Further studies on the substrate spectrum of phytanoyl-CoA hydroxylase: implications for Refsum disease?

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Abstract **Refsum disease is a peroxisomal disorder characterized by adult-onset retinitis pigmentosa, anosmia, sensory neuropathy, ataxia, and an accumulation of phytanic acid in plasma and tissues. Approximately 45% of cases are caused by mutations in phytanoyl-CoA hydroxylase (PAHX), the enzyme catalyzing the second step in the peroxisomal α-oxidation of 3-methyl-branched fatty acids. To study the substrate specificity of human PAHX, different 3-alkyl-branched substrates were synthesized and incubated with a recombinant polyhistidine-tagged protein. The enzyme showed activity not only toward racemic phytanoyl-CoA and the isomers of 3-methylhexadecanoyl-CoA, but also toward a variety of other mono-branched 3-methylacyl-CoA esters with a chain length down to seven carbon atoms. Furthermore, PAHX hydroxylated a 3-ethylacyl-CoA quite well, whereas a 3-propylacyl-CoA was a poor substrate. Hydroxylation of neither 2- or 4-methyl-branched acyl-CoA esters, nor long or very long straight-chain acyl-CoA esters could be detected. The results presented in this paper show that the substrate specificity of PAHX, with regard to the length of both the acyl-chain and the branch at position 3, is broader than expected. Hence, Refsum disease might be characterized by an accumulation of not only phytanic acid but also other 3-alkyl-branched fatty acids.**—Foulon, V., S. Asselberghs, W. Geens, G. P. Mannaerts, M. Casteels, and P. P. Van Veldhoven. **Further studies on the substrate spectrum of phytanoyl-CoA hydroxylase: implications for Refsum disease?** ***J. Lipid Res.*** **2003.** 44: **2349–2355.**

Supplementary key words α-oxidation • 3-methyl-branched fatty acids • phytanic acid • peroxisomes • dioxygenases • stereochemistry

Human phytanoyl-CoA hydroxylase (PAHX) is an iron- and 2-oxoglutarate-dependent dioxygenase that catalyzes a key step in the metabolic degradation of 3-methyl-branched fatty acids such as phytanic acid. Due to the presence of a 3-methyl group, these substrates cannot be degraded by the normal β-oxidation pathway. Instead, they are shortened by one carbon atom in a four-step α-oxidation process, yielding 2-methyl-branched fatty acids, which are substrates for subsequent β-oxidation cycles (1).

In the last decade, the different intermediates and cofactors of the α-oxidation pathway have been characterized (2–9). Evidence has been found that α-oxidation is localized to peroxisomes, and several key enzymes [PAHX (10, 11), and 2-hydroxyphytanoyl-CoA lyase/2-HPCL (12)] have been identified and cloned. Phytanic acid is at present the only known physiological substrate of (hepatic) α-oxidation in humans. It is a conversion product of phytol, the side chain of chlorophyll, and is taken up with the diet in ruminant fat and dairy products. Other potential substrates for α-oxidation are retinoic acid, dolichoic acid, and the terminally oxidized isoprenoid moieties of prenylated proteins, although data supporting this contention are still missing.

In humans, the plasma level of phytanic acid is normally low (<30 μM). Accumulation is typically seen in adult Refsum disease (ARD) and, to a lesser extent, in generalized peroxisome biogenesis disorders. ARD is an autosomal recessive syndrome, clinically characterized by retinitis pigmentosa, peripheral neuropathy, and cerebellar ataxia, usually presenting in the second or third decade of life. Eliminating phytanic acid from the diet usually results in an arrest of the progress of the disease and even in a regression of the peripheral neuropathy, indicating that at least some of the symptoms observed in ARD are directly correlated with the accumulation of this 3-methyl-branched fatty acid in plasma and tissues (13). Older literature refers also to the accumulation of mono- and tri-unsaturated analogs of phytanic acid in sera and urine of patients with ARD (14). These analogs, which are incorporated in phospholipids and neutral lipids, might well be geranylgeraniol-derived products.

In 45% of patients affected with ARD, mutations have been found in the cDNA encoding PAHX, and deficiencies in hydroxylase activity resulting from these mutations

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have been demonstrated (10, 11, 15). Human PAHX, encoded by a gene on chromosome 10p15.1 (11), is a 38.5 kDa peroxisomal matrix protein, containing a cleavable peroxisome-targeting sequence, type 2 (PTS2). In the presence of Fe^{2+}, 2-oxoglutarate, ascorbate, Mg^{2+}, and ATP or GTP, the enzyme catalyzes the conversion of 3-methylacyl-CoA esters to the corresponding 2-hydroxy-3-methylacyl-CoA intermediates. Based on these cofactor and cosubstrate requirements, as well as on the comparison of the amino acid sequence and the predicted three-dimensional structure, PAHX was classified as a 2-oxoglutarate/ascorbate-dependent non-heme ferrous ion dioxygenase. As such, PAHX belongs to a class of enzymes that includes propyl hydroxylase, lysyl hydroxylase, aspartyl β-hydroxylase, and γ-butyrobetaine hydroxylase (16). The O_2-dependent reactions catalyzed by these enzymes are characterized by the fact that one atom of molecular oxygen is incorporated into the substrate, while the other atom of oxygen is incorporated in the cosubstrate 2-oxoglutarate, resulting in the subsequent formation of succinate and the release of CO_2. Most obviously, oxidation of the prime substrate proceeds via a highly oxidizing iron(IV) oxene intermediate.

In a previous study, we showed that both phytanoyl-CoA and 3-methylhexadecanoyl-CoA are efficiently hydroxylated by recombinant PAHX, whereas in vitro, no activity toward 2- or 4-methyl-branched acyl-CoA esters or toward long and very long straight-chain acyl-CoA esters could be detected (9). Normally, reactions catalyzed by dioxygenases are highly stereospecific, occurring with retention of the configuration (17). However, we previously demonstrated that α-oxidation of phytanic acid is not stereoselective with regard to the 3-methyl branch, although closer analysis revealed that the orientation of the β-substituent determines the stereochemistry of the α-hydroxylation (9, 18). Taken together, these observations would indicate that the enzyme recognizes only the branching point, not the length of the branch, and that the catalytic pocket allows access of both isomers. Therefore, we investigated the substrate spectrum of PAHX with regard to the length of both the acyl chain and the branch at position 3.

MATERIALS AND METHODS

Materials

4-Phenyl-2-butanol (99%), 2-undecanol (99%), and methanesulfonyl chloride were from Acros. 3-Phenylbutyric acid (98%), 3-methylpentanoic acid (98%), 2-methylhexanoic acid (99%), citronellic acid (3,7-dimethyloct-6-enoic acid; 98%), 2-octanol (99%), 2-decanol (98%), pyridine, and borane-methyl sulfide were purchased from Aldrich. 4-Methylnonanoic acid (97%) and 4-decanol (97%) were obtained from Avocado. 5-Nonanol (99.5%), diethyl malonate, 1,1′-carbonyldiimidazole, and 2,2-dimethoxypropane were from Fluka. 3-Nonanol (97%) was from Merck.

Synthesis of branched-chain fatty acids

Unless otherwise mentioned, all branched fatty acids (and derivatives) were synthesized as racemic mixtures. 5-Phenyl-3-methylpentanoic acid, 3-methylnonanoic acid, 3-methylundecanoic acid, 3-methyldodecanoic acid, 3-ethylnonanoic acid, 3-propylnonanoic acid, and 3-butylheptanoic acid were obtained by chain elongation of, respectively, 4-phenyl-2-butanol, 2-octanol, 2-decanol, 2-undecanol, 3-nonanol, 4-decanol, and 5-nonanol with diethyl malonate, essentially as described by Spener and Mangold (19). Briefly, diethyl malonate was alkylated with the respective mesylates (prepared from the corresponding alcohols and methanesulfonyl chloride in pyridine) in a ratio of 1:1.15 (v/v). The diethylesters were saponified with 2N NaOH in 10% aqueous ethanol and subsequently extracted under acidic conditions to recover the corresponding dicarboxylic acids. Decarboxylation was performed by pyrolysis at 165°C (120 min) in ethylene glycol. After cooling, water was added and the resulting fatty acids were extracted into chloroform. 5-Phenyl-3-methylpentanoic acid, 3-methylundecanoic acid, 3-methyldodecanoic acid, 3-ethylnonanoic acid, 3-propylnonanoic acid, and 3-butylheptanoic acid were further purified over Sep-Pak NH_2 cartridges and eluted with increasing concentrations of acetic acid in diethylether (2–4%). Purity of the end products was checked by TLC (Silicagel 60, Merck; hexane-diethylether-acetic acid, 60:40:1, v/v/v) after spraying with bromocresol green. R_f values were 0.82 (3-methylundecanoic acid), 0.81 (3-ethylnonanoic acid), 0.79 (3-propylnonanoic acid), and 0.83 (3-butylheptanoic acid), and yields varied between 15% and 30%.

3-Methylheptanoic acid was prepared from 2-methylhexanoic acid, which was first transformed in the corresponding alcohol in tetrahydrofuran (THF) with borane-methylsulfide. The mesylate (generated as described above) was further converted into 2-methylhexyl-1-nitrile in a substitution reaction with KCN. Alkaline oxidation and hydrolysis of the nitrile finally yielded 3-methylheptanoic acid, which was dried in the presence of triethylamine. Purity was checked by TLC (Silicagel 60, Merck; hexane-diethylether-acetic acid, 80:20:1, v/v/v; Rf 0.57).

Synthesis of branched-chain fatty acyl-CoA esters

The CoA esters of 3-methylhexadecanoic acid and its 3*R*- and 3*S*-isomers (9) and of 2-methylhexadecanoic acid (20) were prepared as described previously. Prior to conversion into the corresponding acyl-CoA esters, 3-phenylbutyric acid, 3-methylpentanoic acid, 5-phenyl-3-methylpentanoic acid, 3,7-dimethyloct-6-enoic acid, 3-methylnonanoic acid, 4-methylnonanoic acid, 3-methyldodecanoic acid, 3-methylundecanoic acid, 3-ethylnonanoic acid, 3-propylnonanoic acid, and 3-butylheptanoic acid (250 μmol) were activated with 1.2-fold molar excess 1,1′-carbonyldiimidazole in tetrahydrofuran (for the activation of 4-methylnonanoic acid, dimethoxypropane was added as a moisture scavenger). The activation of 3-methylheptanoate-triethylamine salt was performed in the presence of an equimolar concentration of 2,6-dichlorobenzoic acid in respect to triethylamine. The imidazole derivatives were subsequently dried and dissolved in THF. CoA esterification was initiated at room temperature by the addition of an aqueous solution of CoA-Li_3 in 0.5 M $NaHCO_3$ (pH 8.5); the reaction was allowed to proceed at 4°C. In order to purify 5-phenyl-3-methylpentanoyl-CoA, 3-methylnonanoyl-CoA, 4-methylnonanoyl-CoA, 3-methyldodecanoyl-CoA, 3-methylundecanoyl-CoA, 3-ethylnonanoyl-CoA, 3-propylnonanoyl-CoA, and 3-butylheptanoyl-CoA, each mixture containing ~50 μmol of CoA/CoA ester, were lyophilized, taken up in 10 ml of water, and acidified with perchloric acid. The precipitated acyl-CoAs, collected by centrifugation, were washed once with 0.8% perchloric acid and once with acetone. The residues were dissolved in 5 ml 0.1 M $NaHCO_3$, applied to RP-C18-cartridges, and eluted with increasing concentrations of methanol in water. To purify 3-methylheptanoyl-CoA, 3-methylpentanoyl-CoA, 3-phenylbutyryl-CoA, and 3,7-dimethyloct-6-enoyl-CoA, the reaction mixtures were lyophilized after adding 0.25 ml 1 M ammonium acetate (pH 5). Subsequently, the residue was dissolved in 1 M ammonium acetate (pH

5) and applied to an RP-C18-cartridge. The CoA esters were eluted from the column with increasing concentrations of methanol in 0.5 M ammonium acetate (pH 5).

The following yields (related to CoA input) were obtained: 3-methylheptanoyl-CoA (23.8 mg; 53%), 3-phenylbutyryl-CoA (18.4 mg; 39%), 3-methylpentanoyl-CoA (18 mg; 40%), 3,7-dimethyloct-6-enoyl-CoA (10.1 mg; 21%), 3-methylundecanoyl-CoA (39.6 mg; 80%), 3-ethylnonanoyl-CoA (21 mg; 43%), 3-propylnonanoyl-CoA (28.3 mg; 57%), and 3-butylheptanoyl-CoA (14.7 mg; 30%).

Generation and purification of recombinant human PAHX

Recombinant mature human PAHX was generated and purified as described previously (9), with slight modifications. Briefly, the enzyme was purified from 2 l of culture containing Top10F′ cells (Invitrogen) expressing His-tagged PAHX, 4 h post induction with isopropyl-1-thio-β-D-galactopyranoside. After centrifugation (Kontron A 6.14; 10 min at 8,800 rpm), the cell pellet was dissolved in 40 ml of 20 mM Na-phosphate buffer (pH 7.4) containing 0.5 M NaCl, 10 mM imidazole, and protease inhibitors. Following sonication of the cell suspension and subsequent centrifugation (Kontron A 8.24; 15 min at 11,000 rpm), the supernatant was applied on Ni-NTA (Qiagen) and the poly-His-tagged protein was eluted from the column with 20 mM Na-phosphate buffer (pH 7.4) containing 0.5 M NaCl and 250 mM imidazole. Yield was ~1 mg of protein, and specific activities varied between 216 and 236 nmol/min^{-1}/(mg protein)$^{-1}$ (50 μM 3-methylhexadecanoyl-CoA as substrate; standard conditions).

Hydroxylation of acyl-CoA esters by recombinant human PAHX

Hydroxylation reactions were performed as described previously (9), with some modifications. Incubations (37°C) were started by the addition of 5 μg of purified recombinant human PAHX, appropriately diluted in 50 μl of 20 mM Na-phosphate buffer (pH 7.4) containing 0.5 M NaCl and 250 mM imidazole, to 200 μl of reaction medium. Final concentrations were 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 12.5 μM defatted BSA, 4 mM GTP, 0.2 mM CoA, 2.4 mM $MgCl_2$, 0.1 mM $FeCl_2$, 10 mM L-ascorbate, and 3 mM 2-oxoglutarate (referred to as standard conditions). Substrate concentrations were 50 μM, unless otherwise mentioned. Reactions were stopped after 10 min by adding 25 μl of 1 N H_2SO_4. After addition of 8 nmol of internal standard (octanoyl-CoA or hexadecanoyl-CoA, depending on the substrate used), the samples were extracted with 1,200 μl of isopropanol-heptane, 4:1 (v/v). Subsequently, the supernatant was dried under N_2 at 40°C after addition of 20 μl of 2% (v/v) reduced Triton X-100 and the samples were finally reconstituted in 100 μl of buffer A [CH_3CN-H_2O-0.25 M NH_4OAc (pH 5.0) 10:60:20, v/v/v]. An aliquot (~90 μl) was injected onto a NovaPak C18 column (Waters; 3.9 × 150 mm; 60 Å; 4 μm), and the CoA esters were eluted with increasing concentrations of buffer B [CH_3CN-H_2O-0.25 M NH_4OAc (pH 5.0), 80:10:10, v/v/v] in buffer A. Flow rate was 0.8 ml/min, and effluents were monitored by using an online UV detector (Waters 486) set at 258 nm (see **Fig. 1**).

RESULTS AND DISCUSSION

Kinetic analysis of PAHX-catalyzed conversion of 3-methylacyl-CoA esters

When the activity of purified recombinant human PAHX toward racemic 3-methylhexadecanoyl-CoA was tested at increasing substrate concentrations and a molar substrate-albumin ratio of 2, a plateau was reached from 100 μM onwards (**Fig. 2A**); an apparent K_m of 40.8 μM was calculated. Figure 2B further shows that for a substrate concentration of 50 μM, the reaction was linear for up to 10 min.

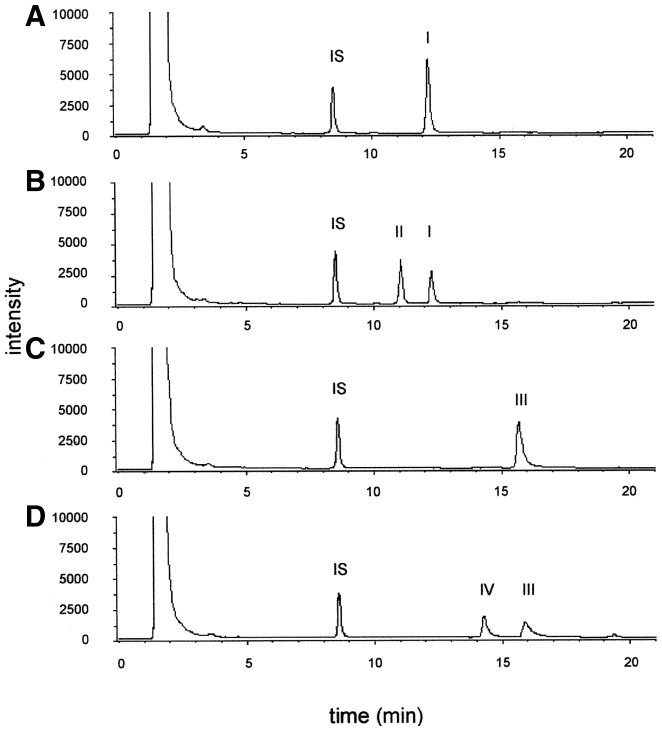

Fig. 1. HPLC analysis of the hydroxylation reactions. Reaction mixtures containing 50 μM 3-methyldodecanoyl-CoA (A, B) or 3-methylhexadecanoyl-CoA (C, D) were incubated for 0 min (A, C) and for 10 min (B, D) with recombinant human phytanoyl-CoA hydroxylase (PAHX) under standard conditions. After addition of octanoyl-CoA (internal standard), the acyl-CoA esters were extracted and subjected to HPLC analysis. The numbered peaks indicate I: 3-methyldodecanoyl-CoA; II: 2-hydroxy-3-methyldodecanoyl-CoA; III: 3-methylhexadecanoyl-CoA; and IV: 2-hydroxy-3-methylhexadecanoyl-CoA. The identity of peaks I, III, and IV was confirmed in separate HPLC analyses using standard compounds.

When assaying the hydroxylation of 3-methylhexadecanoyl-CoA (50 μM) at increasing substrate-albumin ratios (ν), a plateau was reached from a ratio of 4 onwards (Fig. 2C). Interestingly, at this substrate concentration, no hydroxylation of 3-methylhexadecanoyl-CoA could be detected in the absence of albumin ($\nu = \infty$) (Fig. 2C). However, upon lowering the substrate concentration in the absence of albumin, the bulk of 3-methylhexadecanoyl-CoA was converted to its 2-hydroxy intermediate (100% conversion was measured for concentrations up to 5 μM; at a final concentration of 10 μM, 63.5% of the substrate was hydroxylated; at a final concentration of 25 μM, only 44%; data not shown). The conversion of shorter 3-methylacyl-CoA esters (50 μM 3-methylnonanoyl-CoA or 3-methyldodecanoyl-CoA) was much less dependent on the presence of albumin (Fig. 2C).

Taken together, these observations suggest that the enzyme cannot display any activity when the substrate is presented as a micelle. The critical micellar concentration

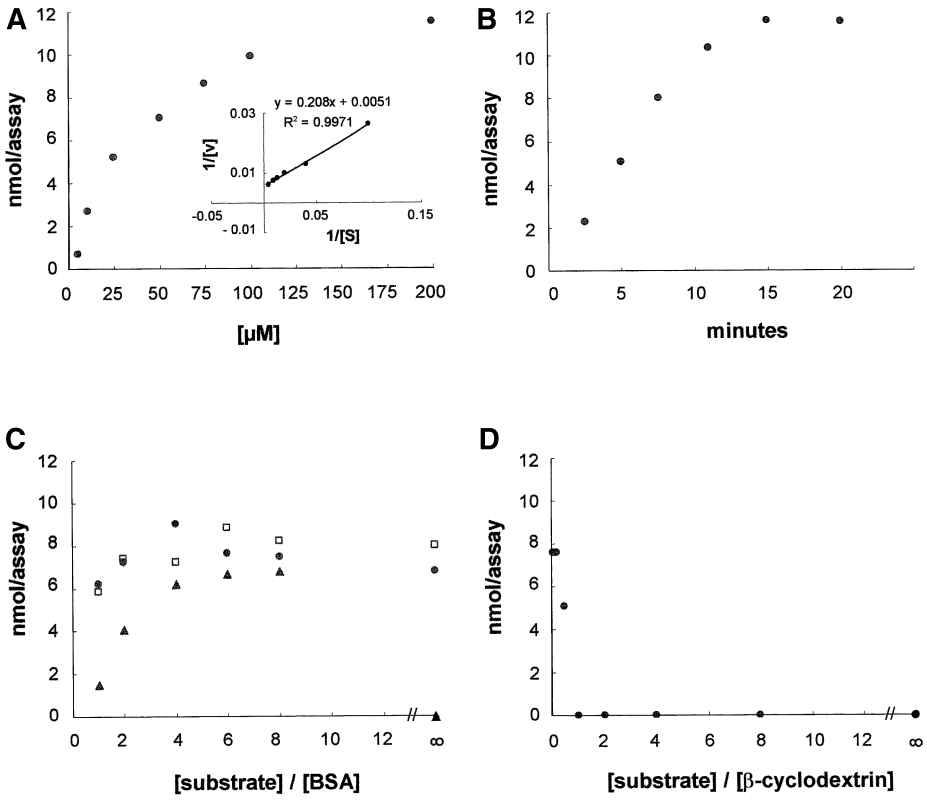

Fig. 2. Functional characterization of recombinant human PAHX. A: Substrate dependence of recombinant human PAHX. Hydroxylase activity was measured at increasing concentrations of 3-methylhexadecanoyl-CoA and a molar substrate:albumin ratio of 2. Results are presented as nmol product generated in the assay. Inset: Lineweaver-Burk transformation of the obtained data. B: Time curve. Conversion of 3-methylhexadecanoyl-CoA was measured at different time intervals with a substrate concentration of 50 μM and a molar substrate:albumin ratio of 4. Final assay volume was 500 μl. C: Dependence of the hydroxylation reaction on albumin. The hydroxylation of 50 μM 3-methylhexadecanoyl-CoA (triangle), 3-methylundecanoyl-CoA (square) and 3-methylnonanoyl-CoA (circle) was assayed in the presence of increasing substrate:albumin ratios. D: Dependence of the hydroxylation of 3-methylhexadecanoyl-CoA on β-cyclodextrin. The hydroxylation of 50 μM 3-methylhexadecanoyl-CoA was measured in the absence of albumin at increasing substrate-β-cyclodextrin ratios.

(CMC) of 3-methylhexadecanoyl-CoA is likely comparable to the CMC of palmitoyl- and stearyl-CoA. As for the former compound, CMC values of 30 μM to 60 μM have been reported (21, 22, 23), in all our experiments performed with 50 μM of 3-methylhexadecanoyl-CoA, the substrate might have been presented in micelles. Albumin, through binding the monomeric CoA-ester, will lower the amount of micelles, and hence stimulate the activity at substrate concentrations above the CMC. At low ν-ratios (ν = 1–3), almost all acyl-CoA is complexed to BSA. Hence, the lower activity under these conditions would further indicate that the enzyme is also not able to act on albumin-bound substrate. At low substrate concentrations, no micelles are formed, explaining the high conversion rates, even in the absence of BSA. For shorter 3-methylacyl-CoA esters, which possess a higher CMC, the hydroxylation rates at 50 μM are less influenced by the concentration of albumin (Fig. 2C).

During this work, Mukherji et al. (24) reported on the use of β-cyclodextrin as required for PHAX measurements as a "solubilizing agent." As acyl-CoA esters are detergents themselves, β-cyclodextrin cannot act as a substrate-solubilizing tool. In the absence of albumin, a beneficial effect of β-cyclodextrin was observed, but rather high amounts were required, i.e., a 2- to 8-fold molar excess over substrate (Fig. 2D). At these concentrations, cyclodextrin will likely capture acyl-CoAs, thereby lowering the amount of micellar substrate. A similar explanation might account for the stimulatory effect of SCP_2, seen at high substrate concentrations by Mukherji et al. (24). On the basis of this observation, these authors suggested that in vivo SCP2 could act as a "solubilizing agent" and that the in vivo substrates for PAHX may be SCP2 complexes. The ARD-like symptoms and biochemical alterations observed in SCP2-deficient mice (25) support this contention. However, a beneficial effect of SCP2 on PAHX activity is seen only at high substrate concentrations, which might never be reached under physiological conditions, rendering this hypothesis rather implausible.

Both a CoA moiety and a branch at position 3 are required for PAHX-dependent hydroxylation

Although PAHX efficiently catalyzes the hydroxylation of phytanoyl-CoA and its chemical substitute, 3-methyl-

hexadecanoyl-CoA, no PAHX-dependent hydroxylation of 3-methylhexadecanoic acid could be observed. This indicates that in α-oxidation, the activation step, which precedes the hydroxylation step (9), is necessary for PAHX-dependent hydroxylation. In contrast to the data reported by Mukherji et al. (24), we were not able to show any PAHX-dependent hydroxylation of straight-chain acyl-CoA esters. Under standard conditions, neither hexadecanoyl-CoA nor octanoyl-CoA was converted to the corresponding 2-hydroxy intermediate (**Fig. 3**). Furthermore, no hydroxylation of either pristanoyl-CoA, 2-methylhexadecanoyl-CoA, or 4-methylnonanoyl-CoA could be detected, corroborating the conclusion that a branch at position 3 is a prerequisite for the activity of PAHX.

Activity of PAHX is dependent on the chain length of the alkyl backbone of the CoA ester

In order to investigate whether PAHX recognizes, in addition to the CoA moiety and the branching point, the length of the alkyl backbone, several mono-branched 3-methylacyl-CoA esters were synthesized and tested as substrates for PAHX. Isovaleryl-CoA (3-methylbutanoyl-CoA), 4-phenyl-3-methylbutanoyl-CoA, and 3-methylpentanoyl-CoA were not hydroxylated by PAHX. However, 3-methylheptanoyl-CoA and 5-phenyl-3-methylpentanoyl-CoA were converted to their 2-hydroxy derivative (Fig. 3). Hence, one can conclude that a chain length of at least seven carbon atoms (straight-chain or generated by the presence of a phenyl group at the ω-carbon atom) is a prerequisite for PAHX-dependent hydroxylation. The optimal chain length appears to be 9–12 carbon atoms (Fig. 3). Other modifications of the alkyl chain (presence of methyl groups or an unsaturated bond at a position behind the 3-methyl branch) seem to have no effect on PAHX-dependent hydroxylation.

Activity of PAHX depends on the chain length of the branch at position 3

To investigate whether PAHX recognizes only the branching point, but not the length of the branch, acyl-CoA esters with an ethyl, propyl, butyl or phenyl branch were designed. 3-Ethylnonanoyl-CoA was hydroxylated at ~60% of the hydroxylation rates for 3-methylnonanoyl-CoA, whereas 3-propylnonanoyl-CoA was, under standard assay

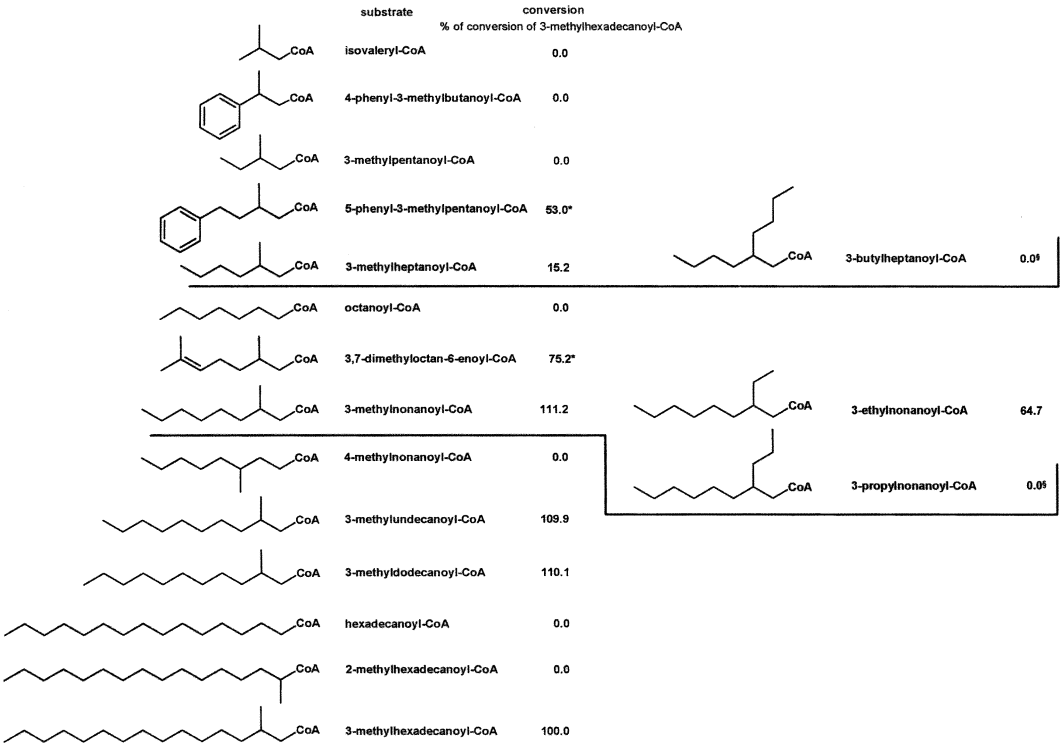

Fig. 3. Dependence of the hydroxylation reaction on the length and composition of the acyl group of the CoA ester. The conversion of different acyl-CoA esters was measured under standard conditions. Results were calculated as nmol product, generated per assay, and are presented as percentage of the hydroxylation of the reference compound, 3-methylhexadecanoyl-CoA. Straight-chain acyl-CoA esters and compounds with a methyl branch are shown at the left, and those with a longer alkyl branch at the right, thereby considering the chain with the lowest number of carbon atoms as the branch. All results are mean values of two independent experiments, except for the results followed by *, which were single determinations. § Although under standard conditions, no hydroxylation of 3-propylnonanoyl-CoA could be detected, experiments performed with 100 μM of substrate at a [S]/[BSA] of 1, and with 20 μg of recombinant protein showed a minor hydroxylation of this compound, with rates up to 5.6% of the hydroxylation rates for 3-methylhexadecanoyl-CoA, measured under the same conditions. 3-Butylheptanoyl-CoA, on the other hand, was not converted, even under these conditions, into the corresponding 2-hydroxy acyl-CoA ester.

conditions, not converted into the corresponding 2-hydroxy intermediate (Fig. 3). When using higher amounts of enzyme at higher substrate concentrations and a molar substrate-albumin ratio of 1, a minor conversion of this compound could be seen (Fig. 3). The fact that 3-butylheptanoyl-CoA is not a substrate for PAHX (not even under the latter conditions) is probably also due to the length of the branch, inasmuch as 3-methylheptanoyl-CoA is converted to the corresponding 2-hydroxy compound, albeit at a low rate (Fig. 3).

Previous studies have revealed that the PAHX-dependent hydroxylation of phytanic acid is not stereoselective with regard to the 3-methyl branch (9). We now provide evidence that a branch at position 3 is a prerequisite for the activity of PAHX, and also demonstrate that an ethyl branch is tolerated. Hence, the enzyme must have a catalytic pocket that predominantly recognizes the branching point and, to a lesser extent, the length of the branch. For the latter, three carbon atoms seem to be the limit. How this fits with the access of both 3-methylhexadecanoyl-CoA isomers (9) remains unsolved. Most likely, the catalytic pocket is large enough to allow entry of a 3-methyl branch in either the *R*- or *S*-configuration.

Next to its physiological substrate, phytanoyl-CoA, (recombinant) PAHX acts on 3-methyl mono-branched acyl-CoAs with a chain length of 17 down to seven carbon atoms. Shorter acyl-CoAs, such as isovaleryl-CoA and 3-methylpentanoyl-CoA, are not substrates. This would be consistent with the notion that peroxisomes (and PAHX) are not involved in the breakdown of the branched amino acid leucine, which is converted to isovaleryl-CoA. On the other hand, an older study by Stokke (26) indicated that 3-methylvaleric acid, but not 3-methylbutyric acid, can undergo α-oxidation in guinea pig kidney slices. Perhaps the guinea pig enzyme is more active toward shorter substrates than recombinant human PAHX.

Concerning the hydroxylation of 3-methyl-branched medium chain acyl-CoAs, the presence of extra methyl branches, double bonds, or bulky ω-substituents seems to be well tolerated. So far, no reports on the α-oxidation of naturally occurring 3-methyl-branched fatty acids with such chain lengths and/or modifications have been made. However, given the prevalence in nature of isoprenoids as geraniol and farnesol, such 3-methyl-branched medium-chain fatty acids are more than likely present in the diet of man. In a thorough analysis of human milk, traces of 3,7,11-trimethyldodecanoic acid were found (27). The latter compound can be generated from 5,9,13-trimethyltetradecanoic acid (which is also present in human milk) by one cycle of β-oxidation but can also be derived from farnesoic acid, inasmuch as in the work-up of the samples, all double bonds were reduced. In addition, 3- and 5-methyl mono-branched medium-chain fatty acids were also shown to be constituents of the lipid fraction of human milk (27).

With regard to the synthetic substrates, some older studies by Stokke, Try, and Eldjarn (28) showing the α-oxidation (one carbon degradation) of 3,6-dimethyloctanoic acid in man and in kidney slices of guinea pig (26) and rat (29), and of 3,9,9-trimethyldecanoic acid in guinea pig liver (30) are called to mind. The high activity of recombinant PAHX toward 3,7-dimethyl-6-octenoyl-CoA, 3-methylnonanoyl-CoA, and 3-methylundecanoyl-CoA that we observed now fully supports this pioneering work. Also, the use of isotopically labeled fatty acids as imaging agents in tomographic studies is worth discussing. In addition to an ω-substitution (iodo-, iodovinyl-, iodophenyl-), a 3-methyl branch is often introduced on these molecules to lower their clearance (31). Our data suggest that the hydroxylation of the CoA esters of such ω-substituted fatty acid derivatives, and likely also their overall α-oxidation, will not be impaired. Hence, the metabolic degradation of these compounds should be taken into account.

Finally, with regard to the substrate spectrum of PAHX, it is of interest to note that even 3-ethylacyl-CoAs can be hydroxylated. The presence of 3-ethyl-branched long-chain acids in diets of mammals has never been documented and seems also unlikely, although such compounds do occur in nature. Butterflies produce a particular form of juvenile hormone (type O; 10,11-epoxy-3,7,11-triethyl-2,6-dodecadienoic acid methylester) containing a 3-ethyl branch (32). Whether the CoA esters of the hydroxylated compounds can be cleaved by 2-HPCL is also not known.

Related to ARD, our findings further suggest that in addition to phytanic acid, other 3-methyl-branched fatty acids will also accumulate. To date, this has received little attention, and in plasma and urine of patients with Refsum disease, only the presence of unsaturated phytanic acid analogs, likely derived from geranylgeraniol, has been reported (14).

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