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Review

Alpha-Oxidation

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Abstract

Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) is a branched chain fatty acid, which is a constituent of the human diet. The presence of the 3-methyl group of phytanic acid prevents degradation by beta-oxidation. Instead, the terminal carboxyl group is first removed by alpha-oxidation. The mechanism of the alpha-oxidation pathway and the enzymes involved are described in this review.
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Keywords: Alpha-oxidation; Phytanic acid; Pristanic acid; Phytol; Refsum disease; Zellweger syndrome

1. Introduction

Although phytanic acid had been isolated from cow milk already in the 1950s [1] and its structure has been known since the 1960s [2,3], at that time this multi-branched fatty acid did not get any special attention. The discovery of high amounts of phytanic acid in kidney, liver, and brain of patients suffering from *Refsum's Syndrome,* however, initiated a long period of research on its metabolism. As phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) has a methyl-residue at the third carbon atom of its backbone, it was immediately recognized that degradation could not occur by beta-oxidation. An alternative catabolic pathway was proposed, involving removal of the alpha carbon unit as CO_2. Accordingly, this process was named alpha-oxidation. After several decades of research, the enzymatic steps involved in this pathway have been resolved now, and two different enzymatic and genetic causes of Refsum Disease have been elucidated. However, even today not all enzymes involved in the alpha-oxidation pathway have been isolated and characterized, nor have all corresponding genes been found. This review provides a description of the current knowledge about the alpha-oxidation process.

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2. Phytanic acid

In studies on the fatty acid composition of bovine milk fat carried out in the early 1950s, a multi-branched C20 fatty acid was isolated [1]. It took 10 years, however, before the structure of this fatty acid could be characterized using analytical techniques including gas chromatography-mass spectrometry and nuclear magnetic resonance [2,3]. These analyses revealed that this fatty acid, which was named phytanic acid, contains 4 methyl residues on a C16 backbone. Accordingly, phytanic acid could be assigned the systematic name 3,7,11,15-tetramethyl-hexadecanoic acid.

2.1. The origin of phytanic acid

Phytanic acid has been found in man (it has been detected in plasma, liver and other organ tissues, milk and sciatic nerve), but also in other mammals, as well as in birds, fish, micro-organisms and their lifeless remains. For instance, phytanic acid was demonstrated to be present in cows (in rumen contents, depot fats, liver), rats (serum), rabbits (serum), pigs (depot fats), deer (depot fats), whales (blubber oil and milk), ovine faeces, fish oil, earthworms, Antarctic and Atlantic krill, crude petroleum, ancient and recent sediments at levels varying around 0.01–0.3% of the total fatty acids. Relatively high amounts of phytanic acid were found in Antarctic krill and

bovine rumen bacteria (1.4 and 2.8% of total fatty acids respectively) and earthworms, which contain 1.9% in winter until as much as 3.5% in summer. Very high levels of phytanic acid (8% of total fatty acids) were found in cows fed for several months with ensilage (for reviews see [4,5]).

2.2. Phytanic acid in man

The presence of phytanic acid in man was first reported in 1963 by Klenk and Kahlke [6] who found phytanic acid in lipid fractions of liver, kidney, and brain of a patient suspected to suffer from Refsum's Syndrome. This neurological syndrome was first described in 1946 by the Norwegian physician Sigvald Refsum [7] and initially called heredopathia atactica polyneuritiformis. Presently, this syndrome is commonly known as Refsum Disease (RD), but is sometimes also referred to as Refsum's Disease or Adult Refsum Disease (ARD).

In later studies it was discovered that phytanic acid accumulation is not a unique feature for Refsum Disease, but also occurs, to a lesser extent, in patients suffering from the group of peroxisome biogenesis disorders (PBDs), including Zellweger Syndrome (ZS), Neonatal Adrenoleukodystrophy (NALD), Infantile Refsum Disease (IRD) and Rhizomelic Chondrodysplasia Punctata type 1 (RCDP1). This observation suggested involvement of peroxisomes in the metabolism of phytanic acid.

2.2.1. Endogenous synthesis

Phytol has a poly-isoprenoid structure and differs from phytanic acid in having a Δ^2 double bond and an alcohol-group rather than a carboxylic acid-group at the first carbon atom. Isoprene units, the building blocks for poly-isoprenoids, are synthesized form three acetate molecules to form mevalonate, which is converted to the isoprene unit isopentenylpyrophosphate. In plant chloroplasts, phytol is synthesized by coupling four isoprene units, followed by reduction of the Δ^6, Δ^{10}, and Δ^{14} double bonds in the backbone of the molecule [8]. As phytanic acid also has a poly-isoprenoid structure it was postulated that phytanic acid could be synthesized in a similar pathway. One of the final steps in this hypothetical pathway would involve the addition of an isoprene unit to farnesol, producing geranylgeraniol. In order to obtain phytanic acid, the double bonds in geranylgeraniol would have to be reduced, and the alcohol-group converted to a carboxylic acid. All precursors for this hypothetical biosynthetic route are available in the human body: farnesol, which plays a role in the farnesylation of proteins, is present in many cell types, and isoprene units, the building blocks for steroids, are also abundant. However, when [2-$^{14}C_1$]mevalonic acid was administered to a patient with Refsum Disease, no incorporation of the label into phytanic acid was found [9–11]. Similar experiments in animals using labelled mevalonic acid and labelled acetate revealed that these potential precursors were not incorporated into phytanic acid [9]. These data have led to the conclusion that phytanic acid is not derived from endogenous synthesis in humans and animals.

2.2.2. Dietary phytol and its conversion to phytanic acid

Phytol (3,7,11,15-tetramethylhexadec-*trans*-2-ene-1-ol) differs in structure from phytanic acid in having a double bond at the first carbon atom and containing an alcohol function instead of a carboxyl-group. Phytol is the alcohol moiety of the chlorophyll molecule and, as an integral part of chlorophyll, it is abundantly present in plants and even more ubiquitous in the marine environment, mainly in planktonic algae [12]. Bound to the chlorophyll molecule, however, humans and rats are not able to absorb this compound effectively [13], which implies that it cannot be a significant dietary precursor of phytanic acid. Phytol can be released from chlorophyll by the action of bacteria present in the rumen of ruminant animals. The resulting free phytol can either be converted to phytanic acid in the rumen of animals or effectively absorbed by both humans and animals [9,13,14].

The conversion of phytol to phytanic acid may proceed via two distinct routes. The first route starts with hydrogenation of phytol yielding dihydrophytol, followed by an oxidation step to form phytanic acid. This route is demonstrated to take place in the rumen of ruminant animals [15–17], and is carried out by resident micro-organisms. The resulting phytanic acid is absorbed by the animal.

The second breakdown pathway was postulated based on the observation that, when free phytol was fed to rats (being a non ruminant), phytanic acid was produced [10], but hardly any dihydrophytol could be detected in plasma and tissues [14]. Further animal studies in various mammals including mouse, rat, rabbit, and chinchilla, showed that upon feeding of phytol not only phytanic acid levels, but also phytenic acid levels increased [10,14]. This has led to the hypothesis that in mammals, phytol is first converted to phytenal, a process which is known to exist in marine bacteria [18], most likely by the action of an alcohol dehydrogenase. Phytenal may then be converted by an aldehyde dehydrogenase to form phytenic acid. Subcellular localization studies in rat have shown that the production of phytenic acid from phytol mainly occurs in mitochondria- and microsome-enriched fractions [19,20]. Based on the observation that in man, the microsomal fatty aldehyde dehydrogenase (ALDH3A2, encoded by the *ALDH3A2* gene) is accepting dihydrophytal as a substrate, van den Brink and co-workers have studied the involvement of this enzyme in the metabolism of phytol. To this end, the ability of both ALDH3A2 deficient cultured fibroblasts (obtained from patients suffering from Sjögren Larssen syndrome (SLS), which is characterized by ALDH3A2 deficiency), and control cell lines was investigated. It was shown that phytenic acid was produced from phytol in fibroblasts from controls, but not in fibroblasts from patients with SLS [21], indicating the involvement of ALDH3A2 in human phytol metabolism.

Muralidharan and Muralidharan [19] have suggested that in mammals, analogous to marine bacteria [18], phytenic acid may be reduced to form phytanic acid, supposedly catalyzed by an NADPH dependent reductase, although the enzyme involved had not been characterized. Recent studies presented evidence that this might not be the only possible conversion. First, it was

demonstrated that the activated form of phytenic acid, phytenoyl-CoA, was converted much more efficiently than free phytenic acid [22], and that phytanoyl-CoA was produced in an NADPH dependent manner. If this breakdown route were true, phytenic acid would need to be activated to phytenoyl-CoA. Based on the finding that free phytanic acid can be activated by long chain acyl-CoA synthetase [23], it was demonstrated that phytenic acid is indeed activated at almost the same reaction rate as phytanic acid, by incubation of the free fatty acids with rat liver homogenate, followed by measurement of phytenoyl-CoA and phytanoyl-CoA formation, respectively [22]. Moreover, it was shown that phytenoyl-CoA is produced in cultured rat hepatocytes grown on a phytol containing medium, demonstrating that phytenoyl-CoA is a true intermediate in the breakdown of phytol.

Recent work by Gloerich and colleagues has led to the identification of the enzyme catalyzing the conversion of phytenoyl-CoA to phytanoyl-CoA [24]. The enzyme involved, human peroxisomal trans-2-enoyl-CoA reductase (PECR), had been identified earlier by Das and co-workers, who proposed it would play a role in fatty acid chain elongation [25], but Gloerich et al. now showed that PECR catalyzes the conversion of phytenoyl-CoA to phytanoyl-CoA in an orthologous expression system in *E. coli* [24].

Taken together, it is likely that the end product of the phytol degradation pathway is not phytanic acid, but rather its activated form, phytanoyl-CoA. This hypothesis is logical in the sense that phytanoyl-CoA is the direct substrate for alpha-oxidation, as will be discussed below.

As can be concluded from the studies described above, phytol bound to chlorophyll as present in plants and vegetables plays no important role as precursor of phytanic acid in non ruminants as chlorophyll is poorly absorbed. Although cooking of vegetables may release some phytol which can be absorbed by humans, this is of little significance when compared to the total amount of dietary phytanic acid (see below), as it comprises less then 10% of the dietary phytanic acid intake. However in ruminants, which display much higher phytanic acid levels in plasma as compared to humans (5–10% and $<0.1\%$ of total fatty acids, respectively [26]) the chlorophyll derived phytol is the major source of phytanic acid. The contribution of free phytol to the total phytanic acid load in the human diet is unclear yet, and should be resolved by phytol measurements in food stuffs. To accomplish this, however, a reliable and preferably inexpensive method to measure phytol in components of the human diet has to be developed, as such a method is currently not available.

2.2.3. Dietary phytanic acid

The average daily intake of phytanic acid in humans is about 50–100 mg, but this amount is strongly dependent on the composition of the diet. Relatively high amounts of phytanic acid are found in dairy products (butter, cheese) and ruminant fats as present in meat products. These levels may vary considerably, dependent on the ruminants' diet: phytanic acid levels may raise from 0.01% of the total fatty acids when feeding on pasture, up to 8% when the cows are fed silage for 6 months, indicating that there is a huge fluctuation during the seasons. Next to the ruminant products, also fish (tuna, cod), fish oil, and vegetable oils are rich in phytanic acid [5,27,28]. Phytanic acid is well absorbed by humans and other mammals (rats, mice), and is rapidly degraded. The elucidation of the metabolic route to degrade phytanic acid will be discussed in detail below.

3. Phytanic acid alpha-oxidation

In studies on phytanic acid alpha-oxidation starting in the 1960s until the mid 1990s, many controversial results have been produced, adding to the confusion about the mechanism and the subcellular localization of the alpha-oxidation pathway.

In vivo experiments in both humans and animals (mostly rats), and studies in intact cell systems including cultured skin fibroblasts, showed that when labelled (either uniformly [U-$^{14}C_{20}$], or specifically at the first carbon atom [1-$^{14}C_1$], or [1-$^{13}C_1$])phytanic acid was administered, production of labelled 2-hydroxyphytanic acid, CO_2 and pristanic acid was detected [10,11,29]. In patients with Refsum Disease, production of 2-hydroxyphytanic acid was deficient [29,30], whereas breakdown of 2-hydroxyphytanic acid and pristanic acid was found to proceed normally [30]. These data have led to the hypothesis that phytanic is first converted to 2-hydroxyphytanic acid and subsequently to pristanic acid [31], and that the first conversion step is deficient in Refsum Disease.

An indication that peroxisomes are at least in part involved in alpha-oxidation was provided by the finding that phytanic acid accumulates in patients suffering from a peroxisome biogenesis disorder, which includes Zellweger Syndrome, Neonatal Adrenoleukodystrophy, and Infantile Refsum Disease. However, cell fractionation studies using human and animal tissues, have resulted in conflicting reports. Most studies elaborated on the concept of the free fatty acid being the substrate for alpha-oxidation, and used CO_2 production as measure of alpha-oxidation activity. In liver from rat and guinea pig, mitochondria were reported to be the main site for alpha-oxidation [32–34], although later the requirement of a cytosolic factor was suggested [35]. Mitochondria were also the apparent alpha-oxidation site in human [36–38] and monkey liver [36]. Using different assay conditions, even the endoplasmic reticulum has been suggested as site for phytanic acid oxidation, at least in rat liver [39]. The first study which reported a peroxisomal involvement was performed in cultured skin fibroblasts [40]. Oxidation was found to be dependent on the presence of ATP, Mg^{2+} and CoA, suggesting that phytanic acid needs to be activated to its CoA-ester prior to oxidation. Remarkably, under the same conditions, a mitochondrial location was found for rat fibroblasts and liver [41]. In search for the activating enzyme, i.e. phytanoyl-CoA ligase, additional studies by the same authors further suggested species dependent localization of this ligase, which they found to be peroxisomal in human liver and fibroblasts, but microsomal and to a lesser extent mitochondrial in the rat [42].

Retrospectively, many of these conflicting findings can now be explained, as in most studies the absolutely essential

components for enzymatic activity were lacking. Briefly, the following two key findings have led to the resolution of the alpha-oxidation pathway in the last decades:

(1) Formic acid and not CO_2 is the primary reaction product of phytanic acid oxidation [43]. The amount of formic acid was shown to be about 9-fold higher as compared to CO_2, which is subsequently formed from formic acid as a secondary product. This finding implied that in all studies in which CO_2 production was used as a measure for enzyme activity, the rates of phytanic acid oxidation have been grossly underestimated. Furthermore, subcellular localization studies became even less reliable, as it is not known whether CO_2 production from formic acid occurs in the same organelle as phytanic acid alpha-oxidation.
(2) Phytanic acid has to be activated to phytanoyl-CoA prior to alpha-oxidation [44]. The authors used [2,3-^{3}H] phytanoyl-CoA as substrate for rat liver peroxisomes and showed that ^{3}H was released. This conversion could only be the result of the 2-hydroxylation of the CoA-ester. In addition, formate was produced in these incubations. Further studies revealed that the hydroxylating enzyme is dependent on the presence of Fe^{2+}, ascorbate, and 2-oxoglutarate [45]. Especially the absolute dependence on 2-oxoglutarate implicated that all previous studies should be considered with great caution, as in none of them this compound had been added to the incubation medium.

After years of debate, consensus has now been reached about the concept of activation of phytanic acid prior to alpha-oxidation, and below the individual steps of this pathway will be described. The subcellular localization of the phytanic acid alpha-oxidation pathway has been established and is predominantly, if not exclusively, peroxisomal.

3.1. Activation and import into peroxisomes of phytanic acid

Despite the fact that Watkins and co-workers provided the first conclusive evidence for the requirement of activation of phytanic acid already in 1994 [44], there is no consensus in literature on the site of phytanic acid activation and the enzymes involved. According to Pahan and Singh, phytanic acid is activated by a distinct acyl-CoA synthetase which they suggested to be localized in peroxisomes in human liver and fibroblasts, but localized in mitochondria and microsomes in rat liver and fibroblasts [42]. Studies by Watkins and co-workers have provided evidence against the existence of a distinct phytanoyl-CoA synthetase [23]. These authors expressed the cDNA encoding the long-chain acyl-CoA synthetase (ACSL1) in an in vitro transcription/translation system, and established that ACSL1 has also affinity for phytanic acid. Interestingly, this enzyme was first thought to be present in peroxisomes, mitochondria and microsomes, but more recent studies by Lewin and co-workers have shown that this is not true: ACSL1 (alias ACS1) is present in peroxisomes and mitochondria-associated membranes (MAM), but not in mitochondria, at least not in rat liver [46]. The catalytic site of this enzyme is exposed to the cytosol which implies that the formation of phytanoyl-CoA occurs in the extra-peroxisomal space, thus requiring a mechanism to shuttle phytanoyl-CoA across the peroxisomal membrane. In literature no data are available on a putative translocator catalyzing the trans-membrane transport of phytanoyl-CoA into peroxisomes.

As an alternative mechanism, phytanic acid could be translocated over the peroxisomal membrane as free fatty acid, followed by activation inside the peroxisome. Human very-long-chain acyl-CoA synthetase (VLACS, encoded by the *SLC27A2* gene) is present in the peroxisomal membrane, has been shown to face the peroxisomal matrix, and is capable of activating phytanic acid [47], demonstrating the feasibility of this route. In order to study this possible role of VLCS in phytanic acid metabolism, the *Slc27a2* knock-out mouse [48] would be a useful tool.

3.2. 2-Hydroxylation by phytanoyl-CoA hydroxylase (PhyH/Pahx)

The first step of the alpha-oxidation pathway involves the 2-hydroxylation of phytanoyl-CoA to form 2-hydroxyphytanoyl-CoA. Studies in rat liver peroxisomes using [1-$^{14}C_1$] phytanic acid by Mihalik et al, showed that indeed [1-$^{14}C_1$]2-hydroxyphytanoyl-CoA was produced, and that this reaction was dependent on Fe^{2+}, ascorbate, and 2-oxoglutarate [45]. The latter compounds are known to be cofactors and co-substrate for the enzyme class of oxygenases. In addition, the incubations yielded labelled formic acid and CO_2, suggesting that in a subsequent step formic acid is produced, which eventually is converted to CO_2, in line with previous studies by Poulos and co-workers [43]. These important findings were confirmed by ourselves [49] and Croes and co-workers [50]. The latter authors showed that in rat liver the model substrates 3-methylheptadecanoic acid and 3-methylhexadecanoic acid also undergo 2-hydroxylation, dependent on Fe^{2+}, ascorbate, and 2-oxoglutarate, and that formic acid and CO_2 are produced [50,51].

The assay system used by Mihalik and co-workers in the studies mentioned above had been optimized for use in purified subcellular (peroxisomal) fractions, where [1-$^{14}C_1$]phytanic acid rather than the true substrate phytanoyl-CoA, was used as pro-substrate, and ATP, Mg^{2+}, CoASH together with purified microsomes were included as a source for synthetase to generate phytanoyl-CoA. However, when this assay-system was applied to activity measurement in total liver homogenates, a rapid hydrolysis of phytanoyl-CoA was observed after addition of liver homogenate to the reaction mixture, indicating that hepatic hydrolases are more abundant in total homogenates when compared to purified peroxisomes and microsomes [52]. This problem was overcome by using phytanoyl-CoA as a substrate, and incubation in the presence of ATP, Mg^{2+}, and CoASH, which proved sufficient to maintain constant levels of phytanoyl-CoA for at least 30 min [49,52]. Subsequent activity measurements in human liver showed that phytanoyl-CoA hydroxylase (PhyH) has high activity in human liver, is localized in peroxisomes, and is deficient in liver biopsies from patients suffering from Zellweger syndrome [49]. A

similar deficiency of PhyH activity was found in liver material from patients with RCDP type 1 [53]. As patients with RCDP type 1 lack Pex7p, and are therefore incapable of importing proteins equipped with a PTS2 target sequence into peroxisomes, this finding strongly suggested that PhyH is a peroxisomal protein and contains a PTS2.

Another important finding was the discovery that PhyH activity is also deficient in a liver specimen from a patient with Refsum Disease, which resolved the molecular mechanism of this disorder [54]. Following up on this finding, *PHYH* (in literature also referred to by its alias *PAHX*), the gene encoding phytanoyl-CoA hydroxylase was cloned [55,56], and it was demonstrated that a large set of patients suffering from Refsum Disease carry sequence variants in *PHYH* [55–58].

Structure based studies on the enzymatic properties of PhyH and its reaction mechanism have been performed initially by Mukherji and co-workers [59]. Sequence comparisons and crystallographic data of the enzyme family of 2-oxoglutarate dependent oxygenases led these authors to predict secondary structure elements of PhyH, including the Fe^{2+} and 2-oxoglutarate binding domains. The Fe^{2+} binding domain comprises the three amino acid sequence HXD, in PhyH at positions 175–177. Site directed mutagenesis of this motif, using p.H175A and p.D177A, abolished hydroxylation of phytanoyl-CoA in vitro [59], and also sequence variants of this domain observed in patients with Refsum Disease, p.Q176K and p.D177G, caused strong reduction of PhyH activity [57]. Also, the p.Q176K variant uncouples the hydroxylation of phytanoyl-CoA from the 2-oxoglutarate utilization, as the 2-oxoglutarate to succinate conversion is reduced to 57% of the control rate, whereas residual hydroxylation activity is only 16% [59]. Furthermore, binding of 2-oxoglutarate was impaired when p.R275 was substituted, strongly suggesting its involvement in the 2-oxoglutarate binding domain. Interestingly, the p.R275Q substitution, which is also observed in two patients with Refsum Disease, results in a complete loss of catalytic activity when 2-oxoglutarate is used as co-substrate, but when 2-oxobutyrate is used in in vitro experiments as alternative co-substrate, PhyH activity is partially restored. Two other substitutions, p.R275A and p.R275W, do not display restoration of enzymatic activity. This 'substrate rescue' may have potential for treatment of patients with Refsum Disease with this specific sequence variation. However, first this concept needs to be further evaluated, starting with in vivo studies.

Another peculiar finding was the observation that two sequence variants, p.G204S and p.N269H, cause the uncoupling of the hydroxylation of phytanoyl-CoA and the conversion of the co-substrate 2-oxoglutarate into succinate and CO_2. In these two variants no hydroxylation occurs, but 2-oxoglutarate is still converted at 40% of its normal reaction rate, basically the same phenomenon as observed with the p.Q176K variant, which is located in the Fe^{2+} binding domain, as described above.

Crystallization studies by McDonough and co-workers have recently revealed the molecular structure of PhyH [60]. This structural knowledge has enabled a better understanding of the enzymatic mechanism of PhyH, and confirmed all hypotheses regarding the Fe^{2+} and 2-oxoglutarate binding domains described above. Moreover, this structural information also allows more detailed predictions on the consequences of sequence variations in Refsum Disease, and possible interventions therein.

3.3. Conversion of 2-hydroxyphytanoyl-CoA to pristanal by 2-hydroxyphytanoyl-CoA lyase

The second step of the alpha-oxidation pathway involves the decarboxylation of 2-hydroxy-phytanoyl-CoA. Initially it has been hypothesized that pristanoyl-CoA would be the product of phytanic acid alpha-oxidation, but surprisingly, pristanic acid was found to be the end product [61]. This finding has led to the postulate that 2-hydroxyphytanoyl-CoA first is converted to the fatty aldehyde pristanal and formyl-CoA by the action of a lyase. Indeed, incubation of human liver homogenate in the presence of 2-hydroxyphytanoyl-CoA yielded pristanal as product [62]. Addition of NAD as cofactor for a putative aldehyde dehydrogenase possibly catalyzing the subsequent dehydrogenation of pristanal, resulted in the production of pristanic acid, which indicated that this proposed pathway might be correct. The enzyme catalyzing the decarboxylation of 2-hydroxyphytanoyl-CoA was named 2-hydroxyphytanoyl-CoA lyase (2-HPCL). This enzyme has been purified by Foulon and co-workers and was found to be localized in peroxisomes in rat liver, and dependent on thiamine pyrophosphate (TPP) and Mg^{2+} [63]. Cloning of the encoding gene (*HACL1*) revealed the presence of an hitherto unrecognized PTS1 signal (-SNM, preceded by a positive charge: in this case RSNM), which directs 2-HPCL to peroxisomes, as transfection experiments with GFP-2-HPCL fusion protein have shown a peroxisomal localization in $Pex5^{+/-}$, and a cytosolic localization in $Pex5^{-/-}$ mouse cell lines.

Although it had previously been demonstrated that formate was produced in this step [43], now it was also demonstrated that in fact formyl-CoA is the primary product of this decarboxylation [63,64]. Formyl-CoA, which was shown to be instable at neutral pH, spontaneously splits into formate and CO_2 [64], explaining the previous observations of formate production in this step. The fate of formate within peroxisomes is unclear. A good candidate for the subsequent oxidation of formate would be catalase. This enzyme detoxifies hydrogen peroxide by catalyzing the conversion of $2H_2O_2$ to $2H_2O+O_2$, but also may act as a more general peroxidase catalyzing the reaction $H_2O_2+RH_2$ to $2H_2O+R$, in which hydrogen peroxide is used to oxidize various compounds (R) such as phenols, alcohols, formaldehyde, and also formic acid. The capability of catalase to oxidize formic acid has already been reported in 1948 by Chance [65].

3.4. Dehydrogenation of pristanal by an aldehyde dehydrogenase

The last step in phytanic acid alpha-oxidation involves the conversion of pristanal to pristanic acid. This reaction is carried out by an NAD(P) dependent fatty aldehyde dehydrogenase, as

was demonstrated in human liver and cultured skin fibroblasts [62,66]. Based on a single experiment in human liver only, it was suggested that this conversion may take place in the endoplasmic reticulum [61]. The possible involvement of the fatty aldehyde dehydrogenase ALDH3A2 was investigated using cell lines from patients with Sjögren Larssen syndrome (SLS), characterized by a microsomal ALDH3A2 deficiency. It was shown that [2,3-^{3}H]phytanic acid oxidation occurred at less than 25% of the control rate [66], indicating a role of ALDH3A2 in alpha-oxidation. This hypothesis is not very appealing, as it would imply the transport of pristanal from peroxisome to endoplasmic reticulum, followed by transport of pristanic acid in the opposite direction for further beta-oxidation in peroxisomes. A more attractive idea would be the existence of an aldehyde dehydrogenase within peroxisomes. This aldehyde dehydrogenase activity in peroxisomes was demonstrated in rat liver [64,67]. Moreover, studies in fibroblast homogenates from patients with SLS showed more than 25% residual activity for pristanal conversion, whereas dehydrogenation of other aldehydes such as octanal was almost completely deficient [67]. These observations indicated that microsomal ALDH3A2 may not be the main catalyst in pristanal degradation.

The existence of many (at least more than ten) different aldehyde dehydrogenases [68], some with a poorly characterized subcellular localization, have complicated detailed

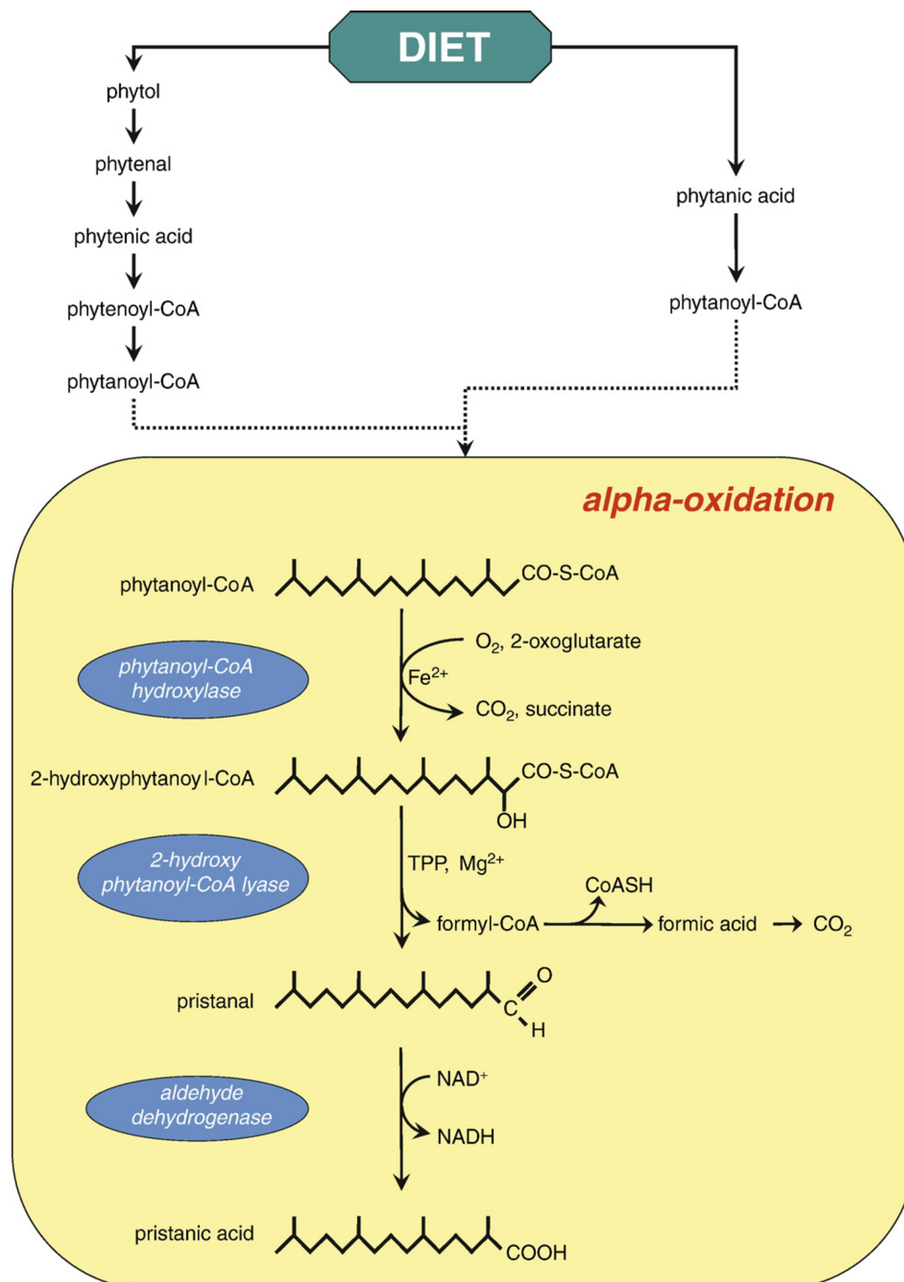

Fig. 1. The phytanic acid alpha-oxidation pathway. The top of this scheme shows the dietary input of phytanic acid. Phytol, also a dietary component, can be converted to phytanoyl-CoA and subsequently undergo alpha-oxidation. See text for details.

studies. Moreover, some of these enzymes have overlapping substrate specificities, and several may be capable of pristanal conversion. Several attempts to purify a peroxisomal pristanal converting aldehyde dehydrogenases have led to the isolation of (microsomal) ALDH3A2 (van den Brink, personal communication). Despite being qualified as a microsomal enzyme, ALDH3A2 is still a possible candidate for pristanal conversion for the following reasons: first, a bimodal localization between peroxisomes and ER has been reported [69,70], although this has been rejected by others [71,72]. Second, different splice variants of ALDH3A2 (encoded by the *ALDH3A2* gene) occur, one of which lacks the targeting sequence for the endoplasmic reticulum, which may well lead to a different subcellular localization. Third, a hypothesis for the biogenesis of peroxisomes involves the budding of ER membranes to form peroxisomal membranes, thus allowing the incorporation of originally ER proteins in peroxisomes. Conclusive evidence for any of the mentioned ideas needs to be provided by future studies.

An overview of the alpha-oxidation pathway is depicted in Fig. 1.

3.5. Stereochemistry of the phytanic acid alpha-oxidation pathway

In contrast to the peroxisomal beta-oxidation system, the phytanic acid alpha-oxidation pathway is not stereo-selective [73]. Indeed, the two naturally occurring stereo-isomers

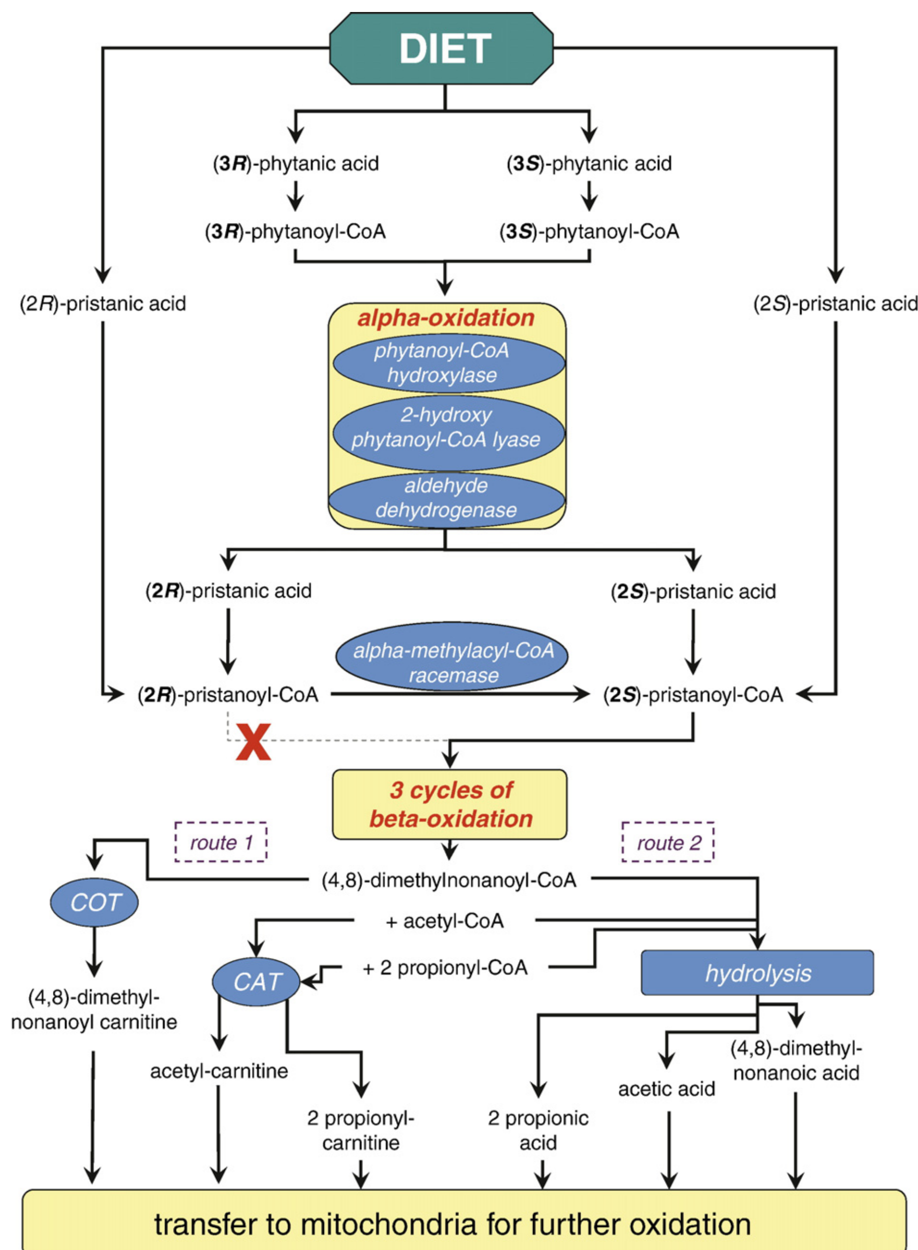

Fig. 2. Schematic representation of the degradation of phytanic acid and pristanic acid in peroxisomes. The products of the peroxisomal beta-oxidation, as depicted in the bottom half of this scheme, may follow route 1 or 2 before transport across the peroxisomal membrane and transfer to the mitochondria. See text for details.

(3***R***,7*R*,11R)- and (3***S***,7*R*,11R)-phytanic acid can be activated to their corresponding coenzyme A esters and alpha-oxidized to produce (2***R***,6*R*,10*R*)- and (2***S***,6*R*,10*R*)-pristanic acid (see Fig. 2).

3.6. Further catabolism of pristanic acid by the peroxisomal beta-oxidation system

Early studies by Poulos and co-workers had already shown that pristanic acid is not oxidized in mitochondria, but in peroxisomes [74]. The first direct indication that pristanic acid is indeed a substrate preferred by peroxisomes came from the observation that pristanic acid levels in plasma are elevated in patients lacking peroxisomes (such as Zellweger Syndrome). Subsequent studies using radio-labelled pristanic acid have confirmed this finding.

The site of activation of pristanic acid has not been established with certainty but may well be different depending on the source of pristanic acid. Indeed, early studies by one of us have shown that pristanic acid can be activated at different subcellular locations including peroxisomes, mitochondria and endoplasmic reticulum. The activity localized in peroxisomes is membrane-bound, with the site of catalytic activity exposed to the cytosol [75]. This synthetase may well be involved in the activation of pristanic acid derived from extra-peroxisomal sources. However, as described above, a substantial part of pristanic acid is generated inside peroxisomes by alpha-oxidation of phytanic acid. It may well be that pristanic acid generated inside peroxisomes is activated by the enzyme very long-chain acyl-CoA synthetase (VLACS, encoded by the *SLC27A2* gene), which has a bimodal distribution in peroxisomes and endoplasmic reticulum, respectively [47]. Available evidence suggests that this enzyme is peripheral membrane-bound, with its catalytic site exposed to the peroxisomal lumen. VLACS may be targeted to peroxisomes by a potential PTS1 signal, -LKL.

In contrast to the alpha-oxidation system, the peroxisomal (and also mitochondrial) beta-oxidation system is stereo selective and only accepts (2*S*)-acyl-CoA esters. This implies that (2*S*)-pristanoyl-CoA can be directly degraded by the peroxisomal beta-oxidation system, in contrast to (2*R*)-pristanoyl-CoA, which first needs to be converted to (2*S*)-pristanoyl-CoA. Peroxisomes contain alpha-methylacyl-CoA racemase (AMACR, encoded by the *AMACR* gene) activity which allows this conversion. Interestingly, AMACR is equipped with two targeting signals, one at the carboxy-terminus and the other at the amino terminus of the protein. The amino-terminal part contains a mitochondrial targeting signal (MTS) whereas the carboxy-terminus contains a peroxisomal targeting signal (PTS1).

Studies by Verhoeven and co-workers [76] have shown that pristanoyl-CoA undergoes three cycles of beta-oxidation in peroxisomes, yielding two molecules of propionyl-CoA, one acetyl-CoA, and one 4,8-dimethylnonanoyl-CoA as end products, which may be transported to the mitochondria for further oxidation via one of the following routes. Route 1 involves the conversion of the acyl-CoAs in peroxisomes to the corresponding carnitine-esters as mediated by the two peroxisomal acyltransferases, i.e. carnitine acyltransferase (CAT, encoded by the *CRAT* gene), which converts acetyl-CoA and propionyl-CoA into their carnitine-esters, and carnitine O-octanoyltransferase (COT, encoded by the *CROT* gene), which converts 4,8-dimethylnonanoyl-CoA into 4,8-dimethylnonanoylcarnitine. Route 2 involves hydrolytic cleavage of the acyl-CoAs to form free fatty acids plus coenzyme A, followed by the transfer of the fatty acids out of the peroxisomes and into mitochondria for further oxidation (see Fig. 2).

4. Concluding remarks

4.1. Additional substrates for alpha-oxidation: straight chain fatty acids

Recent studies suggest that peroxisomes are involved in the breakdown of 2-hydroxylated straight chain fatty acids. 2-Hydroxy straight chain fatty acids are present in various tissues, but most abundant in brain, as constituents of cerebrosides and sulfatides, mostly found in myelin. It is unclear whether PhyH can catalyze the hydroxylation of straight chain fatty acids, as suggested by some [77], or not, as shown by others [73,78]. Alternatively, hydroxylation of straight chain fatty acids may also be carried out by a distinct hydroxylase FA2H in brain [79]. It has been suggested that these 2-hydroxy fatty acid compounds are broken down by alpha-oxidation. Foulon and co-workers now have demonstrated that 2-hydroxy straight chain fatty acids can indeed be alpha-oxidized in both intact and broken cell systems from rat brain and liver, thereby producing a fatty aldehyde, formyl-CoA and CO_2 [80]. Furthermore, the reaction was shown to be dependent on ATP, Mg^{2+}, CoASH, TPP, and NAD, exactly the same cofactors as needed for peroxisomal phytanic acid alpha-oxidation as catalyzed by 2-HPCL and the subsequent aldehyde dehydrogenase. Thus far, only a few substrates with a limited number of chain lengths have been studied. Additional studies will be needed to show the range of substrates and their chain lengths that are degraded by this pathway, but regardless of the results, these data already strongly suggest an hitherto unrecognized role of peroxisomal alpha-oxidation in brain lipid metabolism.

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