Molecular aspects of Refsum disease and the enzymatic degradation of phytol to phytanic acid
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Chapter 1

GENERAL INTRODUCTION
Chapter 1

Introduction

Research on phytanic acid started in the 1950's when dairy products were being investigated for their fatty acid content. These studies revealed that phytanic acid is a major constituent of a variety of food products in the human diet, especially in products derived from grazing animals. In the following decade it was found that phytanic acid is a C_{20} fatty acid, made up of a C_{16} backbone and four additional methyl groups, which makes it a saturated branched chain fatty acid (Fig. 1) (reviewed in 1). The interest in phytanic acid increased considerably when Klenk and Kahlke reported elevated phytanic acid levels in tissues of a patient suffering from Refsum Disease (2). Once it had been established that phytanic acid was also massively elevated in plasma of their patient, an easy biochemical diagnostic test became available. Since then, phytanic acid is generally regarded as the most important pathognomonic marker of the disease. In addition, recent evidence indicates that phytanic acid is directly involved both in the induction of apoptosis in astrocytes (3, 4) as well as in the onset of heart problems (5).

Refsum Disease

Before the finding that phytanic acid accumulates in patients suffering from Refsum disease, diagnosis was carried out on the basis of a number of clinical hallmarks, first described by Sigvald Refsum, a Norwegian neurologist. In 1946, he reported five cases suffering from a similar syndrome that had not been described before (6). Although he proposed the name heredopathia atactica polyneuritiformis, the syndrome is now better known as Refsum disease. The symptoms that were noted included retinitis pigmentosa, peripheral neuropathy, cerebellar ataxia and elevated protein concentrations in the cerebrospinal fluid in the absence of an increased number of cells (7). Additionally, in a recent evaluation, anosmia was found in nearly all Refsum disease patients, while deafness, ichthyosis and cardiac arrhythmias were also regularly present (Fig. 2) (8, 9), and in around a third of the patients short metacarpals or metatarsals were found (10). The onset of symptoms is usually relatively late in life, with most patients presenting in adolescence. The first clinical manifestations often start with night blindness followed by further deteriorating of vision. Although onset of disease may occur much earlier in life, the subtle nature of the first symptoms makes it difficult to delineate the precise age at which the disease started. However, in most cases the symptoms will progress to retinitis pigmentosa, leading to tunnel vision or even complete loss of vision. Occurrence of cardiac arrhythmias can sometimes lead to death or necessitate a cardiac transplant. The incidence of Refsum disease is reported to be quite low although it is possible that the true incidence of the disease may be much higher than currently believed. Indications that Refsum disease is overlooked as a diagnostic entity by many clinicians comes...
from the fact that over 80% of currently known patients were diagnosed inside the United Kingdom where the awareness of Refsum disease is high. At the same time there is no indication of a possible founder effect, since many different mutations in the disease causing gene have been reported (11).

Figure 2. Cumulative incidence of clinical features on presentation of 15 patients with Refsum disease. ◆, Retinitis pigmentosa; ■, anosmia; ▲, neuropathy; ○, deafness; ×, ataxia; ○, ichthyosis. Taken from Wierzbicki et al. (8), with permission.

Accumulation of phytanic acid
The pathogenesis of Refsum disease was thought to follow the increased levels of phytanic acid found in patients. This notion sparked further interest into the origin of phytanic acid. As described above, phytanic acid had already been found in certain food stuffs and research in the 1960’s was directed towards the identification of other sources.

Contribution of endogenous synthesis
Work by Steinberg and colleagues demonstrated that endogenous synthesis of phytanic acid does not take place although this would be quite conceivable because its polyisoprenoid structure is similar to that of farnesol and geranylgeraniol. These latter compounds can be synthesized in mammalian cells by assembly of multiple mevalonate units (12). Phytanic acid only differs from geranylgeraniol by the absence of three double bounds (at positions Δ⁶, Δ¹⁰ and Δ¹⁴) and a carboxyl- instead of an alcohol-group. However, radio-labelled mevalonate administered to rats did not lead to incorporation of label into phytanic acid (13, 14). Therefore, it was concluded that all phytanic acid is derived from dietary sources.

Dietary intake of phytanic acid
Hansen and co-workers first identified phytanic acid in buttermilk in the 1950’s (15, 16) and since then it has been detected in a variety of food sources. Phytanic acid is espe-
Phytanic acid is particularly abundant in ruminant animals including cows and sheep in various tissues such as fat, liver, plasma, milk and rumen content. However, it is also present in some non-ruminants such as rats, pigs and humans. The amount of phytanic acid found ranges from about 0.01 to 0.3% of the total fatty acid pool, but can exceed the 10% mark in milk from cows fed on ensilage, the fermented grass that is used as winter feed for cattle. High amounts were also detected in Antarctic krill (1.4%), the plankton that forms the basis of the oceanic food chain, which accounts for the presence of phytanic acid in for example molluscs, fish oil, whale oil and whale milk (1). In addition to this, high levels of phytanic acid are present in earthworms (up to 3.5%). This raised the hypothesis that phytanic acid was actually derived from a precursor molecule that is very abundant in nature, namely phytol.

Normally, phytol (3,7,11,15-tetrahexadec-2-en-1-ol) is a constituent of the chlorophyll molecule (Fig. 3), a bio-molecule that is involved in the production of energy from light.

![Figure 3. Chlorophyll A. Phytol is esterified to the molecule (depicted in bold).](image)

Since almost all photosynthetic organisms use chlorophyll, phytol is also abundantly present in nature. As a constituent of the large quantities of grasses consumed by ruminant animals, a lot of chlorophyll is taken in from which the phytol moiety is released and converted into phytanic acid. How this process takes place exactly will be discussed later in more detail.

To show that phytol could be converted into phytanic acid, feeding studies were performed in which animals were fed phytol-supplemented diets. Feeding these diets indeed resulted in the accumulation of phytanic acid in rats or rabbits, with levels reaching as high as 50% of total fatty acids (13, 17). This was also true in humans as shown in studies where a dose of radiolabelled phytol was administered to Refsum disease patients after which labelled phytanic acid could be detected in plasma (18, 19). These findings led to the conclusion that phytanic acid is indeed derived from exogenous sources via the diet. The investigators went on to speculate that a reduction of chlorophyll in the diet of Refsum disease patients might be beneficial in reducing the phytanic acid levels, but this was refuted by the finding that phytol bound to chlorophyll is hardly released. This was clearly shown in experiments where a dose of radioactively labelled chlorophyll was administered orally to rats as well as human subjects. Around 95% of the chlorophyll passed through the digestive system intact, leaving only 5% of the phytol available for conversion into phytanic acid (20, 21). Additionally, only around 5% of the phytol present in spinach fed to a healthy control was accounted for in the lymph (20). In con-
Contrast to rats or humans however, animals with a ruminant digestion system are capable of releasing phytol from chlorophyll, which is mediated by the action of bacteria in the gut of these animals. After it is released, phytol is then available to be converted into phytanic acid that is then recovered in the tissues of these animals (17). The implication of this is that the major source of phytanic acid for humans does not come from chlorophyll-bound phytol, but rather from phytic acid present in food products. In surveys of phytanic acid content of a variety of food products high levels were indeed found in goods such as milk, butter and cheese and meat from cows and sheep, while in meat from pigs and poultry hardly any could be detected. Almost no phytanic acid is present in vegetables, while some species of fish and fish oils were found to contain high amounts (22-24). These observations are an important basis for the treatment of Refsum disease patients, since phytanic acid build up can be entirely prevented by prescribing a diet in which phytanic acid is as low as possible. In fact, to date this remains the predominant form of treatment.

**Breakdown of phytanic acid**

While phytanic acid accumulates in Refsum disease patients, other fatty acids are apparently broken down without difficulty. The branched-chain structure of phytanic acid was proposed to be the reason for this. Normal degradation of fatty acids takes place by β-oxidation, but in case of phytanic acid the presence of a methyl-group at the 3-position (Fig. 1) makes this impossible.

**β-Oxidation**

Most fatty acids are degraded in a stepwise manner by β-oxidation, which first requires activation of a fatty acid to its coenzyme A (CoA)-esters and the subsequent shortening of the carbon-chain by two atoms per cycle. Each cycle consists of four enzymatic steps by which an acetyl-CoA molecule is released as well as energy in the form of FADH₂ and NADH (Fig. 4A). β-Oxidation takes place in two distinct compartments inside the cell, the mitochondria and the peroxisomes. The latter organelle is responsible for the degradation of very long-chain fatty acids and when they are reduced to a length of about 16 carbon atoms, they are translocated to the mitochondria for further β-oxidation. In addition to straight-chain fatty acid β-oxidation, peroxisomes are also involved in the β-oxidation of e.g. branched-chain fatty acids and bile acid intermediates (reviewed in 25).

Because the 3-methyl group of phytanic acid obstructs β-oxidation, phytanic acid was proposed to be degraded via a different mechanism.

**α-Oxidation**

Insight into how phytanic acid is degraded came from the detection of pristanic acid in rats that were administered a dose of phytol (26). Pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) possesses a carbon chain that is one carbon atom shorter than phytanic acid, which led to the hypothesis that phytanic acid undergoes one round of α-oxidation. The process of α-oxidation consists of the removal of one carbon atom instead of the two that are removed on β-oxidation (Fig. 4B). The hypothesis was supported by experiments with human subjects that received an artificial substrate, 3,6-dimethyloctanoic acid. It was established that this 3-methyl branched-chain substrate
Figure 4. Mechanism of α- and β-oxidation. (A) Each cycle of β-oxidation consists of an oxidation, a hydration, a second oxidation and finally thiolysis. In the last step an acetyl-CoA is released and the length of the fatty acid carbon chain is reduced by 2 carbon atoms. The resulting acetyl-CoA is then ready for another cycle of β-oxidation. (B) α-Oxidation is initiated by hydroxylation of the 3-methyl acyl-CoA. A lyase reaction releases a formyl-CoA from the molecule, which can be converted into CO₂. Next, an aldehyde dehydrogenase converts the fatty aldehyde into the corresponding acid, which is then activated to its CoA-ester by a synthetase reaction. The resulting 2-methyl acyl-CoA can be further β-oxidized.

undergoes α-oxidation and furthermore that its degradation is deficient in Refsum disease patients (27). Production of pristanic acid was also found in human fibroblasts cultured in the presence of radiolabelled phytanic acid (28). From these studies a mechanism of phytanic acid α-oxidation was proposed (29).

β-Oxidation is initiated by a dehydrogenation reaction that forms a double bond at the 2-3 position. It was shown that did does not occur for the α-oxidation of phytanic acid, since no phytanic acid (3,7,11,15-tetramethylhexadec-2-enoic acid) could be detected in rats injected with radiolabelled phytanic acid (13). Instead, 2-hydroxyphytanic acid formation was seen in these rats, which indicated that hydroxylation of phytanic acid
at the 2 position was the first step of $\alpha$-oxidation. Support came from in vivo studies, which showed that in plasma of human subjects that had been administered $[1-{ }^{13}\text{C}]$phytanic acid, 2-hydroxy $[1-{ }^{13}\text{C}]$phytanic acid could be detected (29, 30). Analogous to $\beta$-oxidation, it was initially proposed that the hydroxyl group is converted into a keto group. Indeed, it was observed that 2-ketophytanic acid was formed from 2-hydroxyphytanic acid in rat kidney homogenates (31, 32). This reaction was also observed in rat and human liver, although it was not dependent on any cofactors (33), which is in contrast to the oxidation reaction in beta-oxidation that is NAD$^+$-dependent. However, these findings remained controversial and were made redundant by the discovery that substrates for $\alpha$-oxidation are CoA-esters and not free acids.

**Activation of phytanic acid**

Experiments where the rate of $\alpha$-oxidation was increased by the addition of ATP, Mg$^{2+}$ and CoA, co-factors of synthetase enzymes (34), suggested that phytanic acid can not only be activated to its CoA-ester, but also that phytanoyl-CoA is the true substrate for the hydroxylase reaction (35). This proved to be a major step forward in the elucidation of the $\alpha$-oxidation mechanism, because for the first time hydroxylation of phytanic acid could be measured directly in cell homogenates.

The enzyme responsible for the activation of phytanic acid to its CoA-ester was first reported to be a distinct phytanoyl-CoA ligase and thought to be present in peroxisomes in human liver and in microsomes and mitochondria in rat liver (36). However, later research showed that phytanic acid can be activated by long-chain acyl-CoA synthetase, which is a known enzyme present in peroxisomes that also has affinity for straight-chain fatty acids (37).

**Hydroxylation of phytanic acid**

The first true step of $\alpha$-oxidation of phytanoyl-CoA is its hydroxylation into 2-hydroxy-phytanoyl-CoA by the enzyme phytanoyl-CoA hydroxylase (PAHX or PhyH). However, as has been noted before, a lot of confusion surrounded the hydroxylation of phytanic acid and its subsequent fate during $\alpha$-oxidation. There were still major problems with measurements in broken cell preparations as was evident from experiments where $\alpha$-oxidation activity was 20-fold lower in post-nuclear supernatant fractions from rat liver compared to intact rat hepatocytes (38). And although some conflicting results had been resolved by the discovery that the CoA-ester was the true substrate for $\alpha$-oxidation, it was not until the hydroxylation of phytanoyl-CoA was investigated in a methodological way that much of the controversy could be expelled (reviewed in detail in 39).

Mihalik and co-workers investigated whether hydroxylase activity in fractionated rat liver could be stimulated by the addition of a selection of cofactors (40). For this they monitored how the rate of conversion of phytanoyl-CoA to 2-hydroxyphytanoyl-CoA was influenced by the addition of various compounds required by enzymes that catalyze similar reactions, such as Cu$^{2+}$ and ascorbate; NAD$^+$; NADH; NADP$^+$; NADPH; crotonase; tetrahydrobiopterin and Fe$^{3+}$; 2-oxoglutarate, Fe$^{2+}$ and ascorbate. The results showed a dramatic increase in activity upon addition of the dioxygenase cofactors 2-oxoglutarate, Fe$^{2+}$ and ascorbate, while other cofactors had little effect. The dependence on these cofactors for activity was corroborated in later studies (41, 42), classifying PAHX as a non-haem iron(II) and 2-oxoglutarate-dependent oxygenase. Iron(II) functions in the active site of these enzymes and the presence of ascorbate helps to keep the iron in the
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+2 valance. 2-Oxoglutarate functions as a cosubstrate and is converted into succinate. Now that activity of the hydroxylase reaction could easily be measured in an isolated assay, measurements could be made in tissues derived from Refsum patients. In this way it was finally unequivocally established that Refsum disease patients were deficient in PAHX, the first step of α-oxidation (43). In addition, a deficiency of PAHX was shown in liver material derived from Zellweger patients (42). Indications for this had been described some years before by Poulos et al. (44). Because patients suffering from Zellweger syndrome are characterized by the complete absence of peroxisomes, this also meant that PAHX was most likely a peroxisomal protein. This was confirmed by the purification and cloning of the enzyme, as will be discussed later.

Decarboxylation of phytanic acid
The second step of phytanic acid α-oxidation involves the shortening of the molecule by one carbon atom. Originally it was presumed that CO₂ was produced in this reaction but this was refuted by Poulos et al. (45), who observed that >90% of radioactivity coming from [1-¹³C] phytanic acid α-oxidation in fibroblasts was present in the water-soluble fraction and not the gaseous fraction. They and others (41) showed that the radioactivity came from formate, or, as was discovered somewhat later, from formyl-CoA, which is now known to be the true product of the decarboxylation of phytanic acid (46) (Fig. 4B). Formyl-CoA can subsequently be broken down to CO₂ by an enzymatic reaction that takes place not in the peroxisomes, but in the cytosol (41). With the release of formyl-CoA, 2-hydroxyphytanoyl-CoA is converted into the aldehyde pristanal (2,6,10,14-tetramethylpentadecanal). Pristanal was already hypothesized to be an intermediate of phytanic acid breakdown as early as the 1960’s (29), but this question remained unresolved for nearly three decades. Support for this hypothesis came from the suggestion that pristanic acid, and not pristanoyl-CoA was the end product of phytanic acid α-oxidation (47) and finally proof was obtained by the detection of the product pristanal itself in incubations of rat liver homogenates with 2-hydroxyphytanoyl-CoA (48).

The enzyme catalyzing this reaction was defined as 2-hydroxyphytanoyl-CoA lyase (HPCL) and was further characterized in an isolated assay in rat liver homogenates. These studies showed a peroxisomal activity for HPCL, which was in line with the emerging hypothesis that the complete process of α-oxidation was localized within the peroxisomes (49).

Shortly afterwards the enzyme was purified from rat liver peroxisomes and cloned. The human cDNA was expressed from which a dependence on thiamine pyrophosphate and Mg²⁺ was discovered (50). In further studies it was observed that HPCL has specificity for substrates that possess a 2-hydroxy and a CoA-moiety, but a 3-methyl group was not necessary for activity. Affinity for hydroxyl substrates is quite unusual for lyases, since mostly a 2-keto carboxyl compound is used as a substrate (51).

Dehydrogenation of pristanal to pristanic acid
The third step of phytanoyl-CoA α-oxidation concerns the conversion of pristanal into pristanic acid. This reaction was first measured in human liver (48) and subsequently in cultured human fibroblasts (52). Since it had previously been suggested that the conversion of 2-hydroxyphytanoyl-CoA into pristanic acid took place at the endoplasmatic reticulum (ER) (47), a candidate enzyme for the dehydrogenation of pristanal was fatty
aldehyde dehydrogenase (FALDH, ALDH10), also present on the ER membrane. To test this, fibroblasts derived from patients suffering from Sjögren-Larsson Syndrome (SLS), which are deficient in FALDH, were investigated for their ability to degrade phytanic acid. It was observed that oxidation of [2,3-3H]phytanic acid was reduced to 25% in SLS fibroblasts. In addition, in SLS cells that were incubated in the presence of [2,3-3H]phytanic acid, a 4-fold increase was detected in the accumulation of radioactivity in N-alkylphosphatidyl ethanolamine (52). Both these findings indicate a possible role of FALDH in the breakdown of phytanic acid. However, this would imply that the pathway of α-oxidation is not completely peroxisomal, but would rely on the translocation of pristanal to the ER to be converted into pristanic acid, which would then in turn have to be transported back to the peroxisomes for further β-oxidation. This unlikely mechanism sparked detailed investigation in the possible involvement of FALDH in α-oxidation.

Incubations using pristanal as a substrate to measure isolated FALDH activity in SLS fibroblast homogenates revealed that there was a >25% residual activity, while with other substrates, such as octadecanal, this was <10% (52, 53). This suggests that another aldehyde dehydrogenase is involved in the conversion of pristanal into pristanic acid. Furthermore, it was shown that a distinct peroxisomal aldehyde dehydrogenase activity exists inside the peroxisome (46, 53).

Because of the large number of aldehyde dehydrogenases known in man (more than ten, reviewed in 54) it does not come as a great surprise that more than one of these can use pristanal for its substrate. Interestingly, none of the aldehyde dehydrogenases described in the review has a peroxisomal localization. However, it cannot be ruled out that FALDH is involved in the breakdown of pristanal, since some evidence exists that it has a double localization inside the cell, namely in peroxisomes as well as in the ER, at least in rats (55, 56), although later studies indicated an exclusively ER localization of FALDH (57, 58). Interestingly, our own unpublished efforts in purifying pristanal dehydrogenase activity from purified rat peroxisomes have twice resulted in the identification of FALDH. This might be explained by a microsomal contamination of the peroxisomes that were used or, alternatively, might result from the presence of ER-membrane proteins in the peroxisomes. Interestingly, recent evidence has been pro-

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*Figure 5.* C-terminal alignments of rat, mouse and human FALDH and splice variants. Normally, splicing occurs between exon 9 and 10 and translation stops on exon 10. Alternative splicing occurs in a minority of transcripts (M. and H. variant for respectively mouse and human alternative transcripts) resulting in the insertion of an extra exon (exon 9') between exon 9 and 10. Exon 9' also contains a termination codon, and translation of the variant transcript results in FALDHv, which is 26 or 27 amino acids longer (for mouse and human respectively) than the original FALDH protein. Amino acids marked by a zigzag line make up the transmembrane anchor domain and those marked by asterisks within arcs effect ER-retention.
duced, supporting a contentious model of peroxisome biogenesis in which newly formed peroxisomes bud off from the ER, which suggests that a direct connection might exist between these two organelles (59, 60).

Localization studies of FALDH are complicated by the existence of two splice-variants in the corresponding ALDH10 gene. Both in humans and in mice it was shown that the last intron is not spliced in about 10% of the ALDH10 transcripts, which leads to an extension of the protein at the C-terminus by 27 and 26 amino acids respectively as depicted in figure 5 (61-63). The C-terminus of FALDH has been shown to be crucial for localization of the enzyme to the ER. Furthermore, a stretch of hydrophobic amino acids was shown to be essential for anchoring the protein to the ER, since deletion of this domain resulted in a cytosolic localization (58). The protein resulting from variant splicing of the ALDH10 gene (FALDHv), still possesses an intact membrane spanning domain (Fig. 5), but the ER-retention signal on the C-terminus is disturbed. A recombinant FALDH protein lacking this signal was shown to be still associated with the ER, although less strongly than the full-length protein (57, 58). Therefore the possibility remains that FALDHv might have an alternative localization in the cell. However, the importance of the amino acids of FALDH located inside the ER matrix, is made more ambiguous because in humans it is distinctly different than that in rats and mice, while the rest of the protein is >85% conserved between these species (Fig. 5). Precise expression studies with the two human variants will have to elucidate the putative role of FALDH in α-oxidation. In addition to this, the recent generation of FALDH-transgenic mice by Rizzo and co-workers (personal communication) might shed more light on the same question. Some reports in literature indicate that a peroxisomal aldehyde dehydrogenase is present in rat liver, induced by clofibrate (64). However, since it is known that FALDH is also induced by clofibrate (65) and, furthermore, that the aldehyde dehydrogenase that was described was membrane-bound, most likely a microsomal contamination was behind the results that were observed. The true identity of the aldehyde dehydrogenase involved in α-oxidation, therefore, remains elusive. Further research will have to establish whether an additional, peroxisomal, aldehyde dehydrogenase metabolizes pristanal into pristanic acid.

Further studies on PAHX

Final proof that the defect underlying Refsum disease was a deficiency of the first step of α-oxidation came from the purification of the enzyme. This was first accomplished by Jansen et al. (66) who purified PAHX from rat liver peroxisomes using column chromatography. From N-terminal sequencing of the purified protein, a cDNA could be obtained from EST-database screens. From the rat cDNA, the human orthologue could also be found by screening the EST-database, which also opened the way to sequencing the PAHX gene of patients suffering from Refsum disease. In this way the first mutations were found, establishing the underlying gene defect of Refsum disease (67).

Targeting of PAHX to peroxisomes

With the resolution of the PAHX cDNA another long-standing debate could be resolved, namely the subcellular localization of α-oxidation. Although most evidence had pointed to a peroxisomal localization, some evidence existed that in the rat mitochondria were involved instead (68). The presence of a peroxisomal targeting signal type 2 (PTS2)
on PAHX, in the absence of a mitochondrial targeting signal, finally resolved this issue (66).

Peroxisomal matrix protein import
Peroxisomal matrix proteins, which are all synthesized on free polyribosomes in the cytosol, are imported into peroxisomes via two routes. They contain either a PTS1 or a PTS2, which can be recognized by receptor proteins in the cytosol. The receptor for PTS1-proteins is peroxin 5 and the receptor for PTS2-proteins is peroxin 7, and both these receptors function in a similar way. As depicted in Fig. 6, the protein that needs to be imported is bound to the receptor in the cytosol, after which the complex is able to dock on the peroxisomal membrane at docking sites made up from other peroxins of protein import, which includes the docking, translocation over the peroxisomal membrane, and finally the release of peroxins 5 or 7 by which the circle is completed (69, 70).

Both receptor peroxins function by recognizing certain amino acids on the target protein. However, the location of these amino acids on the protein as well as the consensus sequence that makes up the PTS differ between PTS1 and PTS2 sequences. A PTS1 is located at the extreme C-terminal end of the protein and is made up from three or occasionally four amino acids. On the other hand, the PTS2 is positioned near the N-terminal part of the protein and is made up of nine amino acids, of which only the outer two on each side are important and the middle five are of no consequence (71). After import into the peroxisome, the part of the protein on which the PTS2 is situated is cleaved off. Therefore the mature protein has a somewhat lower molecular weight than its precursor. In the case of PAHX, the precursor of 38.5 kDa is reduced to 35 kDa inside the peroxisome (66). Interestingly, Mihalik et al. identified PAHX in an EST-database screen for PTS2 proteins, and was able to show it was mutated in Refsum disease by finding mutations in Refsum disease patients (72). 2-Hydroxyphytanoyl-CoA lyase was shown

Figure 6. Simplified cartoon of the import of peroxisomal matrix proteins. See text for details.
to possess a PTS1 sequence (50). Of the multitude of peroxisomal matrix enzymes in mammals, the fast majority is known to possess a PTS1. A defect of peroxin 5 therefore results in a deficiency of most peroxisomal processes. Patients with a deficiency of peroxin 5 suffer from Zellweger syndrome, which is characterized by facial dysmorphisms, ocular abnormalities such as cataracts and retinopathy, sensorineural deafness and mental retardation. Symptoms are present at birth or become apparent soon afterwards and usually patients die within the first year of life, although some patients with milder symptoms can live to early adulthood (73, 74). Zellweger syndrome is classified as a peroxisome biogenesis disorder and can also be caused by defects in other peroxins leading to either a deficiency of PTS1-protein import or a complete inability to generate peroxisomes (75). Since an absence of peroxisomes causes a deficiency of α-oxidation, peroxisome biogenesis disorders are also marked by elevated levels of phytanic acid.

Peroxin 7 deficiency

In contrast to defects in peroxin 5, peroxin 7 deficiency results in the incorrect import of only three peroxisomal proteins, which, besides PAHX, are peroxisomal thiolase and alkyl-dihydroxyacetonephosphate-synthase (ADHAPS). Peroxisomal thiolase is involved in very long-chain fatty acid (VLCA) β-oxidation, but it is believed that its function can be taken over completely by another thiolase, sterol carrier protein X. This is supported by the observation that a deficiency of peroxin 7 does not lead to accumulation of VLCFAs (76, 77). ADHAPS plays a role in the synthesis of plasmalogens, a group of ether-phospholipids especially abundant in brain, but with unknown function. A deficiency of peroxin 7, caused by mutations in the PEX7 gene, leads to rhizomelic chondrodysplasia punctata (RCDP) type 1, which is characterized by growth retardation, profound developmental delay, cataracts, rhizomelia, dysostoses and ichthyosis with death occurring within the first years of the patient’s life (78, 79).

Genetic heterogeneity within Refsum disease

Once the gene causing Refsum disease had been identified, mutation analysis of PAHX was performed in many Refsum disease patients. A recent review summarizes PAHX sequence analysis in 31 unrelated affected families where 29 different variant alleles were observed (11). Somewhat surprisingly, it was found that not in all patients mutations were present, despite a clear deficiency in PAHX activity. A possible explanation for this was provided by the finding of genetic heterogeneity, as concluded from linkage analysis studies in Refsum disease patients’ families (80). In this study it was described that four out of eight families tested did not show significant linkage to the chromosome 10p13 locus where PAHX is located. This finding led to the conclusion that in up to 50% of patients diagnosed with Refsum disease, the PAHX gene itself is not affected, but that the true molecular defect lies at another locus. This percentage may well be an overestimation, however, since from the above-mentioned review of PAHX sequence analyses it appears that in more than 80% of patients mutations in PAHX have been found (11). After analysis of the four families had failed to show linkage to the PAHX locus, investigations went on to find a locus that did show significant linkage. In chapter 2 (and in 81) it is described that two affected families showed linkage to a locus on chromosome 6q22-24. This locus contains the PEX7 gene and subsequent analysis showed that
patients from these families had a defect in peroxisomal import of PTS2-proteins. Subsequent mutation analysis identified mutations in the PEX7 gene, which indicated that these patients had the same molecular defect as RCDP type 1 patients. Most likely, the mild phenotype observed in these patients compared to the much more severe phenotype normally found in RCDP type 1 patients can be explained by the fact that the mutations do not result in complete deficiency of peroxin 7 function, but allowed some residual PTS2-protein import.

Interestingly, indication of genetic heterogeneity was described earlier by Moser et al. (82), who performed complementation analysis with a cell line derived from a Refsum disease patient (patient 9 in the article). Interestingly, this patient was one of the first patients that had been diagnosed and described by Dr. Sigvald Refsum (6). In the study by Moser et al. (82) it was observed that the cell line was able to complement another Refsum disease cell line, showing that it was not a defect in PAHX that was the cause of Refsum disease in this patient. Instead, the cells did not show complementation with a RCDP type 1 cell line and mutations were found in the PEX7 gene (78). Since these findings were part of a larger screen of molecular defects of patients suffering from peroxisome biogenesis defects, the implications for Refsum disease had not been appreciated.

Taken together, these data show that Refsum disease is caused by mutations in PAHX in the majority of patients and by mild mutations in PEX7 in a subgroup of patients. However, there still remains a small number of patients in which no mutations in either of these two genes can be found, leaving open the possibility of additional loci for Refsum disease.

Breakdown of phytol

In contrast to the well-studied breakdown mechanism of phytanic acid, the way in which phytol is broken down is a neglected topic. Studies where phytol was administered to laboratory animals shed light on intermediates of the phytol to phytanic acid conversion, but it was not until 20 years later that a comprehensive model was proposed.

The mechanism of the conversion of phytol into phytanic acid

Phytol is an unsaturated fatty alcohol and therefore it requires two distinct processes for it to be transformed into phytanic acid, namely the reduction of the double bond at the 2,3 position and the oxidation of the alcohol into a carboxyl-group. Which particular intermediates are formed depend on the order in which the reactions occur, as is shown in Fig. 7A.

One of these possible intermediates, phytanic acid, was indeed detected in rats that had been fed on a phytol-enriched diet, while the other, dihydrophytol, was absent (13, 17). This suggested that at least in rats, phytol is first converted to phytanic acid, which then can be reduced to produce phytanic acid. Furthermore, an accumulation of phytanic acid was observed upon injection of phytanic acid in rats, showing that it is a bona fide intermediate of phytol degradation (13). It has to be noted, however, that dihydrophytol can also be converted into phytanic acid (83). Dihydrophytol production has been detected in the rumen of cows (84), but this might well be the result of bacte-
Figure 7. Pathway of phytol breakdown. (A) Possible reaction sequences leading to phytanic acid production. Mechanism I involves first the oxidation of phytol to phytanic acid followed by the reduction of phytanic acid, as observed in rat and humans. Mechanism II involves first the reduction of phytol to dihydrophytol and next the oxidation to phytanic acid, as proposed for cows. (B) The phytol breakdown pathway proposed by Muralidharan and Muralidharan (85).

Production of phytanic acid
The way in which phytol is converted into phytanic acid was studied by Muralidharan and Muralidharan (85). They performed in vitro incubations of rat liver post-nuclear supernatant with phytol and used thin layer chromatography in order to detect the reaction products. In this way they observed production of phytanic acid, while no dihydrophytol production was detected, which confirmed the findings of the in vivo studies. Characterization of the reaction showed optimal rates of phytanic acid production in an incubation mixture consisting of a phosphate buffer, BSA and NAD$^+$ (86), although, peculiarly, their previous study had been based on incubations in the presence of carnitine, fumarate, nicotinamine, Mg$^{2+}$, Fe$^{3+}$, ATP, CoA and NADPH. These compounds proved to be redundant for the reaction however. The effect of NADPH probably lies in contamination of NADP$, which was shown to be capable of acting as co-factor for the reaction, although optimal rates were achieved with NAD$^+$ (86). A cell-fractionation study indicated that phytanic acid production was high in mitochondria and microsome-enriched fractions, while the supernatant showed no activity, implying that cytosolic alcohol dehydrogenase was not involved in the reaction (85, 86). Highest activity was measured in rat liver, with about 10% of that found in kidney and spleen. Some activity was present in brain, heart and lungs, while in intestine and adipose tissue no phytanic acid production could be detected (86). In chapter 3 (and in 87) it is shown that human fibroblasts also have the capacity to convert phytol into phytanic acid, and that phytanic acid is
also an intermediate in this reaction.
The experiments described above characterize the conversion of phytol in phytanic acid. This process, however, is a two-step mechanism. The enzymatic oxidation of an alcohol into an acid usually requires an alcohol dehydrogenase and an aldehyde dehydrogenase, by which an aldehyde is produced as an intermediate. Following this, phytol would be converted first into phytalen and subsequently into phytanic acid (Fig. 7B). Phytanal has already been shown to be a degradation product of phytol in marine bacteria, which can then also be converted into phytanic and phytanic acid (88, 89).

**FALDH is required for the production of phytanic acid**
Since production of phytanic acid was shown to be high in microsomes (86, and our own data), microsomal enzymes were investigated that might catalyze this reaction. In a report by Kelson et al. it was reported that FALDH could convert dihydrophytol into phytanal (90). This prompted investigations of our own to establish whether FALDH was involved in the breakdown of phytol. For this, fibroblast cell lines derived from SLS patients, marked by a deficiency in FALDH, were cultured in a medium containing phytol. As is described in chapter 3 (and in 87), these cells did not show any production of phytanic acid, while in control cells phytanic acid could be readily detected. Furthermore, using similar incubation conditions as Muralidharan and Muralidharan had used (86), homogenates from fibroblasts derived from SLS patients were clearly deficient in the breakdown of phytol. In Chapter 4 and 5 a new assay for the diagnosis of SLS is described based on these findings (see also 91).

FALDH is known to be part of an enzyme complex, fatty alcohol:NAD+ oxidoreductase (FAO), that consists of an alcohol and aldehyde dehydrogenase (92). FAO is involved in the cycling of fatty acids to fatty alcohols by which the availability of fatty alcohols for the incorporation in e.g. ether lipids is regulated (93). By column purification of FAO the two enzyme activities can be separated. This resulted in the cloning and characterization of FALDH (90, 94, 95), but the alcohol dehydrogenase enzyme proved to be unstable and has yet to be identified (96). However, it is likely that the FAO complex as a whole catalyzes the conversion of phytol into phytanic acid.

**Phytol and SLS**
The biochemical hallmark of SLS is the accumulation of long-chain aliphatic alcohols (97) and clinical symptoms include ichthyosis, mental retardation and spastic diplegia or tetraplegia (98, 99). The accumulation of fatty alcohols instead of fatty acids might lie in the instability of the alcohol dehydrogenase when it is separated from FALDH (96) or in the reactivity of aldehydes, but this question has not been satisfactorily addressed. Recently, it was observed that SLS patients accumulate leukotriene B₄, which is also degraded by FALDH (100). Leukotriene B₄ is a pro-inflammatory cytokine and may play a role in the characteristic ichthyosis that is observed in these patients.

After it was found that the degradation of phytol is deficient in SLS patients, it was questioned whether a possible accumulation of phytol might contribute to the symptoms observed in the patients. It was speculated that phytol accumulation was involved in the rise of an unknown lipid peak observed in MRI spectra in some SLS patients (101). However, investigations in our lab have so far not been able to show elevated
levels of phytol in plasma of SLS patients (unpublished data).

**Phytanic acid reductase**

The enzymatic conversion of phytanic acid into phytanic acid has not received any investigation until this moment. According to the model depicted in Fig. 7B, phytanic acid is directly converted into phytanic acid, a process which usually is NADPH dependent. In chapter 6 the characterization of this reaction is investigated and indeed enzyme activity is detected. The rate of phytanic acid reduction was found to be very low however and detailed characterization of the enzyme resulted in serious doubts about its relevance in the in vivo situation. Therefore, other ways of converting phytanic acid into phytanic acid were investigated. In this way we discovered that phytanic acid first needs to be converted to its CoA-ester before it is converted into phytanoyl-CoA. Therefore, the pathway of phytol degradation involves an additional step, catalyzed by an acyl-CoA synthetase. In this new model, phytol is still converted to phytanic acid, mediated by FALDH. Next, phytanic acid is converted to phytanoyl-CoA, which is then reduced to phytanoyl-CoA. Phytanoyl-CoA can then directly be α-oxidized and further degraded.

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General introduction


Chapter 1


