Phytanoyl-CoA hydroxylase: recognition of 3-methyl-branched acyl-CoAs and requirement for GTP or ATP and Mg^{2+} in addition to its known hydroxylation cofactors

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Abstract **Phytanoyl-CoA hydroxylase is a peroxisomal α-oxidation enzyme that catalyzes the 2-hydroxylation of 3-methyl-branched acyl-CoAs. A polyhistidine-tagged human phytanoyl-CoA hydroxylase was expressed in *E. coli* and subsequently purified as an active protein. The recombinant enzyme required GTP or ATP and Mg^{2+}, in addition to its known cofactors Fe^{2+}, 2-oxoglutarate, and ascorbate. The enzyme was active towards phytanoyl-CoA and 3-methylhexadecanoyl-CoA, but not towards 3-methylhexadecanoic acid. Racemic, *R*- and *S*-3-methylhexadecanoyl-CoA were equally well hydroxylated. Hydroxylation of *R*- and *S*-3-methylhexadecanoyl-CoA yielded the (*2S,3R*) and (*2R,3S*) isomers of 2-hydroxy-3-methylhexadecanoyl-CoA, respectively. Human phytanoyl-CoA hydroxylase did not show any activity towards 2-methyl- and 4-methyl-branched acyl-CoAs or towards long and very long straight chain acyl-CoAs, excluding a possible role for the enzyme in the formation of 2-hydroxylated and odd-numbered straight chain fatty acids, which are abundantly present in brain. In conclusion, we report the unexpected requirement for ATP or GTP and Mg^{2+} of phytanoyl-CoA hydroxylase in addition to the known hydroxylation cofactors. Due to the fact that straight chain fatty acyl-CoAs are not a substrate for phytanoyl-CoA hydroxylase, 2-hydroxylation of fatty acids in brain can be allocated to a different enzyme/pathway.**—Croes, K., V. Foulon, M. Casteels, P. P. Van Veldhoven, and G. P. Mannaerts. **Phytanoyl-CoA hydroxylase: recognition of 3-methyl-branched acyl-CoAs and requirement for GTP or ATP and Mg^{2+} in addition to its known hydroxylation cofactors. *J. Lipid Res.* 2000.** 41: **629–636.**

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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 be β-oxidized due to the presence of a methyl-group in the 3-position. They are first shortened by one carbon atom via α-oxidation, a process during which the fatty acid is activated, requiring ATP, Mg^{2+}, and CoA, and subsequently 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). The 2-methyl fatty aldehyde is dehydrogenated to the corresponding 2-methyl fatty acid in an NAD^+-dependent reaction (6, 7). The 2-methyl-branched fatty acid is then further degraded mainly by peroxisomal β-oxidation (8–12).

The human enzymes catalyzing the hydroxylation (phytanoyl-CoA hydroxylase) (13, 14) and cleavage (2-hydroxyphytanoyl-CoA lyase) (15) reactions have recently been purified and cloned.

Human phytanoyl-CoA hydroxylase, which is encoded by a gene located on chromosome 10 (13), is a 41.2 kDa peroxisomal matrix protein containing a cleavable type 2 peroxisome targeting signal (PTS2) (13, 14). The activity of phytanoyl-CoA hydroxylase is severely impaired in peroxisome biogenesis disorders such as Zellweger syndrome (3) and rhizomelic chondrodysplasia punctata (RCDP) (16), and in Refsum's disease (17). While in Refsum's disease an isolated hydroxylase deficiency is seen due to a mutation in the hydroxylase gene (13, 14), the deficiency in RCDP is caused by a mutation in the gene encoding the peroxisomal PTS2 import receptor (Pex7p) resulting in a defective peroxisomal import of the hydroxylase (18–20). In Zellweger syndrome, the whole α-oxidation pathway is probably malfunctioning.

Although Mihalik et al. (13) confirmed that recombinant human phytanoyl-CoA hydroxylase requires Fe^{2+}

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

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and 2-oxoglutarate for its activity, no further studies were performed as to other possible cofactors or to the substrate specificity of the enzyme. The latter aspect is particularly interesting with regard to the origin of the 2-hydroxy fatty acids and the odd-numbered fatty acids that are abundantly present in brain (21), as phytanoyl-CoA hydroxylase might also be involved in the 2-hydroxylation and α-oxidation of straight chain acyl-CoAs. However, the involvement of phytanoyl-CoA hydroxylase in the α-oxidation of very long straight chain fatty acids in brain can be questioned by the fact that the amounts of odd-numbered and 2-hydroxy fatty acids in cerebroside and cerebroside sulfate are normal in Refsum's disease (22). In addition, the 2-hydroxy acid methyl esters obtained from calf brain cerebrosides have the *R*-configuration (23), whereas studies in rat liver have shown that during hepatic α-oxidation of branched chain fatty acids both *R*- and *S*-2-hydroxy isomers can be formed depending on the configuration of the 3-methyl group of the fatty acid (24).

We, therefore, expressed and purified the human phytanoyl-CoA hydroxylase and re-investigated the cofactor requirements of the enzyme. In a second series of experiments its activity towards the CoA-esters of phytanic acid and its synthetic substitute 3-methylhexadecanoic acid (10, 25), of 2-methyl- and 4-methyl-branched chain, long and very long straight chain fatty acids, and towards 3-methyl-branched chain fatty acids was studied.

MATERIALS AND METHODS

Materials

Octadecanoyl-CoA and lignoceroyl-CoA were obtained from Sigma. Adenosine-5′-O-(3-thiotriphosphate), adenylyl-imidodiphosphate, adenylyl-(β,γ-methylene)-diphosphonate, and guanylyl-imidodiphosphate (all tetralithium-salts) were from Roche Diagnostics.

Synthesis of substrates

Phytanic acid (25), unlabeled and 1-^{14}C-labeled *R*- and *S*-isomers of 3-methylhexadecanoic acid (2) were synthesized as described previously. Unlabeled and 1-^{14}C-labeled *rac* 3-methylhexadecanoic acids were prepared by mixing equimolar amounts of both isomers.

The CoA esters of *R*- and *S*-3-methylhexadecanoic acid, *rac* 3-methyl[1-^{14}C]hexadecanoic acid, and phytanic acid were prepared by means of acyl-CoA synthetase (from *Pseudomonas fragi*, Boehringer), essentially as described for the synthesis of sorboyl-CoA (26). Briefly, a reaction medium containing final concentrations of 0.25 mm fatty acid (Na-salt), 50 mm tris(hydroxymethyl)aminomethane (Tris) (pH 7.5), 8 mm $MgCl_2$, 4 mm ATP, 0.4 mm CoA, 10 mm dithiothreitol, 0.1% (v/v) Triton X-100, and 20 U pyrophosphatase, added to enhance the forward reaction, was incubated at 37°C. The reaction was started by addition of 10 U acyl-CoA synthetase (final volume: 20 ml). The reaction was monitored by high pressure liquid chromatography (HPLC) analysis and stopped after 4 h by cooling the reaction mixture on ice. The CoA esters were purified by applying the reaction mixture to a reverse-phase solid-phase-extraction column (C_{18} Bond Elut, 200 mg, Varian) (26). The eluted fractions containing the CoA ester were combined and applied on a NH_2 solid-phase-extraction column (NH_2 Bond Elut column, 500 mg, Waters). The column was washed with 6 ml of methanol–water 8:2 (v/v) and subsequently with 6 ml of methanol–water 8:2 (v/v) containing 1% (v/v) acetic acid to elute the remaining fatty acids, and with 6 ml of methanol–water 8:2 (v/v) containing 0.5 m HCl to elute the CoA esters. Purity of the CoA esters was checked by reversed phase HPLC (A_{258}) and by TLC (solvent n-butanol–acetic acid–water, 5:2:3, v/v) .

Rac 3-methylhexadecanoyl-CoA used in later experiments was prepared by activation (27) of *rac* 3-methylhexadecanoic acid, which was obtained as a reaction product in the synthesis of 2-hydroxy-3-methylhexadecanoic acid (6), and was purified as described before (28).

2-Hydroxy-3-methylhexadecanoic acid, 2-hydroxy-3-methylhexadecanoyl-CoA (6), and 2-hydroxyoctadecanoyl-CoA (28), *rac* 2-methylhexadecanoyl-CoA (29), and *rac* 4,8,12-trimethyltridecanoyl-CoA (30) were prepared as described previously.

Generation and purification of recombinant human phytanoyl-CoA hydroxylase

A 1 kb amplicon, containing the open reading frame (13, 14) of human phytanoyl-CoA hydroxylase mRNA was generated by PCR on a human liver cDNA library (31) with *Pfu* DNA polymerase (Promega) and gene-specific primers containing appropriate restriction sites (underlined): forward primer 5′ CGGGGT ACCAGCCATGGAGCAGCTTC; reverse primer 5′ CCCAAGCTT ATTTCAAAGATTGGTTCTTTC; denaturation at 94°C for 30 s, annealing at 52°C for 30 s, extension at 72°C for 45 s; 30 cycles.

The *Kpn* I–*Hind* III restricted fragment was subcloned in the pQE-31 vector (Qiagen) and *E. coli* Top10F′cells (InVitrogen) were transformed with the resultant plasmid. Colonies, growing on a medium containing ampicillin and tetracyclin, were checked by restriction analysis and DNA-sequencing (ALF-sequencer, Pharmacia; fluorescent vector primers pQE-F and pQE-R, cycle sequencing kit, Pharmacia).

Positive clones were grown overnight and expression of the recombinant protein was induced by adding isopropyl-1-thio-β-d-galactopyranoside (final concentration 1 mm, 400 ml culture medium). Cells were harvested 4 h post induction by centrifugation at 2,600 *g* for 20 min and the pellet was dissolved in 10 ml of Na-phosphate buffer (20 mm, pH 8) containing 500 mm NaCl, 10 mm imidazole, and 1 mg/ml lysozyme. After sonication of the cell suspension and subsequent centrifugation at 12,500 *g* for 20 min, the supernatant was applied on Ni-NTA Agarose (Qiagen) and the polyhistidine-tagged protein was eluted from the column with Na-acetate buffer (100 mm, pH 8) containing 250 mm imidazole. Analysis of the eluate by SDS-PAGE (12% polyacrylamide) revealed one single Coomassie-stained band with a molecular mass of approximately 41 kDa.

The yield was 300 to 1000 μg of protein with a specific activity of 50 to 80 nmol·min^{-1}·(mg protein)$^{-1}$ using 100 μm 3-methylhexadecanoyl-CoA as substrate.[2] Hydroxylase activity with this substrate depended on the substrate concentration and on the molar substrate/albumin ratio. Optimal rates were observed for molar substrate/albumin ratios between 2 and 8, and substrate concentrations between 50 and 200 μm (with a molar substrate/albumin ratio of 4).

Incubation conditions and analytical procedures

α-Oxidation of 3-methylhexadecanoic acid and 3-methylhexadecanoyl-CoA in a light mitochondrial fraction of rat liver and in permeabilized rat hepatocytes. A light mitochondrial fraction was prepared from livers of overnight fasted male Wistar rats, weighing between 120 and 200 g, in 0.25 m sucrose containing 0.1% (v/v) ethanol and 5 mm 3-(N-morpholino)propanesulfonic acid (pH 7.2) by differen-

[2] The Ni-NTA Agarose column was reused for subsequent purifications. Higher protein yields but lower specific activities were obtained when using new Ni-NTA Agarose.

tial centrifugation as described before (32). Incubations (37°C) of the light mitochondrial fraction with 0.05 mm 1-^{14}C-labeled 3-methylhexadecanoic acid or 3-methylhexadecanoyl-CoA (specific radioactivity for both 6.75 Ci/mol) were performed as described before (4). Different sets of cofactors were tested as mentioned in the text. The reactions were stopped after 10 min by addition of 100 μl glacial acetic acid and the reaction mixtures were analyzed for the oxidation products, $^{14}CO_2$ (10) and [^{14}C]formate (33), and for the formation of 2-hydroxy-3-methyl[1-^{14}C]hexadecanoyl-CoA (2). Permeabilized and washed rat hepatocytes were prepared and incubated as described before (2) with [1-^{14}C]3-methylhexadecanoyl-CoA as a substrate. Production of $^{14}CO_2$ (10) and [^{14}C]formate (33) was measured.

Hydroxylation of acyl-CoA esters by recombinant human phytanoyl-CoA hydroxylase. Incubations (37°C) were started by addition of 50 μl of purified enzyme (5–20 μg of protein), appropriately diluted in Na-acetate buffer (100 mm, pH 8) containing 250 mm imidazole, to 200 μl reaction medium containing final concentrations of 50 mm Tris (pH 7.5), 100 mm KCl, 0.050 mm defatted bovine serum 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 as standard α-oxidation conditions. The presence of imidazole did not affect hydroxylation rates (data not shown). Substrate concentrations were 0.1 mm phytanoyl-CoA, *rac R-* or *S*-3-methylhexadecanoyl-CoA, *rac* 2-methylhexadecanoyl-CoA, *rac* 4,8,12-trimethyltridecanoyl-CoA, octadecanoyl-CoA or lignoceroyl-CoA. For the competition experiment a substrate concentration of 50 μm *rac* 3-methylhexadecanoyl-CoA was used (control) and 50 μm of either hexadecanoyl-CoA or *rac* 2-methylhexadecanoyl-CoA was added in the test conditions. As a dependence of the hydroxylase activity on the molar acyl-CoA/albumin ratio was observed, the final total acyl-CoA/albumin ratio in the competition experiment was adjusted to 4 for each condition. Reactions were terminated by addition of 25 μl of 1 N H_2SO_4. After addition of 12 nmol internal standard (hexadecanoyl-CoA for incubations with phytanoyl-CoA, 3-methylhexadecanoyl-CoA, octadecanoyl-CoA and 2-methylhexadecanoyl-CoA; 2-hydroxyhexadecanoyl-CoA for incubations with 4,8,12-trimethyltridecanoyl-CoA; octadecanoyl-CoA, for incubations with lignoceroyl-CoA), the samples were extracted with 1200 μl isopropanol–heptane 4:1 (v/v) (modified from Lazo, Contreras, and Singh (34)). The supernatant was evaporated under N_2 at 40°C after addition of 20 μl of reduced Triton X-100 2% (v/v) (2). After reconstituting the residue in 100 μl of water and adjusting the pH to 7.5–8 with 1 N NaOH in order to dissolve the acyl-CoA esters, an aliquot (~90 μl) was injected onto a Nova-Pak C_8 HPLC column (3.9 × 150 mm, 4 μm, 60 Å; Waters). The CoA esters were eluted with an increasing gradient of buffer A (acetonitrile–200 mm ammonium acetate in water (pH 5.5) 9:1 (v/v)) in buffer B (20 mm ammonium acetate in water (pH 5.5)). Incubations with phytanoyl-CoA, 3-methylhexadecanoyl-CoA, 2-methylhexadecanoyl-CoA, 4,8,12-trimethyltridecanoyl-CoA, and octadecanoyl-CoA were analyzed using the following gradient: isocratic 45% (v/v) buffer A, 1 min; linear gradient 45–80% (v/v) buffer A over 20 min. Incubations with lignoceroyl-CoA were analyzed using a different gradient: isocratic 54% (v/v) buffer A, 1 min; linear gradient 54–64% (v/v) buffer A over 5 min; linear gradient 64–70% (v/v) buffer A over 2 min; linear gradient 70–91% (v/v) buffer A over 12 min. The flow rate was 1 ml/min. Column effluents were monitored by using an on-line ultraviolet detector (Waters 486) set at 258 nm.

Determination of the configuration of the 2-hydroxy isomers formed during hydroxylation of R- and S-3-methylhexadecanoyl-CoA by recombinant human phytanoyl-CoA hydroxylase. Recombinant human phytanoyl-CoA hydroxylase was incubated in quadruplicate with 0.1 mm *R-* or *S*-3-methylhexadecanoyl-CoA under standard α-oxidation conditions (see previous paragraph). After termination of the reaction with H_2SO_4, 10 nmol of 2-hydroxyoctadecanoyl-CoA was added as internal standard. The four samples of the respective isomers were pooled, alkalized with NaOH, and hydrolyzed (45 min, 75°C). The samples were cooled, acidified with HCl, and extracted twice with 2 ml of diethylether. The dried residues of the diethylether extracts containing the fatty acids were methylated with acidic methanol and converted to their *R*-2-methoxy-2-fluoromethylphenylacetyl derivatives and analyzed by gas chromatography (GC) as described before (24) but with a slightly modified temperature program: 80–220°C at a rate of 10°C/min, 220–280°C at a rate of 2°C/min.

Hydroxylation of rac 3-methylhexadecanoic acid by recombinant human phytanoyl-CoA hydroxylase. Recombinant human phytanoyl-CoA hydroxylase was incubated with 0.1 mm *rac* 3-methylhexadecanoic acid under standard α-oxidation conditions (see above), except that CoA was omitted from the reaction mixture to exclude every possibility of fatty acid activation. The reaction volume was increased to 2 ml to increase the sensitivity of the detection. After termination of the reaction by addition of 2 ml of an icecold mixture of methanol–glacial acetic acid 98:2 (v/v), 40 nmol of 2-hydroxyoctadecanoic acid was added as internal standard. The samples were extracted with diethylether. The dried residues of the diethylether extracts containing the fatty acids were derivatized as described in the previous paragraph.

RESULTS AND DISCUSSION

Human phytanoyl-CoA hydroxylase requires GTP or ATP and Mg^{2+} in addition to Fe^{2+}, 2-oxoglutarate, and ascorbate

The standard set of cofactors for the α-oxidation of 3-methyl-branched fatty acids consists of ATP, Mg^{2+}, and CoA, thought to be required for the initial activation of the fatty acid, and Fe^{2+}, 2-oxoglutarate, and ascorbate, required for the subsequent hydroxylation step (1, 2). However, when a light mitochondrial fraction from rat liver was incubated with *rac* 3-methyl[1-^{14}C]hexadecanoic acid and *rac* 3-methyl[1-^{14}C]hexadecanoyl-CoA, the total 2-hydroxylation rate (production of CO_2 plus formate plus 2-hydroxy-3-methylhexadecanoyl-CoA) obtained with *rac* 3-methylhexadecanoic acid under standard α-oxidation conditions was attained with *rac* 3-methylhexadecanoyl-CoA only when ATP and Mg^{+} were present in addition to Fe^{2+}, 2-oxoglutarate, and ascorbate (28.6 versus 30.3 $nmol \cdot min^{-1} \cdot (g\ liver)^{-1}$ for 3-methylhexadecanoic acid and 3-methylhexadecanoyl-CoA, respectively). Because recovery of 3-methylhexadecanoyl-CoA was almost complete (83%) after incubation in the presence of Fe^{2+}, 2-oxoglutarate, and ascorbate only, lack of activity due to hydrolysis of the substrate could be excluded (data not shown). Omission of ATP from the incubations with 3-methylhexadecanoyl-CoA resulted in negligible 2-hydroxylation rates (**Table 1**).

To further investigate the nucleotide-dependent stimulation, recombinant human phytanoyl-CoA hydroxylase was generated, purified, and incubated with *rac* 3-methylhexadecanoyl-CoA under standard α-oxidation conditions. HPLC analysis of the acyl-CoA extract revealed a peak around 5.3 min (**Fig. 1, panel B**), eluting at the same time as the peak containing the (*2S,3R*) and (*2R,3S*) isomers of the chemically synthesized 2-hydroxy-3-methylhexadecanoyl-

TABLE 1. Cofactor requirements for the 2-hydroxylation of 3-methyl[1-^{14}C]hexadecanoyl-CoA in a light mitochondrial fraction from rat liver

Cofactors	CO_2 + Formate	2-Hydroxy-3-methyl-hexadecanoyl-CoA	Total 2-Hydroxylation
	nmol·min^{-1}·(g liver)$^{-1}$		*% of control*
Standard conditions (control)	8.47/8.48[a]	22.36/22.06[a]	100
Fe^{2+}, 2-oxoglutarate, ascorbate	0.39	0.47	3
Fe^{2+}, 2-oxoglutarate, ascorbate, CoA	0.58	0.54	4
Fe^{2+}, 2-oxoglutarate, ascorbate, Mg^{2+}	0.21	0.33	2
Fe^{2+}, 2-oxoglutarate, ascorbate, Mg^{2+}, CoA	0.17	0.23	1
Fe^{2+}, 2-oxoglutarate, ascorbate, ATP	11.04	15.02	84
Fe^{2+}, 2-oxoglutarate, ascorbate, ATP, CoA	7.78	18.91	87
Fe^{2+}, 2-oxoglutarate, ascorbate, Mg^{2+}, ATP	12.38	17.87	99

CO_2, formate and 2-hydroxy-3-methylhexadecanoyl-CoA production were measured in a light mitochondrial fraction of rat liver incubated with 0.05 mm *rac* 3-methyl[1-^{14}C]hexadecanoyl-CoA in the presence of Fe^{2+} (0.1 mm), 2-oxoglutarate (3 mm), ascorbate (10 mm), Mg^{2+} (2.4 mm), ATP (4 mm) and CoA (0.2 mm) (control; duplicate values) or in the presence of the cofactors mentioned (molar substrate/BSA ratio of 2). Reactions were terminated after 10 min. Total 2-hydroxylation represents the sum of CO_2, formate, and 2-hydroxy-3-methylhexadecanoyl-CoA. The production of CO_2 plus formate and 2-hydroxy-3-methylhexadecanoyl-CoA during incubation with 0.05 mm *rac* 3-methyl[1-^{14}C]hexadecanoic acid in standard conditions was 8.73 and 19.79 nmol·min^{-1}·(g liver)$^{-1}$, respectively.

[a] In another experiment, control values were 9.19/9.37 and 21.10/22.57, respectively.

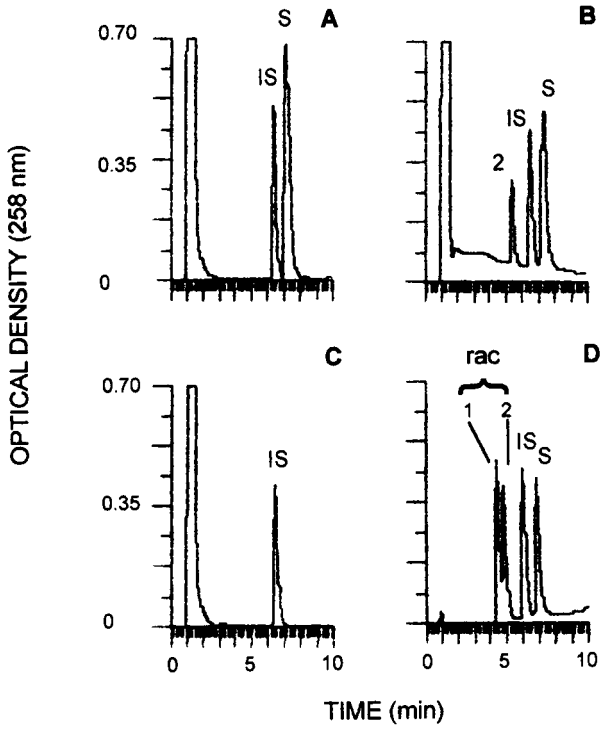

Fig. 1 HPLC analysis of 2-hydroxy-3-methylhexadecanoyl-CoA formed during hydroxylation of *rac* 3-methylhexadecanoyl-CoA by recombinant human phytanoyl-CoA hydroxylase. Recombinant human phytanoyl-CoA hydroxylase was incubated with *rac* 3-methylhexadecanoyl-CoA (substrate, S) for 0 min (panel A) and for 10 min (panel B) or without substrate for 10 min (panel C) under standard α-oxidation conditions. After addition of hexadecanoyl-CoA (internal standard, IS), the acyl-CoAs were extracted and subjected to HPLC analysis. Panel D shows the elution profile of a mixture of substrate, internal standard and *rac* 2-hydroxy-3-methylhexadecanoyl-CoA (eluting in two peaks containing the (*2S,3S*)- and (*2R,3R*)-isomers (peak 1) and the (*2R,3S*)- and (*2S,3R*)-isomers (peak 2) (24)).

CoA (Fig. 1, panel D) (24). These data show that the recombinant human phytanoyl-CoA hydroxylase was functionally active towards *rac* 3-methylhexadecanoyl-CoA, and that only two ((*2S,3R*) and (*2R,3S*)) of the four possible isomers of 2-hydroxy-3-methylhexadecanoyl-CoA were formed.

Consistent with the findings described above, recombinant human phytanoyl-CoA hydroxylase was not able to hydroxylate *rac* 3-methylhexadecanoyl-CoA when only Fe^{2+}, 2-oxoglutarate, and ascorbate were present (**Table 2**). Table 2 further shows that human phytanoyl-CoA hydroxylase needs ATP and Mg^{2+} in addition to the classic hydroxylation cofactors, Fe^{2+}, 2-oxoglutarate, and ascorbate. ATP could not be replaced by CTP, UTP, or ITP (all 4 mm) (results not shown), whereas substitution of ATP with GTP (4 mm) resulted in even higher hydroxylation rates (Table 2). The presence of Mg^{2+} led to a slight increase of the hydroxylation rates regardless of whether the nucleotide present was ATP or GTP. Substitution of Mg^{2+} with other divalent cations such as Ca^{2+} and Mn^{2+} (both 2.4 mm) reduced the hydroxylation rates by 24 and 66%, respectively, compared to the optimal conditions. AMP (4 mm), ADP (4 mm), NAD^+ (2 mm), and FAD (5 μM) could not replace ATP (results not shown). Enzyme activity was reduced by 96% when ATP was replaced by pyrophosphate (results not shown). Compared to the synthetic substrate, hydroxylation of phytanoyl-CoA was approximately 2-fold lower but similar findings were obtained: no hydroxylation in the absence of ATP/GTP and a 3-fold increase in hydroxylation rates when using GTP instead of ATP (data not shown).

The nature of the ATP/GTP dependence is unclear. The lack of activity in the absence of nucleotides does not seem to be related to hydrolysis of the substrate or the product. As for the experiment with the light mitochon-

TABLE 2. Cofactor requirements for the 2-hydroxylation of 3-methylhexadecanoyl-CoA by the recombinant human phytanoyl-CoA hydroxylase

Cofactors	2-Hydroxy-3-methylhexadecanoyl-coA	
	% of Control	% of Optimal Condition
Standard α-oxidation conditions (control condition)	100	
Fe^{2+}, 2-oxoglutarate, ascorbate	4	
Fe^{2+}, 2-oxoglutarate, ascorbate, Mg^{2+}	6	
Fe^{2+}, 2-oxoglutarate, ascorbate, ATP	75	
Fe^{2+}, 2-oxoglutarate, ascorbate, Mg^{2+}, ATP	94	
Fe^{2+}, 2-oxoglutarate, ascorbate, Mg^{2+}, AMP-PCP	23	
Fe^{2+}, 2-oxoglutarate, ascorbate, Mg^{2+}, AMP-PNP	88	
Fe^{2+}, 2-oxoglutarate, ascorbate, Mg^{2+}, thioATP	52	
Fe^{2+}, 2-oxoglutarate, ascorbate, Mg^{2+}, GTP, CoA (optimal condition)	349	100
Fe^{2+}, 2-oxoglutarate, ascorbate, Mg^{2+}, GTP		87
Fe^{2+}, 2-oxoglutarate, ascorbate, Mg^{2+}, GMP-PNP		74
Fe^{2+}, 2-oxoglutarate, ascorbate, GTP		81

The production of 2-hydroxy-3-methylhexadecanoyl-CoA was measured during incubation of recombinant human phytanoyl-CoA hydroxylase with 0.1 mm *rac* 3-methylhexadecanoyl-CoA in the presence of Fe^{2+} (0.1 mm), 2-oxoglutarate (3 mm), ascorbate (10 mm), Mg^{2+} (2.4 mm), ATP (4 mm), and CoA (0.2 mm) (control condition) or in the presence of the cofactors mentioned (molar substrate/BSA ratio of 2). Reactions were terminated after 10 min. Results are expressed as % of control. The amount of 2-hydroxy-3-methylhexadecanoyl-CoA measured under standard α-oxidation conditions with this batch of hydroxylase was 77.4 $nmol \cdot min^{-1} \cdot (mg\ protein)^{-1}$. Values are based on single determinations, but similar findings were obtained with other batches of hydroxylase.

drial fraction, 82% of the initial amount of 3-methylhexadecanoyl-CoA was recovered after the incubation with the recombinant hydroxylase in the absence of ATP. Also, 2-hydroxy-3-methylhexadecanoyl-CoA, when added to the incubation mixture, was quantitatively recovered in the extracts (99%). The finding that ATP and Mg^{2+} are also required for 2-hydroxylation in a light mitochondrial fraction and in permeabilized hepatocytes (cfr. infra), which contain the mature form of phytanoyl-CoA hydroxylase (14), proves that the requirement for these cofactors is not due to the polyhistidine tag or the cleavable peroxisome targeting signal type 2 present in the recombinant human phytanoyl-CoA hydroxylase. The fact that adenosine-5′-O-(3-thiotriphosphate) (thioATP), adenylylimidodiphosphate (AMP-PNP), adenylyl-(β,γ-methylene)-diphosphonate (AMP-PCP) and guanylyl-imidodiphosphate (GMP-PNP) (all 4 mm) could replace ATP and GTP, respectively, at least to a certain extent (Table 2), indicates that hydrolysis of the nucleotide was not required for enzyme activity. We were also not able to find a known GTP or an ATP-binding motif when scanning the human protein sequence against the Prosite database (35) and the recombinant hydroxylase was not retained on an ATP-agarose column (data not shown). As far as we know, stimulation of other dioxygenases by nucleotide triphosphates has not been reported.

Regardless of the mechanism involved in the ATP/GTP activation, the hydroxylation step is the first example of an intra-peroxisomal GTP-dependent reaction. So far, nucleotide pools, with the exception of protein-bound FAD and CoA, have not been demonstrated in isolated peroxisomes, most likely due to the presence of a pore-forming protein (36). In the intact cell, however, the peroxisomal membrane permeability appears to be regulated and the presence of different membrane transporters has been documented (31, 37). A 34 kDa integral membrane protein, belonging to the family of solute transporters, was recently identified in human and mouse (31). This PMP34 might assist in the transport of FAD (31), but ATP or GTP could be other candidate ligands.

In an attempt to reveal a physiological role for GTP in α-oxidation, we measured the production of $^{14}CO_2$ and [1-^{14}C]formate in permeabilized and washed rat hepatocytes, incubated with 3-methyl[1-^{14}C]hexadecanoyl-CoA, in the presence of Fe^{2+}, 2-oxoglutarate, ascorbate, Mg^{2+}, CoA, and thiamine pyrophosphate (15), with or without ATP/GTP. Maximal rates (37.26 $nmol \cdot min^{-1} \cdot (10^8\ cells)^{-1}$ were obtained with ATP and GTP (4 mm each). Rates in the presence of 4 mm ATP or GTP alone were 31.69 and 17.50 $nmol \cdot min^{-1} \cdot (10^8\ cells)^{-1}$, respectively. In the absence of ATP or GTP, poor rates were observed (0.20 $nmol \cdot min^{-1} \cdot (10^8\ cells)^{-1}$). Although interpretation of these data might not be straightforward due to uncertainties with regard to the translocation of the CoA-ester, these data point indeed towards a role for GTP in α-oxidation.

Hydroxylation of 3-methylacyl-CoA by recombinant human phytanoyl-CoA hydroxylase yields (*2S,3R*) and (*2R,3S*) 2-hydroxy-3-methylacyl-CoA

As described above, recombinant human phytanoyl-CoA hydroxylase was active towards *rac* 3-methylhexadecanoyl-CoA. HPLC analysis of the acyl-CoA fractions obtained from incubations with *R*- and *S*-3-methylhexadecanoyl-CoA showed that both isomers were equally well hydroxylated (103 and 106%, respectively, compared to the rates obtained for the racemate). In order to determine which of the four possible isomers of 2-hydroxy-3-methylhexadecanoyl-CoA were formed, the incubation mixtures were subjected to alkaline hydrolysis and after acidification the fatty acids were extracted, methylated, derivatized with *R*-2-methoxy-2-fluoromethylphenylacetic acid chloride (MTPA) (24), and analyzed by GC. **Figure 2** shows that after incubation of human phytanoyl-CoA hydroxylase with *R*- or *S*-3-methylhexadecanoyl-CoA, only one isomer of 2-hydroxy-3-methylhexadecanoic acid was recovered for each substrate, the formed isomers coeluting with the synthetic isomers II and IV, respectively. As described before (24), isomers II and IV are the (*2S,3R*) and (*2R,3S*) isomers of 2-hydroxy-3-methylhexadecanoic acid, demonstrating that hydroxylation of *R*- and *S*-3-methylhexadecanoyl-CoA by human phytanoyl-CoA hydroxylase yields the (*2S,3R*) and (*2R,3S*) isomers of 2-hydroxy-3-methylhexadecanoyl-CoA, respectively. These data are consistent with our previous findings obtained on the stereochemistry of the α-oxidation of 3-methyl-branched fatty acids in rat liver (24) and confirm that the insertion of the

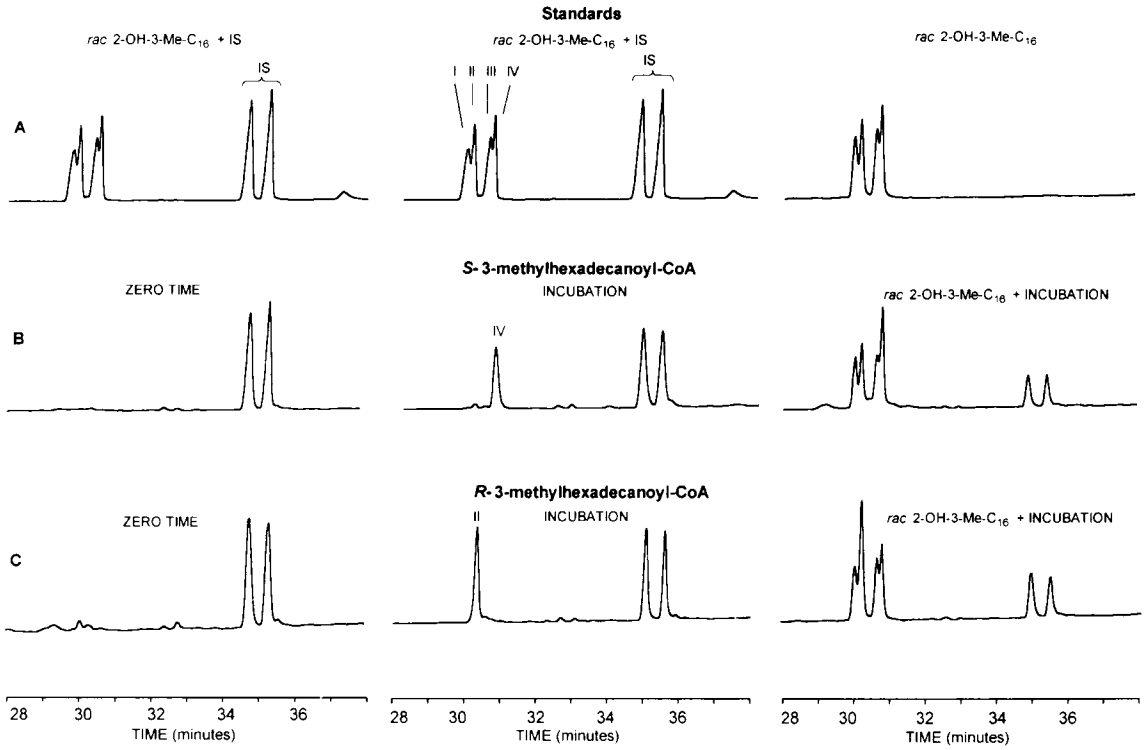

Fig. 2 GC analysis of the 2-hydroxy-3-methylhexadecanoyl-CoA stereoisomers formed during hydroxylation of *R*- and *S*-3-methylhexadecanoyl-CoA by recombinant human phytanoyl-CoA hydroxylase. Recombinant human phytanoyl-CoA hydroxylase was incubated with *R*- and *S*-3-methylhexadecanoyl-CoA. After alkaline hydrolysis, the resulting 2-hydroxy acids were extracted, methylated, derivatized with *R*-2-methoxy-2-trifluoromethylphenylacetyl chloride, and analyzed by GC. Trace A shows a standard of derivatized *rac* 2-hydroxy-3-methylhexadecanoic acid in the presence (left and middle panels) or absence (right panel) of derivatized 2-hydroxyoctadecanoic acid (internal standard, IS). Trace B and C show the GC chromatograms of the derivatives obtained after incubation for 0 min (left panels) and 10 min (middle panels) with *S*- (trace B) and *R*-3-methylhexadecanoyl-CoA (trace C). In the right panel of trace B and C the standard of derivatized *rac* 2-hydroxy-3-methylhexadecanoic acid is superimposed on the stereoisomer of 2-hydroxy-3-methylhexadecanoic acid formed during hydroxylation of *S*- and *R*-3-methylhexadecanoyl-CoA, respectively. IS, internal standard; I, (*2S,3S*) 2-hydroxy-3-methylhexadecanoyl-CoA; II, (*2S,3R*) 2-hydroxy-3-methylhexadecanoyl-CoA; III, (*2R,3R*) 2-hydroxy-3-methylhexadecanoyl-CoA; IV, (*2R,3S*) 2-hydroxy-3-methylhexadecanoyl-CoA.

2-hydroxyl group is governed by the configuration of the 3-methyl group.

Human phytanoyl-CoA hydroxylase does not hydroxylate 3-methyl-branched fatty acids, long and very long straight chain acyl-CoAs or 2-methyl- and 4-methyl-branched acyl-CoAs

In order to see whether human phytanoyl-CoA hydroxylase is able to hydroxylate 3-methyl-branched fatty acids, the enzyme was incubated with *rac* 3-methylhexadecanoic acid under standard α-oxidation conditions, except that CoA was omitted to exclude every possibility of fatty acid activation. After termination of the reaction by acidification the assay mixtures were extracted with diethylether, methylated, and derivatized with MTPA (24). GC analysis showed that no 2-hydroxy-3-methylhexadecanoic acid was formed (data not shown), demonstrating that human phytanoyl-CoA hydroxylase is not able to hydroxylate 3-methyl-branched fatty acids. The observation that human phytanoyl-CoA hydroxylase is able to hydroxylate a 3-methylacyl-CoA but not the corresponding 3-methyl-branched fatty acid, confirms our earlier conclusions drawn from experiments in rat liver (2) that during hepatic α-oxidation of 3-methyl-branched fatty acids activation precedes hydroxylation.

In contrast to the activity shown towards 3-methylhexadecanoyl-CoA, phytanoyl-CoA hydroxylase did not show any hydroxylation activity towards octadecanoyl- and lignoceroyl-CoA when incubated under standard α-oxidation conditions, indicating that the enzyme recognizes 3-methyl-branched acyl-CoAs but not straight chain acyl-CoAs (data not shown). This lack of activity towards long and very long straight chain acyl-CoAs implies that the very long straight chain 2-hydroxy fatty acids, which are abundantly found in brain sphingolipids (21), cannot result from hydroxylation by phytanoyl-CoA hydroxylase and that hydroxylation of 3-methyl-branched fatty acids during hepatic α-oxidation and hydroxylation of very long chain fatty acids in brain are two different processes. The fact that multiple human tissue Northern blot analysis failed to show the presence of the mRNA of phytanoyl-CoA hydroxylase in brain (result not shown) supports this conclusion. Additionally, the results imply that phytanoyl-CoA hydroxylase and the classic α-oxidation pathway are also not involved in the oxidative decarboxylation of even-numbered straight chain fatty acids to odd-numbered straight chain fatty acids.

Furthermore, recombinant human phytanoyl-CoA hydroxylase was not able to hydroxylate *rac* 2-methylhexadecanoyl-CoA or *rac* 4,8,12-trimethyltridecanoyl-CoA, which shows that a methyl-branch in position 3 is an absolute requirement for the hydroxylase activity of the enzyme. However, the production of 2-hydroxy-3-methylhexadecanoyl-CoA from *rac* 3-methylhexadecanoyl-CoA was reduced by 26 and 44% in the presence of an equimolar concentration of hexadecanoyl-CoA or *rac* 2-methylhexadecanoyl-CoA, respectively (data not shown), suggesting that phytanoyl-CoA hydroxylase is able to bind straight chain and 2-methyl-branched acyl-CoAs, despite the fact that they are no substrate. This observation would explain the accumulation of phytanic acid seen in the plasma of certain patients with isolated peroxisomal β-oxidation defects as an accumulation secondary to the accumulation of pristanic acid (pristanoyl-CoA) (38).

CONCLUSIONS

Our present data show that human phytanoyl-CoA hydroxylase *1*) requires GTP or ATP and Mg^{2+} besides the already known cofactors for hydroxylation; *2*) requires a methyl group in position 3 for its activity, ruling out a role for the enzyme in the formation of straight chain 2-hydroxy fatty acids and odd-numbered fatty acids, which are abundantly present in brain; *3*) inserts a hydroxyl-group at position 2 of a 3-methyl-branched acyl-CoA in a reaction that is controlled by the configuration of the 3-methyl group; and *4*) is inhibited by straight chain and 2-methyl-branched chain acyl-CoAs, possibly explaining the accumulation of phytanic acid seen in certain disorders caused by a deficient peroxisomal β-oxidation.

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