methylhexadecanoyl-CoA can be formed during α-oxidation of racemic 3-methyl branched fatty acids. Reverse-phase HPLC resolved synthetic *rac* 2-hydroxy-3-methylhexadecanoyl-CoA in two peaks (**Fig. 4**, panel B). When peroxisomes were incubated with *rac* 3-methylhexadecanoic acid under standard conditions, HPLC of the CoA-ester fraction revealed that the physiologically formed 2-hydroxy-3-methylhexadecanoyl-CoA (Fig. 4, panel C) coeluted with the second peak of the synthetic *rac* 2-hydroxy-3-methylhexadecanoyl-CoA (Fig. 4, panel D).

The development of an analytical method capable of separating the four isomers was indispensable to determine which isomers were physiologically formed. Separation of the methyl esters of the four isomers of 2-hydroxy-3-methylhexadecanoic acid was achieved by GC after derivatization of the hydroxy group with the chiral compound MTPA. Based on their order of elution during GC analysis, the stereoisomers were designated I–IV (**Fig. 5**). Analysis of the two peaks collected after HPLC revealed that the first HPLC peak consisted of the stereoisomers I and III and the second HPLC peak of stereoisomers II and IV (results

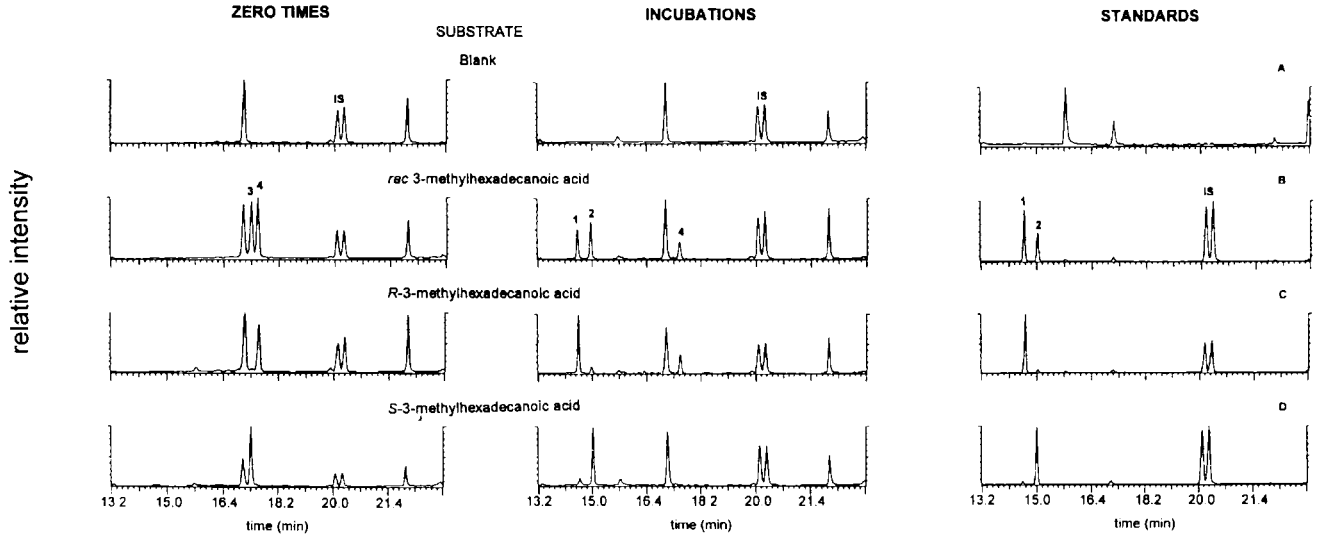

Fig. 3. GC–MS analysis of the stereoisomers of 2-methylpentadecanoic acid formed during peroxisomal α-oxidation of *rac*, *R*- and *S*-3-methylhexadecanoic acid. Purified peroxisomes were incubated for 0 (left panel) or 10 min (middle panel) without (blank) or with *rac*, *R*- or *S*-3-methylhexadecanoic acid under standard α-oxidation conditions. After extraction, the resulting 2-methylpentadecanal was converted to 2-methylpentadecanoic acid by reincubation with purified peroxisomes in the presence of NAD^+. The fatty acids were extracted after addition of *rac* 3-methylheptadecanoic acid (internal standard, IS), derivatized with *R*-1-phenylethylamine and subjected to GC–MS analysis. The mass spectra were recorded in full scan, but for interpretation single ion mode was used at *m*/*z* 359, 373, and 387. Elution of standards is shown in the right panel: (A) blank, (B) *rac* 2-methylpentadecanoylphenylethylamide, (C) *R*-2-methylpentadecanoylphenylethylamide, and (D) *S*-2-methylpentadecanoylphenylethylamide. The numbered peaks indicate 1: *R*-2-methylpentadecanoylphenylethylamide; 2: *S*-2-methylpentadecanoylphenylethylamide; 3: *S*-3-methylhexadecanoylphenylethylamide; and 4: *R*-3-methylhexadecanoylphenylethylamide.

not shown). Taken together these results indicate that maximally two of the four possible isomers of 2-hydroxy-3-methylhexadecanoyl-CoA, i.e., isomers II and IV, are formed in peroxisomes incubated with *rac* 3-methylhexadecanoic acid. In order to investigate this further, purified peroxisomes were incubated with *R*- or *S*-3-methylhexadecanoic acid under standard conditions. The CoA-esters of triplicate samples were extracted and analyzed by HPLC. The 2-hydroxy-3-methylhexadecanoyl-CoA peak was collected, hydrolyzed, methylated, and derivatized with MTPA. Figure 5 shows that isomers II and IV are formed during α-oxidation of *R*- and *S*-3-methylhexadecanoic acid, respectively, which confirms the results of the HPLC-analysis (see above).

However, it might still be possible that all four isomers of 2-hydroxy-3-methylhexadecanoyl-CoA are formed, but that only isomers I and III are further metabolized, resulting in an accumulation of isomers II and IV. In order to verify this possibility, purified peroxisomes were incubated with synthetic *rac* 2-hydroxy-3-methyl[1-^{14}C]hexadecanoyl-CoA. After termination of the reaction and addition of 2-hydroxyoctadecanoyl-CoA as an internal standard, the samples were hydrolyzed and the fatty acids were extracted, methylated, derivatized with MTPA, and analyzed by GC. As can be seen from **Table 1** peroxisomes are capable of metabolizing all four isomers of 2-hydroxy-3-methyl[1-^{14}C]hexadecanoyl-CoA, although each isomer seems to be metabolized to a different extent. Whether this variation reflects a difference in the affinity for the different isomers or is simply due to the different amount of each isomer initially present could not be demon-

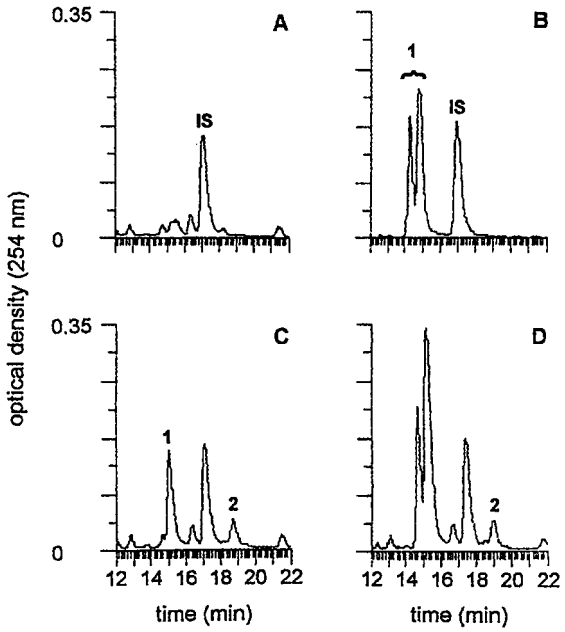

Fig. 4. HPLC analysis of the 2-hydroxy-3-methylhexadecanoyl-CoA intermediate formed during peroxisomal α-oxidation of *rac* 3-methylhexadecanoic acid. Purified peroxisomes were incubated for 0 min with *rac* 2-hydroxy-3-methylhexadecanoyl-CoA (trace B) and for 10 min without (trace A) or with (trace C and D) *rac* 3-methylhexadecanoic acid under standard α-oxidation conditions. After addition of hexadecanoyl-CoA (internal standard, IS), the acyl-CoAs were extracted and subjected to HPLC analysis without (trace A, B, and C) or with the addition of 7.5 nmol synthetic *rac* 2-hydroxy-3-methylhexadecanoyl-CoA (trace D). The numbered peaks indicate 1: 2-hydroxy-3-methylhexadecanoyl-CoA and 2: 3-methylhexadecanoyl-CoA.

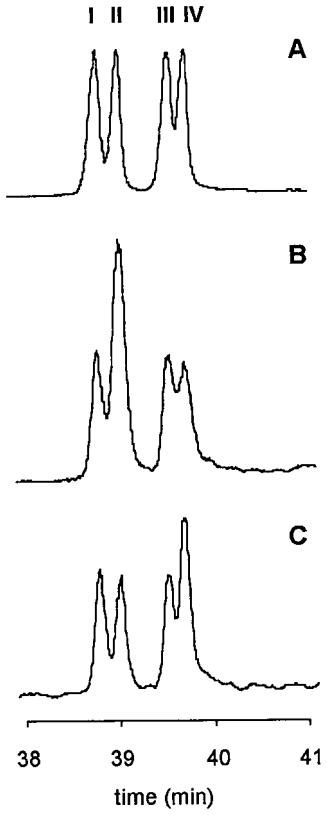

Fig. 5. GC analysis of the 2-hydroxy-3-methylhexadecanoyl-CoA stereoisomers formed during peroxisomal α-oxidation of *R*- and *S*-3-methylhexadecanoic acid. Purified peroxisomes were incubated for 10 min with *R*- or *S*-3-methylhexadecanoic acid under standard α-oxidation conditions. The acyl-CoA esters were extracted and analyzed by HPLC. The 2-hydroxy-3-methylhexadecanoyl-CoA peak was collected and hydrolyzed. The resulting 2-hydroxy-3-methylhexadecanoic acid was extracted, methylated, and derivatized with *R*-2-methoxy-2-trifluoromethylphenylacetic acid chloride. Trace A shows a standard of derivatized synthetic *rac* 2-hydroxy-3-methylhexadecanoic acid. In trace B and C the standard of derivatized synthetic *rac* 2-hydroxy-3-methylhexadecanoic acid is superimposed on the stereoisomer of 2-hydroxy-3-methylhexadecanoic acid formed during α-oxidation of *R*- and *S*-3-methylhexadecanoic acid, respectively.

strated. Indeed, although the synthetic 2-hydroxy-3-methylhexadecanoic acid contained the four isomers in equal amounts (see also Fig. 5, panel A), the synthetic *rac* 2-hydroxy-3-methylhexadecanoyl-CoA contained 18, 34, 16, and 32% of isomers I, II, III, and IV, respectively, probably due to a stereoselectivity during the activation reaction in the synthesis. The amount of 2-hydroxy-3-methylhexadecanoyl-CoA metabolized was well in agreement with the amount of formate formed during the reaction (10.9 versus 10.3 nmol, respectively, see Table 1), indicating that the decrease of the four isomers of 2-hydroxy-3-methylhexadecanoyl-CoA was indeed due to metabolism through the α-oxidation pathway and not through another process.

Determination of the configuration of the hydroxy group of 2-hydroxy-3-methylhexadecanoic acid

Rat kidney cortex contains *L*-α-hydroxyacid oxidase which catalyzes the oxidation of (long chain) *L*-α-hydroxy-

TABLE 1. Metabolism of 2-hydroxy-3-methylhexadecanoyl-CoA in purified peroxisomes

Reaction Time	2-Hydroxy-3-methylhexadecanoic Acid Recovered					Formate Formed
	Peak 1	Peak 2	Peak 3	Peak 4	Total I–IV	
min	*nmol*					*nmol*
0	3.4	6.4	3.0	6.0	18.8	0
10	1.5	1.9	2.0	2.5	7.9	10.3

Purified peroxisomes were incubated for 10 min with *rac* 1-^{14}C-labeled 2-hydroxy-3-methylhexadecanoyl-CoA and the production of formate was measured. After addition of 2-hydroxyoctadecanoyl-CoA (internal standard) and alkaline hydrolysis, the fatty acids of duplicate samples were extracted, methylated, derivatized with *R*-2-methoxy-2-trifluoromethylphenylacetic acid chloride and subjected to GC-analysis. The amount of each stereoisomer of 2-hydroxy-3-methylhexadecanoic acid was calculated by using the internal standard.

acids to produce the corresponding α-ketoacids and hydrogen peroxide (29). In order to check whether the oxidase retains its activity towards a 2-hydroxy acid containing a 3-methyl branch, rat kidney cortex homogenate was incubated with 2-hydroxy-3-methylhexadecanoic acid and the production of hydrogen peroxide was measured. Hydrogen peroxide formation in the presence of 2-hydroxyhexadecanoic acid was measured as a control. The apparent V_{max} and K_m values obtained for *rac* 2-hydroxy-3-methylhexadecanoic acid were 0.74 U/g kidney cortex and 0.32 mm, respectively, as compared to 1 U/g kidney cortex and 0.13 mm for *rac* 2-hydroxyhexadecanoic acid, suggest-

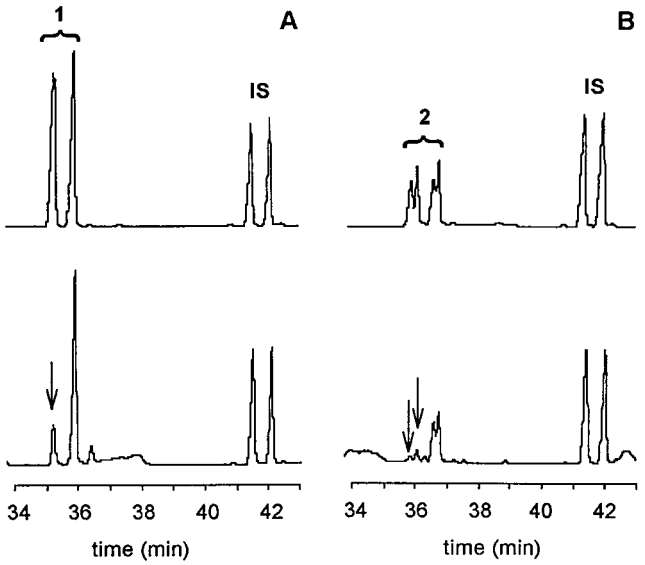

Fig. 6. GC analysis of the stereoisomers of 2-hydroxy-3-methylhexadecanoic acid after incubation with rat kidney cortex homogenate. Rat kidney cortex homogenate was incubated with 2-hydroxyhexadecanoic acid (panel A) and 2-hydroxy-3-methylhexadecanoic acid (panel B). After termination of the reaction at 0 (top trace) or 10 min (bottom trace), *rac* 2-hydroxyoctadecanoic acid was added as an internal standard (IS), the fatty acids were extracted, methylated, derivatized with *R*-2-methoxy-2-trifluoromethylphenylacetic acid chloride, and analyzed by GC. The numbered peaks indicate 1:2-hydroxyhexadecanoic acid and 2: 2-hydroxy-3-methylhexadecanoic acid. The arrows indicate the stereoisomers that are decreased upon incubation.

ing that the 3-methyl branch hinders the oxidase reaction only moderately.

Whether the presence of the 3-methyl branch alters the stereoselectivity of the *L*-α-hydroxyacid oxidase was investigated by incubating rat kidney cortex homogenate with *L*-, *D*-, and *D-allo*-2-hydroxy-3-methylvaleric acid followed by measurement of the production of hydrogen peroxide. The *L-allo* isomer was not commercially available. At a substrate concentration of 1 mm, the oxidase activity was 1.0 U/g kidney cortex with *L*-2-hydroxy-3-methylvaleric acid, while the oxidase activity with both *D*-isomers was negligible. These results demonstrate that the presence of a 3-methyl group does not alter the selectivity of the oxidase towards the *L*-isomer of the 2-hydroxy acids.

Consequently, the configuration of the 2-hydroxy group in the four isomers of 2-hydroxy-3-methylhexadecanoic acid can be determined by incubating *rac* 2-hydroxy-3-methylhexadecanoic acid with rat kidney cortex homogenate. *Rac* 2-hydroxyhexadecanoic acid was used as a control. After termination of the reaction by acidification, 2-hydroxyoctadecanoic acid was added as an internal standard. The fatty acids were then extracted, methylated, derivatized with MTPA, and analyzed by GC. When 2-hydroxyhexadecanoic acid was incubated with rat kidney cortex homogenate, the isomer that eluted first from the GC column disappeared, indicating that this is the *L*-isomer of 2-hydroxyhexadecanoic acid (**Fig. 6**, panel A). This finding contrasts with the assumption of Beneytout, Tixier, and Rigaud (28) that for 2-hydroxy fatty acid enantiomers the *D*-form is the first compound to be eluted. For 2-hydroxy-3-methylhexadecanoic acid, the first two peaks disappeared upon incubation, demonstrating that peaks I and II are the two *L*-2-hydroxy isomers, while peaks III and IV are the two *D*-2-hydroxy isomers (Fig. 6, panel B).

Following the Cahn-Ingold-Prelog convention (*R*,*S*-convention) (30), *L*-2-hydroxy fatty acids and *D*-2-hydroxy fatty acids can be assigned the 2*S*- and 2*R*-configurations, respectively. Taken together with the data discussed in the preceding sections, these results lead to the conclusion that the absolute configurations of isomer II and IV are (2*S*,3*R*) and (2*R*,3*S*), respectively. Consequently, the isomers I and III must have the (2*S*,3*S*) and (2*R*,3*R*) configuration, respectively.

DISCUSSION

In the present study we investigated the stereochemistry of α-oxidation in rat liver. Our results show that the α-oxidation rates of *R*- and *S*-3-methylhexadecanoic acid are

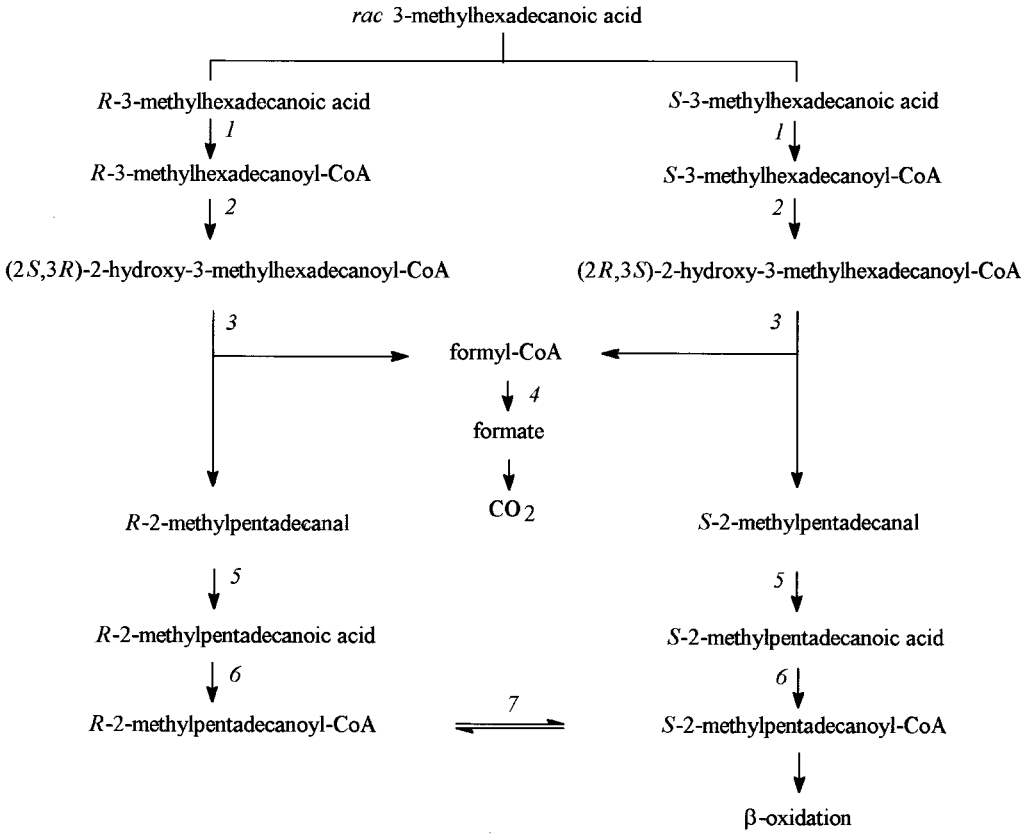

Fig. 7. α-Oxidation pathway of 3-methyl-branched fatty acids. The scheme in the figure depicts the α-oxidation pathway of 3-methyl-branched fatty acids and the stereochemical configuration of the intermediates involved. The numbers indicate the enzymes catalyzing the different steps: 1: acyl-CoA synthetase; 2: 3-methylacyl-CoA 2-hydroxylase (phytanoyl-CoA hydroxylase); 3: 2-hydroxy-3-methylacyl-CoA lyase (2-hydroxyphytanoyl-CoA lyase); 4: formyl-CoA hydrolase (or transferase); 5: fatty aldehyde dehydrogenase; 6: acyl-CoA synthetase; and 7: 2-methylacyl-CoA racemase. Whether 1 and 6 are different enzymes remains to be determined (34–36).

comparable in intact hepatocytes as well as in rat liver homogenates and that the subcellular localization of the α-oxidation of both isomers is peroxisomal. Although the oxidation rates found for the *R*-isomer seem to be slightly lower than those observed for the *S*-isomer, the maximal difference observed was 24% at a substrate concentration of 50 μm which is lower than the difference of 50% obtained in mitochondria for this concentration range by Tsai et al. (21) with phytanic acid as the substrate.

Earlier results of Huang et al. (16) and Fingerhut, Schmitz, and Conzelmann (17) suggested that there was no racemization of the 3-methyl group. The fact that *R*- and *S*-2-methylpentadecanoic acid are formed in purified peroxisomes incubated with *R*- and *S*-3-methylhexadecanoic acid, respectively, clearly shows that the initial configuration of the 3-methyl group is preserved throughout the whole α-oxidation process and unequivocally demonstrates that no racemization of the 3-methyl branch occurs. Consequently, it can be concluded that α-oxidation is not stereoselective with regard to the 3-methyl group.

In contrast, our results show that the configuration of the methyl group in position 3 determines the side of 2-hydroxylation. Only two of the four possible isomers of 2-hydroxy-3-methylhexadecanoyl-CoA are detected when purified peroxisomes are incubated with *rac* 3-methylhexadecanoic acid. These isomers have the (2*S*,3*R*) and (2*R*,3*S*) configurations. The fact that purified peroxisomes are capable of metabolizing all four stereoisomers excludes the possibility that four isomers are formed but that due to their poor metabolism and subsequent accumulation only two of them are detected. This means that physiologically only the (2*S*,3*R*) and (2*R*,3*S*) isomers are formed, demonstrating that the hydroxylation at position 2 occurs at the opposite side of the methyl group in position 3, probably as a result of a lesser degree of steric hindrance. This confirms the data of Tsai et al. (21) who concluded that the introduction of the hydroxy group at position 2 is stereospecific and determined by the configuration of the methyl group at position 3. They did not, however, determine the absolute configurations of the isomers of 2-hydroxyphytanate formed during α-oxidation of phytanic acid.

In conclusion, this study excludes that racemization of the 3-methyl branch occurs during the α-oxidation of 3-methyl branched fatty acids. It further shows that α-oxidation is not stereoselective with regard to the 3-methyl group, but that the configuration of the methyl group determines the direction of the 2-hydroxylation and hence the configuration of the isomers of the 2-hydroxyacyl-CoA intermediate formed. The α-oxidation pathway of 3-methyl branched fatty acids and its stereochemistry as revealed by the present work are summarized in **Fig. 7**. Interestingly, if the α-oxidation of very long straight-chain fatty acids in brain would occur through the same pathway, one would expect to find both isomeric forms of the 2-hydroxy fatty acids. In contrast, Tatsumi, Kishimoto, and Hignite (31) reported that the 2-hydroxy acid methyl esters obtained from calf brain cerebrosides have the *R*-configuration. In addition, analysis of the lipid composition of the nervous system showed that the amounts of odd-numbered and hydroxy fatty acids in cerebroside and cerebroside sulfate were normal in Refsum's disease (32), which is caused by a deficiency of the 3-methylacyl-CoA 2-hydroxylase (phytanoyl-CoA hydroxylase) (33). These findings suggest that hepatic α-oxidation of 3-methyl-branched fatty acids and α-oxidation of very long chain fatty acids in brain are two different processes.

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