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Utilization of Sterol Carrier Protein-2 by Phytanoyl-CoA 2-Hydroxylase in the Peroxisomal α Oxidation of Phytanic Acid

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Summary

Since it possesses a 3-methyl group, phytanic acid is degraded by a peroxisomal α-oxidation pathway, the first step of which is catalyzed by phytanoyl-CoA 2-hydroxylase (PAHX). Mutations in human PAHX cause phytanic acid accumulations leading to Adult Refsum's Disease (ARD), which is also observed in a sterol carrier protein 2 (SCP-2)-deficient mouse model. Phytanoyl-CoA is efficiently 2-hydroxylated by PAHX in vitro in the presence of mature SCP-2. Other straight-chain fatty acyl-CoA esters were also 2-hydroxylated and the products isolated and characterized. Use of SCP-2 increases discrimination between straight-chain (e.g., hexadecanoyl-CoA) and branched-chain (e.g., phytanoyl-CoA) substrates by PAHX. The results explain the phytanic acid accumulation in the SCP-2-deficient mouse model and suggest that some of the common symptoms of ARD and other peroxisomal diseases may arise in part due to defects in SCP-2 function caused by increased phytanic acid levels.

Introduction

Phytanic acid is a branched-chain isoprenoid fatty acid derived from chlorophyll and is part of the human diet. It cannot be degraded by the normal fatty acid β-oxidation pathway due to the presence of a 3-methyl group, and instead a preliminary α-oxidation pathway within peroxisomes excises its C-2 methylene group to give pristanic acid [1, 2]. This can be further degraded by the β-oxidation pathway, initially in peroxisomes and subsequently in mitochondria [3]. The enzymes of the α-oxidation pathway [4] are found in many different types of organisms, suggesting that it is an important general detoxification mechanism. Defects in the α-oxidation pathway can result in a spectrum of neurological syndromes in humans, including Adult Refsum's Disease (ARD). Symptoms of ARD include deterioration of vision due to retinitis pigmentosa followed by blindness, anosmia, deafness, peripheral neuropathy, ataxia, ichthyosis, and skeletal dysplasias, with premature death eventually occurring due to cardiac arrhythmias. The only available treatment for ARD involves reduction of dietary phytanic acid, which ameliorates some symptoms (e.g., ataxia, ichthyosis), but visual or olfactory damage appear to be irreversible [2, 5].

The individual steps of the α-oxidation pathway have only recently been elucidated. Initially, phytanic acid is converted to its coenzyme A ester by a nonspecific ligase [6]. Phytanoyl-CoA (**1**) is then 2-hydroxylated in a reaction catalyzed by an iron(II) and 2-oxoglutarate-dependent oxygenase, phytanoyl-CoA 2-hydroxylase (PAHX) [7–10]. A thiamine pyrophosphate-dependent lyase cleaves 2-hydroxyphytanoyl-CoA into pristanal [11, 12] and formyl-CoA [13]. Pristanal is converted to pristanic acid by an NADPH-dependent oxidoreductase [14]. Reesterification with coenzyme A by LCFA-CoA synthetase [15] or very-long-chain fatty-acyl-CoA synthetase [16] to give pristanoyl-CoA (**2**) and epimerization [17, 18] allows further degradation by the "normal" β-oxidation pathway [3]. Like all other characterized peroxisomal proteins, the enzymes in the α-oxidation pathway are initially synthesized with N- or C-terminal peroxisomal targeting sequences (PTS-1 [19, 20] or PTS-2 [21]) that are required for importation into peroxisomes.

In ARD around 45% of cases are associated with defects in phytanoyl-CoA 2-hydroxylase (PAHX) [7, 9, 22, 23], with the remainder associated with defects in peroxisomal protein import via peroxin 7 [24, 25]. PAHX may also be implicated both in aspects of signal transduction [26] and blood coagulation [27]. The biochemical effects of some clinically observed mutations of PAHX can be rationalized on the basis of primary sequence comparisons, secondary structure predictions, and active site motifs apparent from the crystal structures of bacterial oxygenases [4].

Naturally occurring phytanoyl-CoA (**1**) exists as a mixture of its (3*S*)- and (3*R*)-epimers, both of which are substrates for PAHX [4, 28]. Only one of the two possible diastereoisomers is formed by PAHX-catalyzed oxidation of either the (3*S*)- or (3*R*)-epimer [29]. In vivo substrate analog studies [30] suggest that the absolute stereochemistry of the diastereomeric 2-hydroxyphytanoyl-CoA products formed from the (3*R*)- and (3*S*)-epimers is (2*S*, 3*R*) and (2*R*, 3*S*), respectively. 2-oxoglutarate-dependent oxygenases are believed to catalyze prime substrate oxidation via a ferryl [Fe(IV)=O/Fe(III)-O·] intermediate in a radical rebound process [31]. We considered it improbable that a PAHX-phytanoyl-CoA (**1**) binding mode with a rigid three-point attachment of coenzyme A, methyl group, and fatty side chain could lead to the observed stereoselectivity. Thus, we proposed that PAHX binds its phytanoyl-CoA substrate by

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the 3-methyl group and either the coenzyme A or fatty side chain [28].

Phytanoyl-CoA (**1**) is amphipathic and forms micelles in aqueous environments; hence, β cyclodextrin has been used as a solubilizing agent during previous in vitro experiments with PAHX [8]. In humans, phytanic acid is transported in lipoproteins, with most being found associated with cholesterol in low-density lipoprotein (LDL) [32]. We considered the possibility that the isoprenoid side chain of phytanoyl-CoA (**1**) either binds to the peroxisomal membrane or is solubilized by a coprotein during the in vivo reaction catalyzed by PAHX. A precedent for the latter possibility is seen in retinoid metabolism, were biological activity is mediated via complexes with cellular retinoid binding proteins [33]. A human acyl-CoA binding protein (ACBP) has been reported [34], but it is apparently localized in the cytosol. Although ACBP selectively binds acyl-CoA esters, there is no reported evidence for its presence in the peroxisome.

Sterol carrier protein 2 (SCP-2) is located within the peroxisome and is involved in β oxidation of fatty acyl-CoA esters [35, 36]. SCP-2-deficient mice accumulate phytanic acid [37] and have a similar phenotype in some respects to ARD with the presence of neuropathy, hypolipidaemia, and cardiac arrhythmias when fed a diet containing phytanic acid. However, they lack certain key features of human ARD including retinitis pigmentosa, anosmia, ataxia, ichthyosis, and deafness [2, 36]. However, in this case it cannot be completely excluded that phytanic acid accumulation arises as a consequence of secondary defects in SCPx (3-ketoacyl-CoA thiolase/SCP-2) function in β oxidation [37]. Phytanoyl-CoA (**1**) is known to bind much more tightly to SCP-2 (K_d = 250 nM) than phytanic acid, pristanic acid, or pristanoyl-CoA (**2**) [37]. In addition, binding of phytanoyl-CoA (**1**) to SCP-2 reduces the rate of its hydrolysis to phytanic acid [38]. We therefore considered the possibility that SCP-2 is a solubilizing protein for substrates of the α-oxidation pathway. Here we demonstrate that SCP-2 acts as a solubilizing coprotein during the in vitro PAHX-catalyzed reaction and propose an analogous in vivo role for SCP-2.

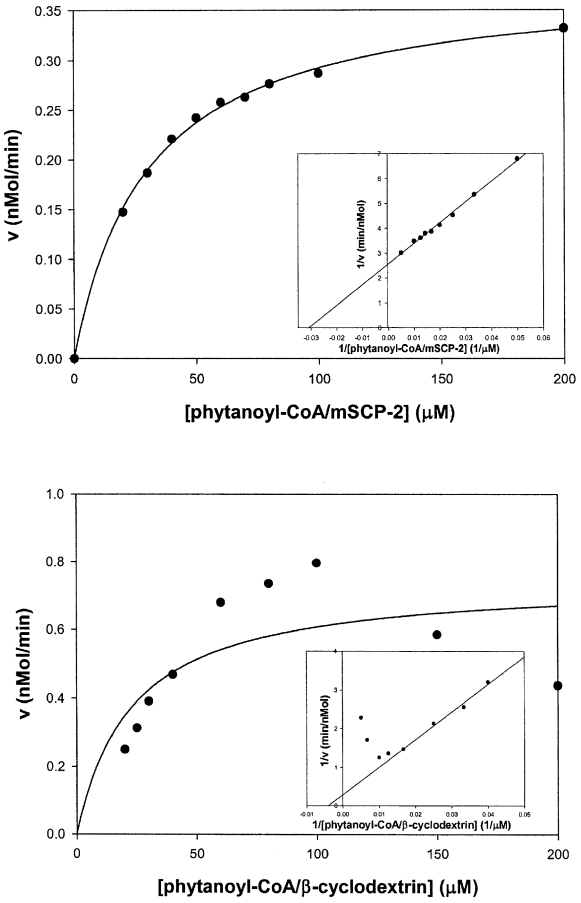

Figure 1. Kinetic Analyses of PAHX-Catalyzed Conversion of Phytanoyl-CoA

(Top) In the presence of mature SCP-2 and (bottom) in the presence of β cyclodextrin. Note that at high substrate concentrations in (bottom graph), the rate of reaction is reduced (noncompetative substrate inhibition) compared to that expected, leading to nonlinearity in the double reciprocal plot (inset).

Results and Discussion

Expression and Purification of Sterol Carrier Protein 2

The pro- and mature forms of human SCP-2 were cloned and expressed in *E. coli* at ~20% of total cell protein. A convenient large-scale protein purification procedure employing cation-exchange chromatography was developed, giving protein at >99% purity (by SDS-PAGE). The identity of mature SCP-2 was confirmed by ESI MS (13,242 Da calculated; 13,243 ± 0.96 Da observed). Recombinant mature PAHX was obtained at >95% purity (by SDS-PAGE) as previously described [4].

Hydroxylation of Phytanoyl-CoA

The activity of highly purified mature PAHX was analyzed for both 2-oxoglutarate conversion [39] and oxidative modification of phytanoyl-CoA (**1**) [28] by separate assays. (3*R*, 3*S*)-phytanoyl-CoA conversion was stimulated by the presence of mature SCP-2 compared to the level observed with β cyclodextrin [4, 28], the previously used in vitro solubilizing agent. The identity of the epimeric 2-hydroxyphytanoyl-CoA product obtained in the presence of SCP-2 was confirmed by coelution of the product peak with authentic product upon HPLC analysis and ESI MS analysis. The reaction apparently displayed Michaelis-Menten kinetics in the presence of SCP-2 (Figure 1, top), and the following apparent steady-state parameters were derived: K_M = 29.5 ± 1.7 μM; K_{cat} = 0.40 ± 0.01 s^{-1}; K_{cat}/K_M = 1.34 × 10^4 M^{-1} s^{-1}. Product formation was linear over a 10 min time course in the presence of SCP-2. In contrast, non-Michaelis-Menten kinetics were observed in the presence of β cyclodextrin, and apparent K_M values for phytanoyl-CoA varied between 7 and 39 μM. At higher phytanoyl-CoA (**1**) concentrations, apparent noncompetitive substrate inhibition occurred in the presence of β cyclodextrin (Figure 1, bottom, inset). It is unclear what is the basis of the observed inhibition, but it may be due to improper solubilization. No reduction of activity at higher sub-

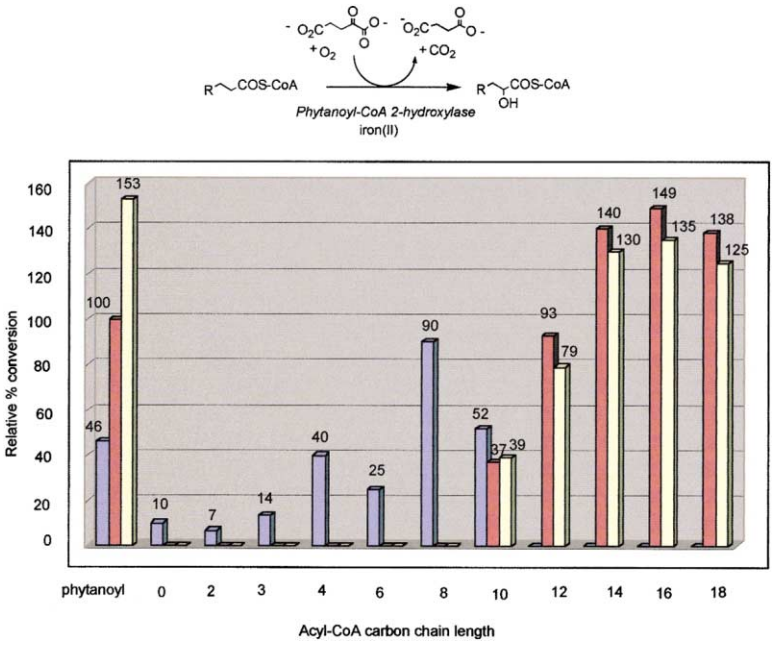

Figure 2. Stimulation of 2-Oxoglutarate Conversion by Straight-Chain Acyl-CoA Esters as a Function of Chain Length

Levels of conversion are normalized to that observed for phytanoyl-CoA in the presence of β cyclodextrin (100%, 18.06 nmol/min/mg) under standard assay conditions (final substrate concentration in assay = 50 μM). A ~10% conversion level was observed for PAHX in the absence of any "prime" substrate ("uncoupled turnover"). A chain length of zero corresponds to coenzyme A, a chain length of 2 corresponds to acetyl-CoA, etc. Results of incubations are without addition of a solubilizing factor (blue), with β cyclodextrin (red), or with SCP-2 (yellow). Product formation (if any) was observed by HPLC analyses for all acyl-CoA esters that gave significant stimulation of 2-oxoglutarate conversion (C-4 to C-10 without solubilizing agent and C-12 to C-18 with β cyclodextrin/SCP-2).

strate concentrations was observed when SCP-2 was used as the solubilizing agent (Figure 1, top).

Hydroxylation of Straight-Chain Fatty Acyl-CoA Esters

Some iron(II) and 2-oxoglutarate-dependent oxygenases have been shown to accept a number of different prime substrates, and the choice of substrate can determine the type of oxidative reaction performed [10]. 2-oxoglutarate-dependent oxygenases that have been shown to convert unnatural substrates including proline hydroxylase [40], DAOCS [41], DAOC/DAC synthase [42], and CAS [43]. Little work has been reported on the substrate selectivity of PAHX, although 3-methylhexadecanoic acid (probably as its CoA ester **3**) is hydroxylated by permabilized cells [30, 44], presumably in a PAHX-mediated process. We therefore investigated whether PAHX was able to hydroxylate straight-chain fatty acyl-CoA esters by assaying for 2-oxoglutarate conversion (Figure 2) and acyl-CoA 2-hydroxylation (or other oxidative modification) by HPLC (data not shown). Three conditions were investigated: without any solubilizing agent, in the presence of β cyclodextrin, and in the presence of SCP-2.

In the absence of any solublizing agent, there was no evidence for conversion of phytanoyl-CoA (**1**) within the limits of detection for the HPLC assay, although some (uncoupled) 2-oxoglutarate conversion was observed (Figure 2, far left). However, some straight-chain acyl-CoA esters (C-4 to C-8) stimulated 2-oxoglutarate conversion and were efficiently hydroxylated as judged by HPLC analysis. The minimum chain length for activity under standard assay conditions appears to be four carbons, as no hydroxylation was observed with propionyl-CoA (C-3) or acetyl-CoA (C-2). Decanoyl-CoA showed considerably reduced 2-oxoglutarate conversion compared to octanoyl-CoA, and this probably reflects the reduced solubility of the former substrate in the absence of SCP-2 or β cyclodextrin. Acyl-CoA esters with chain lengths longer than C-10 were not analyzed in the absence of solubilizing agents due to their insolubility in aqueous solvents.

In the presence of β cyclodextrin, phytanoyl-CoA (**1**) was converted to 2-hydroxyphytanoyl-CoA [4, 28]. Straight-chain acyl-CoA esters with side chains of 12–18 carbons stimulated 2-oxoglutarate conversion (Figure 2) in the presence of β cyclodextrin, and 2-hydroxyl product formation was observed by HPLC analysis.

In contrast to previous negative reports using whole or permabilized hepatocytes [30, 45], hexadecanoyl-CoA (C-16 **4**) was converted in vitro via PAHX catalysis. ^{1}H NMR and ESI MS analyses confirmed the structure of the product as 2-hydroxyhexadecanoyl-CoA. Kinetic analysis of hexadecanoyl-CoA in the presence of SCP-2 (Figure 3, top) gave the following apparent kinetic parameters: $K_M = 25.1 \pm 1$ μM; $K_{cat} = 0.044 \pm 0.0005$ s^{-1}; $K_{cat}/K_M = 1.75 \times 10^3$ M^{-1} s^{-1}. In the presence of β cyclodextrin, the apparent K_M value varied between 40 and 50 μM, and the kinetics approximated a Michaelis-Menten curve (Figure 3, bottom). Thus, the in vitro catalytic efficiency (as measured by K_{cat}/K_M) of PAHX in the presence of SCP-2 for hexadecanoyl-CoA (**4**) is approximately eight times less than for phytanoyl-CoA (**1**), with a lower K_{cat} value accounting for the majority of the difference. A V/K competitive experiment using equimolar quantities of phytanoyl-CoA (**1**) and hexadecanoyl-CoA (**4**) in the presence of SCP-2 confirmed the more efficient conversion of phytanoyl-CoA (**1**) compared to hexadecanoyl-CoA (**4**), with significant conversion of the latter only being observed after the former was almost completely converted (Figure 4).

Heptadecanoyl-CoA (C-17) and arachidoyl-CoA (C-20) were also converted to the anticipated 2-hydroxylated products (as judged by HPLC and ESI MS analyses). These observations demonstrate that PAHX does not discriminate between substrates with an even or

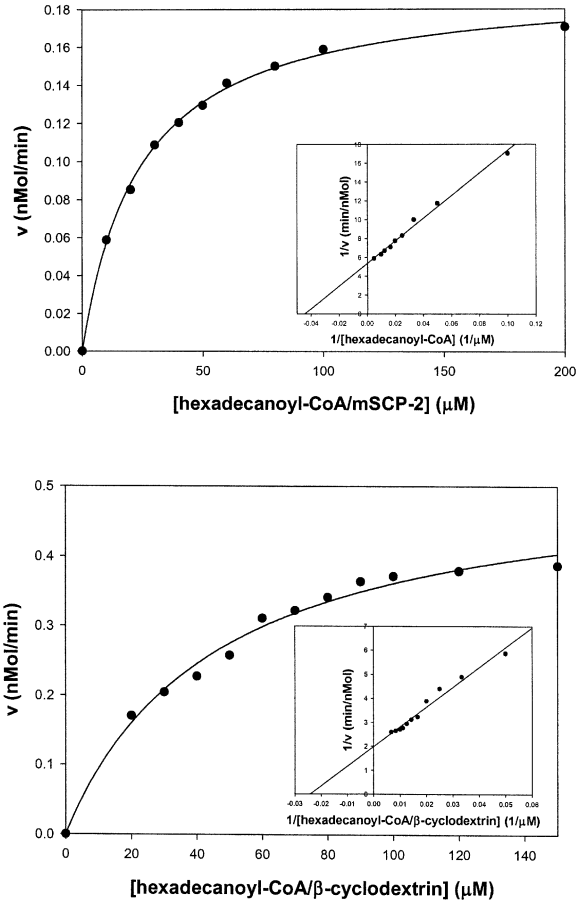

Figure 3. Kinetic Analyses of PAHX-Catalyzed Conversion of Hexadecanoyl-CoA

(A) In the presence of mature SCP-2 and (B) in the presence of β cyclodextrin.

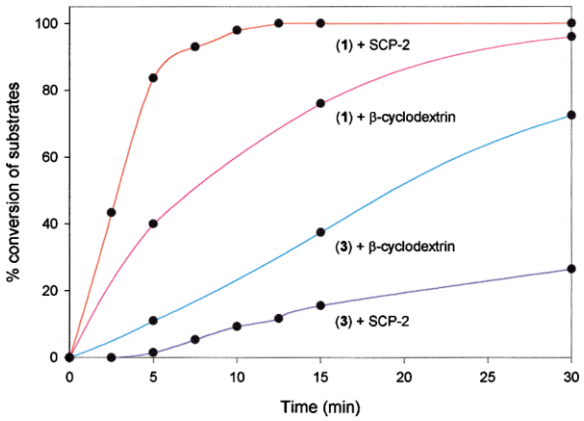

Figure 4. Comparison of the Efficiency of Conversion of Phytanoyl-CoA (1) and Hexadecanoyl-CoA (3) by PAHX

Approximately 0.56 μg were used per assay. Two experiments were performed using a (1:1) mixture of (1) and (3) (50 μM of each) in the presence of mature SCP-2 [(1) red and (3) cyan] and β cyclodextrin [(1) pink and (3) blue], respectively. Product formation was followed by stopped HPLC assays of mixtures incubated for the required length of time.

odd number of methylene groups and that some acyl-CoA esters with extended side chains can be hydroxylated.

Preferential metabolism of hexadecanoyl-CoA (4) by the β-oxidation pathway (which is estimated to be 10–15 times greater than the rate of α oxidation [46]), together with observed substrate selectivity, may account for the previous reports that hexadecanoyl-CoA (4) was not an in vivo substrate for PAHX [30, 44]. Thus, peroxisomal α oxidation of straight-chain fatty acids may only occur to a significant extent when phytanic acid intake in the diet is relatively low. However, it is possible that in vivo behavior could be considerably different, depending on the effect of the peroxisomal membrane or other proteins on the behavior of the acyl-CoA substrates and proteins.

Hydroxylation of Branched Short-Chain Fatty Acyl-CoA Esters

We reasoned that if there was a methyl but not an (extended) fatty side-chain binding site within the enzyme, then PAHX may accept branched short-chained acyl-CoA esters as substrates. Thus, several branched-chain acyl-CoA esters were tested as substrates for PAHX (Figure 5). Isovaleryl-CoA (5) was converted to the expected 2-hydroxylated product (Figure 5), the identity of which was confirmed by ^{1}H NMR and ESI-MS analyses. This substrate displayed non-Michaelis-Menten kinetics, although the reason for this is unclear. PAHX-mediated oxidation of isovaleryl-CoA was reduced by the presence of SCP-2 in the standard assay conditions, as judged by conversion of 2-oxoglutarate to succinate and CO_2. This result contrasts with the effect of SCP-2 on conversion of the long-chain fatty acyl-CoA derivatives and is consistent with its role as a solubilizing agent. Conversion of isovaleryl-CoA is probably reduced in the presence of SCP-2 because the latter impedes access of the former to the active site of PAHX. This result also suggests that SCP-2 is not behaving as an allosteric activator. PAHX is probably not important in amino acid metabolism, even though isovaleryl-CoA (5) may be present in peroxisomes as a product of leucine metabolism [47], as phytanoyl-CoA (1) is likely to be a preferential substrate.

In contrast to isovaleryl-CoA (5), isobutanoyl-CoA (6) was not found to be a substrate for PAHX (Figure 5). This result is consistent with the exclusion of pristanic acid from the α-oxidation pathway due to the presence of a α-methyl group. The importance of the α-methylene group is also illustrated by the observation that 2-methylhexadecanoyl-CoA (7) was not a substrate for PAHX [44].

Metabolism of Unsaturated Substrates

Certain 2-oxoglutarate-dependent oxygenases can oxidize unsaturated substrate analogs to epoxides [40–42]. Thus, commercially available *trans*-3-methyl-2-butenoyl-CoA (8) was assessed as a substrate for PAHX. 2-oxoglutarate conversion was stimulated by its presence (Figure 6), but no oxidized product was observed. Analogous uncoupling of prime substrate oxidation from 2-oxoglutarate conversion has been observed for sub-

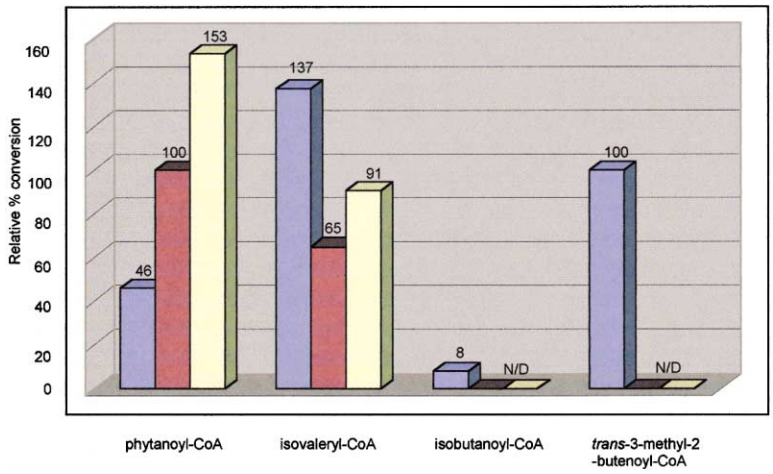

Figure 5. Stimulation of 2-Oxoglutarate Conversion by Branched-Chain Acyl-CoA Esters

Levels of conversion are normalized to that observed for phytanoyl-CoA in the presence of β cyclodextrin (100%, 18.06 nmol/min/mg) under standard assay conditions (final substrate concentration in assay = 50 μM). A 5% conversion level was observed for PAHX in the absence of any prime substrate. Conditions shown are without solubilization agent (blue), with β cyclodextrin (red), or with SCP-2 (yellow). A 2-hydroxylated product was observed only when using isovaleryl-CoA as a substrate. There was no evidence for oxidized product formation with *trans*-3-methyl-2-butenoyl-CoA or isobutanoyl-CoA. Incubation of phytanoyl-CoA did not give any product in the absence of β cyclodextrin or SCP-2. N/D indicates not determined.

strate analogs with other 2-oxoglutarate-dependent oxygenases [48, 49].

The results suggest that other α, β-unsaturated fatty acids, e.g., phytenic acid, may not be directly metabolized by the α-oxidation pathway. They might be discriminated against as substrates for PAHX because of the constraints imposed by the geometry of the double bond. Further, the anticipated epoxide products are potential alkylating agents and are rare in nature. Substrates with remote double bonds are known to bind tightly to SCP-2 [50], but it is unclear if they can be metabolized by the α-oxidation pathway.

Applications of Substrate Selectivity of PAHX

The results demonstrate that wild-type mature PAHX can accept a number of alternative substrates to phytanoyl-CoA. Although phytanoyl-CoA (**1**) can be obtained by chemical [28] or enzymatic [51] synthesis, its amphipathic properties make it difficult to handle. Diagnostic assays using commercially available alternative substrates (Figure 6), such as hexadecanoyl-CoA (**4**) or isovaleryl-CoA (**5**), are therefore potentially useful. The former requires solubilization, and so the latter is potentially a better candidate for diagnostic assays. In diagnostic assays, the possibility that PAHX mutations [4] will affect conversion of substrate analogs differently to phytanoyl-CoA (**1**) must be considered, since differential effects of mutations on the oxidation of natural and unnatural substrates has been observed for related oxygenases [52].

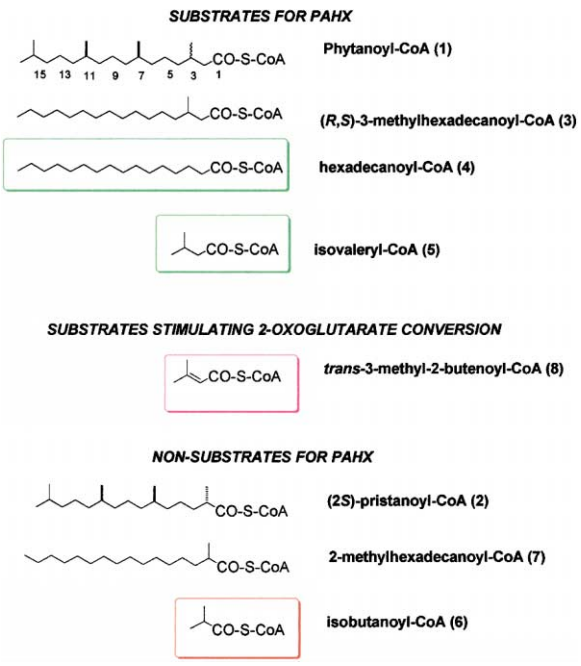

Figure 6. Acyl-CoA Esters Tested as Substrates for PAHX

Enclosed compounds are those tested as substrates in this work.

Biological Implications

The demonstration that PAHX can catalyze the hydroxylation of straight-chain fatty acyl-CoA esters, together with the demonstration of a functional role for SCP-2 in both α and β fatty acid oxidation pathways raises questions about how various metabolites are channeled into the appropriate pathways. While discrimination between 3-methyl and 2-methyl substrates can be relatively easily explained, it is less easy to explain how metabolism of straight-chain fatty acyl-CoA esters (e.g., very-long-chain fatty acids) is regulated, since the in vitro results suggest they can be processed by either α- or β-oxidation pathways. The results suggest that discrimination may be in part mediated by interactions between SCP-2 and the enzymes involved. The presence of SCP-2 conjugates (e.g., SCPx [53]) physically linking the coprotein and associated β-oxidation enzyme (e.g., 3-ketoacyl-CoA thiolase) may make this process more efficient. It is also possible that the enzymes of both α and β oxidation are organized into multienzyme complexes, and the overall structures and relationships of these complexes may contribute to channeling of intermediates into the appropriate pathways.

The α-methylacyl-CoA "racemase" (Figure 7), which catalyzes epimerization of (2*R*)-pristanoyl-CoA into (2*S*)-pristanoyl-CoA [54], also links the α- and β-oxidation pathways. Epimerization is also required subsequent to the involvement of PAHX, in the β-oxidation pathway, to further metabolize the phytanoyl-CoA-derived inter-

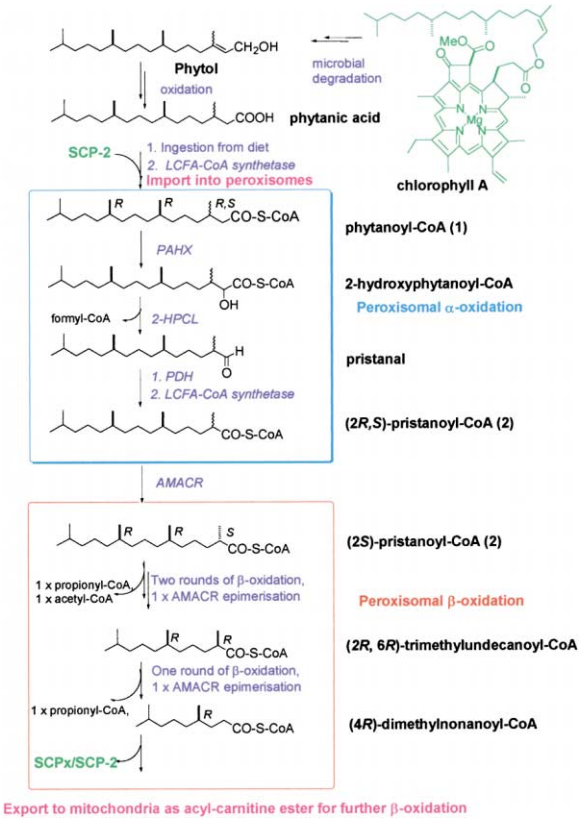

Figure 7. Proposed Involvement of SCP-2 in the Peroxisomal α and β Oxidation of Phytanic and Pristanic Acids

Phytanoyl-CoA may be imported from the cytosol into the peroxisome as the SCP-2 complex (as shown) followed by further metabolism. However, the possibility that the SCP-2 complex is formed subsequent to phytanoyl-CoA importation into the peroxisome cannot be excluded. Release of the chain-shortened acyl-CoA/carnitine ester from SCP-2 is anticipated upon export to mitochondria. It has been suggested that pristanic acid is converted to pristanoyl-CoA by long-chain fatty acyl-CoA synthetase [15] or very-long-chain fatty acyl-CoA synthetase [16]. Abbreviations: LCFA-CoA synthetase, long-chain fatty-acyl-CoA synthetase; PAHX, phytanoyl-CoA 2-hydroxylase; 2HPCL, 2-hydroxyphytanoyl-CoA lyase; PDH, pristanal dehydrogenase; AMACR, α-methylacyl-CoA racemase.

mediates with *R*-methyl groups originally at C-7 and C-11 of phytanic acid. It seems plausible that SCP-2 (or its conjugates) is required for activity of all the enzymes of the α-oxidation pathway and α-methylacyl-CoA "racemase," as well as those in the β-oxidation pathway [54]. It is also possible that peroxisomal metabolism of fatty acids is terminated at about the level of C-11 (dimethylnonanoyl-CoA) (Figure 7) [3], because at this point fatty acids (and their coenzyme A esters) become significantly soluble in an aqueous environment, and binding to SCP-2 is reduced [55, 56].

Significance

Consideration of the characteristics of phytanoyl-CoA (1) and the stereochemical aspects of the PAHX reaction led to the proposal that the in vivo substrate for PAHX was a protein complex. Several pieces of evidence suggested that SCP-2 could be the small protein binding factor, the most compelling being the biochemical changes and some symptoms typical of ARD observed in the SCP-2-deficient mouse model [37]. The in vitro results suggest that the in vivo substrates for PAHX (and by extrapolation all other enzymes of the α-oxidation pathway) may be SCP-2 complexes.

Abnormal elevation of phytanic acid is diagnostic of ARD (and some other peroxisomal disorders), but the mechanisms by which the clinical symptoms are manifested are unclear. The results in this paper suggest that some of the common symptoms may result from a functional reduction in the amount of SCP-2 due to excessive accumulation of a ligand, e.g., phytanic acid in ARD or (2*R*)-pristanic acid in α-methylacyl-CoA racemase deficiency [54]. Direct binding of phytanic (or pristanic) acid to alternative proteins, activation of peroxisomal proliferation elements [57], and direct toxic actions effects may also be important. Indeed, phytanic acid has been shown to activate the PPARα receptor in SCP-2-deficient mice [58], and upregulation and binding of phytanic acid to liver fatty acid binding protein has been demonstrated [59]. Understanding the roles of these effects in disease pathology and the development of symptoms is essential if effective treatments for these disorders are to be achieved.

Experimental Procedures

Chemicals

All chemicals were supplied by the Sigma-Aldrich Chemical Co. (Poole, Dorset, UK), unless otherwise stated, and were of analytical grade or higher. Fatty acyl-CoA esters were purchased from Larodan Lipids (Malmö, Sweden). (3*R,S*, 7*R*, 11*R*)-phytanoyl-CoA was synthesized as previously reported [28]. Chromatography systems and columns were obtained from Amersham Biosciences (Little Chalfont, Bucks., UK) or Bio-Rad (Hemel Hempstead, Herts., UK). Protein purification was performed on an FPLC Bio-pilot system. CM-Sepharose was packed into a XK50/20 column (5 × 10 cm, 200 ml). Recombinant mature PAHX was purified to >95% purity (by SDS-PAGE analysis) as previously described [4]. HPLC columns were obtained from Phenomenex (Macclesfield, Cheshire, UK). Oligonucleotides were obtained from Sigma-Genosys. Other molecular biology reagents were obtained from Stratagene (Amsterdam Zuidoost, The Netherlands) or New England Biolabs Ltd. (Hitchen, Herts., UK).

Cloning and Expression of SCP-2

The pro-SCP-2 gene was amplified from the IMAGE clone 1872828 (http://image.llnl.gov) using the following primers: forward 5′-GAATTCCATATGGGTTTTCCGGAAGCC-3′; reverse 5′-GGATCCAAGCTTTCAGAGCTTAGCGTTGCC-3′. Mature SCP-2 was amplified using the forward primer 5′-GAATTCCATATGAGCTCTGCAAGTGATGG-3′ and the same reverse primer as before. Amplification products were cloned into the pGEM-T vector and sequenced. The required fragments were subcloned into the pET-24a vector using the 5′ *Nde 1* and 3′ *Hind III* restriction sites. Clones were expressed in *E. coli* BL21 (DE3) by induction with 0.5 mM IPTG for 4 hr. Both pro- and mature SCP-2 were expressed as ~20% of the total cell protein. Large-scale growths of recombinant *E. coli* clones were carried out as previously described for PAHX [4].

Purification of SCP-2

All manipulations were performed on ice or at 4°C. Fractions were analyzed by SDS-PAGE using 15% (w/v) resolving and 3% (w/v) stacking gels. Protein concentrations were determined by the method of Bradford [60]. Iso-electric points of pro- and mature SCP-2 were determined with a Bio-Rad analytical flat bed IEF using precast PhastGel™ IEF 3-9 gels (Amersham Biosciences). The mo-

lecular weight of mature SCP-2 was determined by ESI MS (13,242 Da calculated; 13,243 ± 0.96 Da observed).

Cells expressing mature SCP-2 (*E. coli* BL21(DE3)/pET24a, 10 g) were thawed and resuspended in 500 ml of 20 mM MOPS-NaOH, 10% (v/v) glycerol (pH 7.2), 1 mM benzamidine-HCl, 1 mM PMSF, 0.5 mM 1,10-*o*-phenanthroline, and 2 mM DTT. After 15 min stirring, the solution was sonicated for 5 × 30 s, power 5 with 60 s cooling (W-380 sonicator, Utrasonic). DNA was precipitated with 1% (w/v) streptomycin sulfate and 0.1% (w/v) PEI. After 10 min stirring, the sample was centrifuged (JA-14 rotor, 12000 rpm, 22,000 × g, 10 min) and loaded onto a CM-sepharose column equilibrated in the above buffer containing 0.1 mM 1,10-*o*-phenanthroline. The column was washed with 1000 ml buffer, and protein was eluted with a 0–700 mM NaCl gradient over 900 ml with 25 ml fractions collected. Fractions 12–18 (330–505 mM NaCl) were pooled and concentrated in 300 and 50 ml Amicon stirred cells using YM-10 membranes. Purified SCP-2 was exchanged into 10 mM Tris-HCl, 10% (v/v) glycerol (pH 7.5) with an Econo-pak column (Bio-Rad), concentrated to 10 mg/ml and stored at −80°C.

Enzyme Assays and Kinetic Analysis

Assays were performed essentially as previously described [4], in the presence or absence of β cyclodextrin or equimolar quantities of various fatty acyl-CoA derivatives and mature SCP-2. Substrates were incubated for 30 min on ice with mature SCP-2 to ensure full complex formation. Acyl-CoA esters with side chains up to ten carbon atoms did not require solubilization with β cyclodextrin or SCP-2. Determination of kinetic parameters used 10–200 μM phytanoyl-CoA (0.56 μg PAHX per assay), 20–150 μM hexadecanoyl-CoA (2.71 μg PAHX per assay), or 10–2000 μM isovaleryl-CoA (3.64 μg PAHX per assay). Kinetic parameters were determined by direct fitting to a rectangular hyperbola after initial Lineweaver-Burke analysis. Values are reported ± standard error, with K_{cat} calculated using a mass for PAHX as determined by ESI MS (35,452 ± 3 Da).

Product Characterization

Incubations of substrates with PAHX (50 reactions, each containing 20 μg enzyme) were as previously described [4, 28]. NMR assignments are using the previously reported numbering system [28]. Assignments used: s, singlet; d, doublet; t, triplet; m, multiplet; br, broad; and Me, methyl (for Supplemental Data, please contact the *Chemistry & Biology* Production Department at chembiol@cell.com). The phytanoyl-CoA (**1**) substrate was HPLC purified as previously described [28]: ^{1}H NMR (500 MHz; D_2O: CD_3CN, 2:1 [v/v]) (coenzyme A moiety) δ 0.75 (s, 3H, 2′′-Me); 0.90 (s, 3H, 2′′-Me); 2.41 (m, 2H, 2′′′-CH_2); 2.90–2.95 (m, 2H, 2′′′′-CH_2); 3.30 (m, 2H, 1′′′′-CH_2); 3.40 (m, 2H, 1′′′-CH_2); 3.54 (m, 1H, 1′′-CH_2); 3.85 (m, 1H, 1′′-CH_2); 4.02 (1H, s, 3′′-CH); 4.24 (br s, 2H, 5′-CH_2); 4.55 (s, 1H, 4′-CH); 4.82 (2H, m, 2′-CH and 3′-CH); 6.12 (d, *J* = 7 Hz, 1H, 1′-CH); 8.23 (s, 1H, 8-CH); 8.52 (br s, 1H, 2-CH); (phytanoyl moiety) 0.85–0.95 (m, 15H, 5 × Me); 1.00–1.40 (m, 20H, 9 × CH_2, 2 × MeCH); 1.55 (m, 1H, CH(Me)$_2$); 1.95 (m, 1H, CHCH$_2$COS-); 2.41 (m, 1H, 1 of CH_2COS-); 2.58 (m, 1H, 1 of CH_2COS-). A COSY ^{1}H NMR spectrum was consistent with the assigned structure; m/z (-ve ESI MS) = 1061.45 ± 0.20 (M); calc. 1061.40 (M).

Incubations with phytanoyl-CoA (Figure 6; **1**) (~1.2 mg) were carried out in the presence of β cyclodextrin. An efficient (>95%) conversion under standard conditions was observed (as judged by HPLC analyses). 2-hydroxyphytanoyl-CoA was isolated as previously described [28, 61]. Retention times were 16.0 and 18.0 min for product and substrate, respectively. The sample for ESI MS analysis was repurified using NH_4HCO_3 as eluent: ^{1}H NMR (500 MHz; D_2O: CD_3CN, 2:1 [v/v]) (coenzyme A moiety) δ 0.75 (s, 3H, 2′′-Me); 0.7–0.9 (m, 3H, 2′′-Me) 2.35 (br s, 2H, 2′′′-CH_2); 2.8–2.95 (m, 2H, 2′′′′-CH_2); 3.20 (br s, 2H, 1′′′′-CH_2); 3.35 (m, 2H, 1′′′-CH_2); 3.45–3.55 (m, 1H, 1′′-CH_2); 3.55–3.65 (m, 1H, 1′′-CH_2); 3.80 (br s, 1H, 3′′-CH); 4.15 (br s, 2H, 5′-CH); 4.45 (s, 1H, 4′-CH); 6.0 (s, 1H, 1′-CH); 8.2 (s, 1H, 8-CH); 8.55 (br s, 1H, 2-CH); (phytanoyl moiety) δ 0.7–0.85 (m, 15H, 5 × Me); 0.95–1.4 (m, 20H, 9 × CH_2, 2 × MeCH-); 1.4–1.5 (m, 1H, CHCOHCOS-) 3.95 (1H, br t, *J* = 7 Hz, CHOH). A COSY ^{1}H NMR spectrum was consistent with the assigned structure; (-ve ESI MS) 1077.62 ± 0.20 (M); calc. 1077.1 (M).

Incubations with isovaleryl-CoA (~1.2 mg) were carried out in the absence of β cyclodextrin or mature SCP-2. An efficient (>95%) conversion of substrate was observed (as judged by HPLC) under standard assay conditions. 2-hydroxyisovaleryl-CoA was isolated by HPLC (ODS [250 × 4.6 mm], 0.6 ml/min, λ = 254 nm). The column was eluted with 30 mM $NH_4H_2PO_4$, 10% (v/v) acetonitrile (pH 5.5), for 10 min followed by a gradient to 15 mM $NH_4H_2PO_4$, and 60% (v/v) acetonitrile (pH 5.5), over 5 min and 10 min at 60% (v/v) acetonitrile. Retention times were 10.0 and 17.0 min for product and substrate, respectively. The sample for ESI MS analysis was repurified using NH_4HCO_3 as eluent: ^{1}H NMR (500 MHz; D_2O) (coenzyme A moiety) δ 0.6 (s, 3H, 2′′-Me); 0.75 (s, 3H, 2′′-Me); 2.30 (t, *J* = 6.5 Hz, 2H, 2′′′-CH_2); 2.90 (t, *J* = 6.5 Hz, 2H, 2′′′′-CH_2); 3.20 (t, *J* = 6.5 Hz, 2H, 1′′′′-CH_2); 3.35 (t, *J* = 6.5 Hz, 2H, 1′′′-CH_2); 3.40 (m, 1H, 1 of 1′′-CH_2); 3.70 (m, 1H, 1 of 1′′-CH_2); 3.90 (s, 1H, 3′′-CH); 4.10 (br s, 2H, 5′-CH); 4.45 (s, 1H, 4′-CH); 4.60 (2H, 2′-CH and 3′-CH under HOD suppression); 6.15 (d, *J* = 7 Hz, 1H, 1′-CH); 8.1 (s, 1H, 8-CH); 8.4 (s, 1H, 2-CH); (isovaleryl moiety) δ 0.7 (d, *J* = 7.0 Hz, 3H, Me); 0.8 (d, *J* = 7.0 Hz, 3H, Me); 2.05–2.15 (m, 1H, (Me)$_2$CH); 3.95 (d, *J* = 4.0 Hz, 1H, CHOHCOS). A COSY ^{1}H NMR spectrum was consistent with the assigned structure; m/z (-ve ESI MS) = 867.27 ± 0.23 (M); calc. 866.7 (M).

Incubations with octanoyl-CoA (~1.2 mg) were carried out in the absence of β cyclodextrin or mature SCP-2. An efficient (>95%) conversion under standard assay conditions was observed (as judged by HPLC analysis). 2-hydroxyoctanoyl-CoA was isolated as previously described for 2-hydroxyphytanoyl-CoA [28]. Retention times were 11.6 and 13.2 min for product and substrate, respectively. The sample for ESI MS analysis was repurified using HPLC with NH_4HCO_3 as eluent: The coenzyme A moiety exhibited almost identical signals to those reported for isovaleryl-CoA; ^{1}H NMR (500 MHz; D_2O) (octanoyl moiety) δ 0.85 (t, *J* = 6.5 Hz, 3H, Me); 1.35–1.40, 1.50–1.60, 1.65–1.80 (m, 7H, 3 × CH_2; 1 of CH_2CHOH); 2.15–2.20 (m, 1H, 1 of CH_2CHOH); 4.2 (b tr, 1H, CHOHCOS). A COSY ^{1}H NMR spectrum was consistent with the assigned structure; m/z (-ve ESI MS) = 909.42 ± 0.2 (M); calc. 909.7 (M).

Incubations with hexadecanoyl-CoA (~1.2 mg) were carried out in the presence of β cyclodextrin. An efficient (>95%) conversion under standard conditions was observed (as judged by HPLC analyses). 2-hydroxyhexadecanoyl-CoA was isolated as previously described for 2-hydroxyphytanoyl-CoA [28]. Retention times were 17.4 and 19.6 min for product and substrate, respectively. The sample for ESI MS analysis was repurified using NH_4HCO_3 as eluent. The coenzyme A moiety exhibited almost identical signals to those reported for phytanoyl-CoA: ^{1}H NMR (500 MHz; D_2O: CD_3CN, 2:1 [v/v]) (hexadecanoyl moiety) δ 0.85 (t, *J* = 6.5 Hz, 3H, Me); 1.10–1.30, 1.30–1.45, 1.50–1.60, 1.65–1.80 (m, 26H, 13 x CH_2); 4.20 (br t, 1H, CHOHCOS). A COSY ^{1}H NMR spectrum was consistent with the assigned structure; m/z (-ve ESI MS) = 1021.57 ± 0.23 (M); calc. 1021.9 (M).

Incubations with heptadecanoyl-CoA (~1.2 mg) were carried out in the presence of β cyclodextrin. A >95% conversion was observed (as judged by HPLC). 2-hydroxyheptadecanoyl-CoA was isolated as previously described for 2-hydroxyphytanoyl-CoA [28]. Retention times were 18.8 and 20.4 min for product and substrate, respectively. The sample for ESI MS analysis was repurified using HPLC with NH_4HCO_3 as eluent: m/z (-ve ESI MS) = 1035.40 ± 0.20 (M); calc. 1036.0 (M).

Incubations with octadecanoyl-CoA (~1.2 mg) were carried out in the presence of β cyclodextrin. A >95% conversion was observed (as judged by HPLC). 2-hydroxyoctadecanoyl-CoA was isolated as previously described for 2-hydroxyphytanoyl-CoA [28]. The retention time was 19.2 min for the product. The sample for ESI MS analysis was repurified using HPLC with NH_4HCO_3 as eluent. The coenzyme A moiety exhibited almost identical signals to those reported for phytanoyl-CoA: ^{1}H NMR (500 MHz; D_2O: CD_3CN, 2:1 [v/v]) (octadecanoyl moiety) δ 0.85 (br t, 3H, Me); 1.00–1.20, 1.40–1.50, 1.50–1.60 (m, 30H, 15 × CH_2); 4.20 (br t, *J* = 7.0 Hz, 1H, CHOHCOS). A COSY ^{1}H NMR spectrum was consistent with the assigned structure; m/z (-ve ESI MS) = 1049.49 ± 0.14 (M); calc. 1050.0 (M).

Incubations with arachidoyl-CoA (C-20) (~1.2 mg) were carried out in the presence of β cyclodextrin. A >95% conversion was observed (as judged by HPLC). 2-hydroxyarachidoyl-CoA was isolated as previously described for 2-hydroxyphytanoyl-CoA [28]. Retention times were 20.4 and 21.4 min for product and substrate,

respectively. The sample for ESI MS analysis was repurified using HPLC with NH_4HCO_3 as eluent: m/z (-ve ESI MS) = 1077.59 ± 0.25 (M); calc. 1077.1 (M).

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References

1. Verhoeven, N.M., Wanders, R.J.A., Poll-The, B.T., Saudubray, J.M., and Jakobs, C. (1998). The metabolism of phytanic acid and pristanic acid in man: a review. J. Inherit. Metab. Dis. *21*, 697–728.
2. Wanders, R.J.A., Jakobs, C., and Skjeldal, O.H. (2001). Refsum disease. In The Metabolic and Molecular Bases of Inherited Disease, Volume II, C.R. Scriver, ed. (New York: McGraw-Hill), pp. 3303–3321.
3. Verhoeven, N.M., Roe, D.S., Kok, R.M., Wanders, R.J.A., Jakobs, C., and Roe, C.R. (1998). Phytanic acid and pristanic acid are oxidised by sequential peroxisomal and mitochondrial reactions in cultured fibroblasts. J. Lipid Res. *39*, 66–74.
4. Mukherji, M., Chien, W., Kershaw, N.J., Clifton, I.J., Schofield, C.J., Wierzbicki, A.S., and Lloyd, M.D. (2001). Structure-function analysis of phytanoyl-CoA 2-hydroxylase mutations causing Refsum's Disease. Hum. Mol. Genet. *10*, 1971–1982.
5. Gibberd, F.B., and Wierzbicki, A.S. (2000). Heredopathia atactica polyneuritiformis: Refsum's disease. In Handbook of Ataxia Disorders, R. Klockgether, ed. (New York: Marcel Dekker), pp. 235–256.
6. Watkins, P.A., Howard, A.E., Gould, S.J., Avigan, J., and Mihalik, S.J. (1996). Phytanic acid activation in rat liver peroxisomes is catalysed by long-chain acyl-CoA synthetase. J. Lipid Res. *37*, 2288–2295.
7. Jansen, G.A., Ofman, R., Ferdinandusse, S., Ijlst, L., Muijsers, A.O., Skjeldal, O.H., Stokke, O., Jakobs, C., Besley, G.T., Wraith, J.E., et al. (1997). Refsum Disease is caused by mutations in the phytanoyl-CoA hydroxylase gene. Nat. Genet. *17*, 190–193.
8. Mihalik, S.J., Rainville, A.M., and Watkins, P.A. (1995). Phytanic acid α-oxidation in rat liver peroxisomes. Production of α-hydroxyphytanoyl-CoA and formate is enhanced by dioxygenase cofactors. Eur. J. Biochem. *232*, 545–551.
9. Mihalik, S.J., Morrell, J.C., Kim, D., Stackster, K.A., Watkins, P.A., and Gould, S.J. (1997). Identification of PAHX, a Refsum Disease gene. Nat. Genet. *17*, 185–189.
10. Prescott, A.G., and Lloyd, M.D. (2000). The iron(II) and 2-oxo-acid-dependent dioxygenases and their role in metabolism. Nat. Prod. Rep. *17*, 367–383.
11. Jansen, G.A., Verhoeven, N.M., Denis, S., Romeijn, G., Jakobs, C., ten Brink, H.J., and Wanders, R.J. (1999). Phytanic acid α-oxidation: identification of 2-hydroxyphytanoyl-CoA lyase in rat liver and its localisation in peroxisomes. Biochim. Biophys. Acta *1440*, 176–182.
12. Foulon, V., Antonenkov, V.D., Croes, K., Waelkens, E., Mannaerts, G.P., Van Veldhoven, P.P., and Casteels, M. (1999). Purification, molecular cloning and expression of 2-hydroxyphytanoyl-CoA lyase, a peroxisomal thiamine pyrophosphate-dependent enzyme that catalyses the carbon-carbon bond cleavage during α-oxidation of 3-methyl-branched fatty acids. Proc. Natl. Acad. Sci. USA *96*, 10039–10044.
13. Croes, K., Van Veldhoven, P.P., Mannaerts, G.P., and Casteels, M. (1997). Production of formyl-CoA during peroxisomal α-oxidation of 3-methyl-branched fatty acids. FEBS Lett. *407*, 197–200.
14. Verhoeven, N.M., Schor, D.S.M., ten Brink, H.J., Wanders, R.J.A., and Jakobs, C. (1997). Resolution of the phytanic acid α-oxidation pathway: identification of pristanal as product of the decarboxylation of 2-hydroxyphytanoyl-CoA. Biochem. Biophys. Res. Commun. *237*, 33–36.
15. Wanders, R.J.A., Denis, S., van Roermund, C.W.T., and ten Brink, H.J. (1992). Characteristics and subcellular localisation of pristanoyl-CoA synthetase in rat liver. Biochim. Biophys. Acta *1125*, 274–279.
16. Steinberg, S.J., Wang, S.J., Kim, D.G., Mihalik, S.J., and Watkins, P.A. (1999). Human very-long-chain acyl-CoA synthetase: cloning, topography, and relevance to branched-chain fatty acid metabolism. Biochem. Biophys. Res. Commun. *257*, 615–621.
17. Schmitz, W., Fingerhut, R., and Conzelmann, E. (1994). Purification and properties of an α-methylacyl-CoA racemase from rat liver. Eur. J. Biochem. *222*, 313–323.
18. Schmitz, W., Albers, C., Fingerhut, R., and Conzelmann, E. (1995). Purification and characterization of an α-methylacyl-CoA racemase from human liver. Eur. J. Biochem. *231*, 815–822.
19. Gould, S.J., Keller, G.A., Hosken, N., Wilkinson, J., and Subramani, S. (1989). A conserved tripeptide sorts proteins to peroxisomes. J. Cell Biol. *108*, 1657–1664.
20. Miyazawa, S., Osumi, T., Hashimoto, T., Ohno, K., Miura, S., and Fujiki, Y. (1989). Peroxisomal targeting signal of rat liver acyl-coenzyme A oxidase residues at the carboxy terminus. Mol. Cell. Biol. *9*, 83–91.
21. Swinkels, B.W., Gould, S.J., Bodnar, A.G., Rachubinski, R.A., and Subramani, S. (1991). A novel, cleavable peroxisomal targeting signal at the amino terminus of the rat 3-ketoacyl-CoA thiolase. EMBO J. *10*, 3255–3262.
22. Jansen, G.A., Wanders, R.J.A., Watkins, P.A., and Mihalik, S.J. (1997). Phytanoyl-coenzyme A hydroxylase deficiency—the enzyme defect in Refsum's Disease. N. Eng. J. Med. *337*, 133–134.
23. Jansen, G.A., Hogenhout, E.M., Ferdinandusse, S., Waterham, H.R., Ofman, R., Jakobs, C., Skjeldal, O.H., and Wanders, R.J. (2000). Human phytanoyl-CoA hydroxylase: resolution of the gene structure and the molecular basis of Refsum's Disease. Hum. Mol. Genet. *9*, 1195–1200.
24. van den Bring, D.M., Waterham, H.R., Hoogenhout, E., Jansen, G., Mooijer, P.A.W., Gibberd, F.B., Feher, M.D., Wierzbicki, A.S., Skjeldahl, O., and Wanders, R.J.A. (2001). Genetic heterogeneity with Refsum disease: discovery of its underlying basis through the identification of mutations in the PEX7 gene which is also mutated in rhizomelic chondrodysplasia punctata type 1. J. Inherit. Metab. Dis. *24* (*Suppl. 1*), 185–O.
25. Wierzbicki, A.S., Mitchell, J., Lambert-Hammill, M., Hancock, M., Greenwood, J., Sidey, M.C., de Belleroche, J., and Gibberd, F.B. (2000). Identification of genetic heterogeneity in Refsum's Disease. Eur. J. Hum. Genet. *8*, 649–651.
26. Chambraud, B., Radanyi, C., Camonist, J.H., Rajkowski, K., Schumacher, M., and Baulieu, E.-E. (1999). Immunophilins, Refsum disease, and lupus nephritis: the peroxisomal enzyme phytanoyl-CoA α-hydroxylase is a new FKBP-associated protein. Proc. Natl. Acad. Sci. USA *96*, 2104–2109.
27. Chen, C., Wang, Q., Fang, X., Xu, Q., Chi, C., and Gu, J. (2001). Roles of phytanoyl-CoA alpha-hydroxylase in mediating the expression of human coagulation factor VIII. J. Biol. Chem. *276*, 46340–46346.
28. Kershaw, N.J., Mukherji, M., MacKinnon, C.H., Claridge, T.D., Odell, B., Wierzbicki, A.S., Lloyd, M.D., and Schofield, C.J. (2001). Studies on phytanoyl-CoA 2-hydroxylase and synthesis of phytanoyl-coenzyme A. Bioorg. Med. Chem. Lett. *11*, 2545–2548.
29. Tsai, S.C., Steinberg, D., Uhlendorf, B.W., and Fales, H.M. (1973). Studies on the stereospecificity of mitochondrial oxidation of phytanic acid and of α-hydroxyphytanic acid. J. Biol. Chem. *248*, 1091–1097.
30. Croes, K., Casteels, M., Dieuaide Noubhani, M., Mannaerts, G.P., and Van Veldhoven, P.P. (1999). Stereochemistry of the α-oxidation of 3-methyl fatty acids in rat liver. J. Lipid Res. *40*, 601–609.
31. Hanauske-Abel, H.M., and Günzler, V. (1982). A stereochemical

concept for the catalytic mechanism of prolyl hydroxylase: applicability to classification and design of inhibitors. J. Theor. Biol. *94*, 421–455.
32. Wierzbicki, A.S., Sankaralingam, A., Lumb, P., Hardman, T.C., Sidey, M.C., and Gibberd, F.B. (1999). Transport of phytanic acid on lipoproteins in Refsum's Disease. J. Inherit. Metab. Dis. *22*, 29–36.
33. Napoli, J.L. (1999). Interactions of retinoid binding proteins and enzymes in retinoid metabolism. Biochim. Biophys. Acta *1440*, 139–162.
34. Knudsen, J., Jensen, M.V., Hansen, J.K., Faergeman, N.J., Neergaard, T.B.F., and Gaigg, B. (1999). Role of acyl-CoA binding protein in acyl-CoA transport, metabolism and cell signalling. Mol. Cell. Biochem. *192*, 95–103.
35. Seedorf, U., Ellinghaus, P., and Nofer, J.R. (2000). Sterol carrier protein-2. Biochim. Biophys. Acta *1486*, 45–54.
36. Wouters, F.S., Bastiaens, P.I.H., Wirtz, K.W.A., and Jovin, T.M. (1998). FRET microscopy demonstrates molecular association of non-specific lipid transfer protein (nsL-TP) with fatty acid oxidation enzymes in peroxisomes. EMBO J. *17*, 7179–7189.
37. Seedorf, U., Raabe, M., Ellinghaus, P., Kannenberg, F., Fobker, M., Engel, T., Denis, S., Wouters, F., Wirtz, K.W., Wanders, R.J., et al. (1998). Defective peroxisomal catabolism of branched fatty acyl coenzyme A in mice lacking the sterol carrier protein-2/sterol carrier protein-X gene function. Genes Dev. *12*, 1189–1201.
38. Jolly, C.A., Chao, H., Kier, A.B., Billheimer, J.T., and Schroeder, F. (2000). Sterol carrier protein-2 suppresses microsomal acyl-CoA hydrolysis. Mol. Cell. Biochem. *205*, 83–90.
39. Lee, H.-J., Lloyd, M.D., Harlos, K., and Schofield, C.J. (2000). The effect of cysteine mutations on the activity of recombinant deacetoxycephalosporin C synthase from *S. clavuligerus*. Biochem. Biophys. Res. Commun. *267*, 445–448.
40. Baldwin, J.E., Field, R.A., Lawrence, C.C., Lee, V., Robinson, J.K., and Schofield, C.J. (1994). Substrate specificity of proline 4-hydroxylase: chemical and enzymatic synthesis of 2*S*, 3*R*, 4*S*-epoxyproline. Tetrahedron Lett. *35*, 4649–4652.
41. Baldwin, J.E., Adlington, R.M., Crouch, N.P., Hill, R.L., Wood, M.E., and Lloyd, M.D. (1997). Competing pathways in the oxidation of Z-3-ethylidine cephalosporin C by the enzyme deacetoxycephalosporin C synthase (DAOCS). Bioorg. Med. Chem. Lett. *7*, 593–596.
42. Baldwin, J.E., Adlington, R.M., Crouch, N.P., and Pereira, I.A.C. (1992). Epoxide formation in the enzymic conversion of [4-^{2}H] exomethylene cephalosporin C by deacetoxy-/deacetylcephalosporin C synthase. J. Chem. Soc. Chem. Commun. 1448–1451.
43. Lloyd, M.D., Merritt, K.D., Lee, V., Sewell, T.J., Wha-Son, B., Baldwin, J.E., Schofield, C.J., Elson, S.W., Baggaley, K.H., and Nicholson, N.H. (1999). Product-substrate engineering by bacteria: studies on clavaminate synthase, a trifunctional dioxygenase. Tetrahedron *55*, 10201–10220.
44. Croes, K., Foulon, V., Casteels, M., Van Veldhoven, P.P., and Mannaerts, G.P. (2000). 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. *41*, 629–636.
45. Croes, K., Casteels, M., De Hoffmann, E., Mannaerts, G.P., and Van Veldhoven, P.P. (1996). α-oxidation of 3-methyl-substituted fatty acids in rat liver—production of formic acid instead of CO_2, cofactor requirements, subcellular localisation and formation of a 2-hydroxy-3-methylacyl-CoA intermediate. Eur. J. Biochem. *240*, 674–683.
46. Huang, S., Van Veldhoven, P.P., Vanhoutte, F., Parmentier, G., Eyssien, H.J., and Mannaerts, G.P. (1992). α-oxidation of 3-methyl-substituted fatty acids in rat liver. Arch. Biochem. Biophys. *296*, 214–223.
47. Gerbling, H. (1993). Peroxisomal degradation of 2-oxoisocaproate—evidence for free acid intermediates. Bot. Acta *106*, 380–387.
48. Rao, N.V., and Adams, E. (1978). Partial reaction of prolyl hydroxylase. (Gly-Pro-Ala)n stimulates α-ketoglutarate decarboxylation without prolyl hydroxylation. J. Biol. Chem. *253*, 6327–6330.
49. Wu, M., Moon, H.-S., Pirskanan, A., Myllyharju, J., Kivirikko, K.I., and Begley, T.P. (2000). Mechanistic studies on prolyl-4-hydroxylase: the vitamin C requiring uncoupled oxidation. Bioorg. Med. Chem. Lett. *10*, 1511–1514.
50. Dansen, T.B., Westerman, J., Wouters, F.S., Wanders, R.J., van Hoek, A., Gadella, T.W., Jr., and Wirtz, K.W. (1999). High-affinity binding of very-long-chain fatty acyl-CoA esters to the peroxisomal non-specific lipid-transfer protein (sterol carrier protein-2). Biochem. J. *339*, 193–199.
51. Jansen, G.A., Mihalik, S.J., Watkins, P.A., Jakobs, C., Moser, H.W., and Wanders, R.J.A. (1998). Characterisation of phytanoyl-coenzyme A hydroxylase in human liver and activity in patients with peroxisomal disorders. Clin. Chim. Acta *271*, 203–211.
52. Lee, H.-J., Lloyd, M.D., Harlos, K., Clifton, I.J., Baldwin, J.E., and Schofield, C.J. (2001). Kinetic and crystallographic studies on deacetoxycephalosporin C synthase (DAOCS). J. Mol. Biol. *308*, 937–948.
53. Antonenkov, V.D., van Veldhoven, P.P., Waelkins, E., and Mannaerts, G.P. (1997). Substrate specificities of 3-oxoacyl-CoA thiolase A and sterol carrier protein2/3-oxoacyl-CoA thiolase purified from normal rat liver peroxisomes. Sterol carrier protein 2/3-oxoacyl-CoA thiolase is involved in the metabolism of 2-methyl-branched fatty acids and bile acids intermediates. J. Biol. Chem. *272*, 26023–26031.
54. Ferdinandusse, S., Denis, S., Clayton, P.T., Graham, A., Rees, J.E., Allen, J.T., McLean, B.N., Brown, A.Y., Vreken, P., Waterham, H.R., and Wanders, R.J.A. (2000). Mutations in the gene encoding peroxisomal alpha-methylacyl-CoA racemase cause adult onset sensory motor neuropathy. Nat. Genet. *24*, 188–191.
55. Frolov, A., Miller, K., Billheimer, J.T., Cho, T.H., and Schroeder, F. (1997). Lipid specificity and location of sterol carrier protein-2 fatty acid-binding site: a fluorescence displacement and energy transfer study. Lipids *32*, 1201–1209.
56. Frolov, A., and Schroeder, F. (1998). Acyl coenzyme A binding protein. Conformational sensitivity to long chain fatty acyl-CoA. J. Biol. Chem. *273*, 11049–11055.
57. Kersten, S., Desvergne, B., and Wahli, W. (2000). Roles of PPARs in health and disease. Nature *405*, 421–424.
58. Ellinghaus, P., Wolfrum, C., Assmann, G., Spener, F., and Seedorf, U. (1999). Phytanic acid activates the peroxisomal proliferator-activated receptor α (PPARα) in sterol carrier protein-2/sterol carrier protein x-deficient mice. J. Biol. Chem. *274*, 2766–2772.
59. Wolfrum, C., Ellinghaus, P., Fobker, M., Seedorf, U., Assmann, G., Borchers, T., and Spener, F. (1999). Phytanic acid is a ligand and transcriptional regulator of murine fatty acid binding protein. J. Lipid Res. *40*, 708–714.
60. Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. *72*, 248–254.
61. Mukherji, M., Kershaw, N.J., MacKinnon, C.H., Clifton, I.J., Schofield, C.J., Wierzbicki, A.S., and Lloyd, M.D. (2001). 'Chemical co-substrate rescue' of phytanoyl-CoA 2-hydroxylase mutants causing Refsum's Disease. J. Chem. Soc. Chem. Commun. 972–973.