Abstract
Phytanic acid (PA) is an epimeric metabolite of the isoprenoid side chain of chlorophyll. Owing to the presence of its epimeric β-methyl group, PA cannot be metabolized by β-oxidation. Instead, it is metabolized in peroxisomes via α-oxidation to give pristanic acid, which is then oxidized by β-oxidation. PhyH (phytanoyl-CoA 2-hydroxylase, also known as PAHX), an Fe(II) and 2OG (2-oxoglutarate) oxygenase, catalyses hydroxylation of phytanoyl-CoA. Mutations of PhyH ablate its role in α-oxidation, resulting in PA accumulation and ARD (adult Refsum’s disease). The structure and function of PhyH is discussed in terms of its clinical importance and unusual selectivity. Most point mutations of PhyH causing ARD cluster in two distinct groups around the Fe(II)- and 2OG-binding sites. Therapeutically, possibilities for the treatment of Refsum’s disease involving PhyH are discussed.

Introduction
The chlorophyll molecule comprises a magnesium-chelating porphyrin ring and a C20 isoprenoid fatty acid side chain, phytol. There has been extensive work carried out on the metabolism of the porphyrin ring, but less on that of the fatty acid side chain. Given the enormous mass of chlorophyll on Earth, it is perhaps surprising that more work on how Nature metabolizes phytol has not been carried out. Mammals assimilate phytol from the diet and convert it into phytanoyl-CoA, and/or directly take up PA (phytanic acid) (or phytanoyl-CoA). The average human intake of PA in the Western diet is 50–100 mg/day, of which about half is absorbed [1].

The presence of an epimeric β-methyl group means that phytanoyl-CoA cannot be used directly as an energy source by metabolism through the β-oxidation pathway (Figure 1). The β-oxidation of fatty acids proceeds via a sequence of desaturation, hydration, oxidation to the β-ketone and retro-Claisen reaction. Although the first two of these steps could theoretically accommodate a β-methyl group, and may do so in cells, oxidation to the ketone is impossible, at least without rearrangement. Instead, Nature has developed at least two alternative pathways for the degradation of β-substituted fatty acids, such as PA. Studies on the metabolic disorder ARD (adult Refsum’s disease), which is caused by increased levels of PA, have led to the definition of α- and ω-oxidation pathways for PA metabolism (for reviews, see [2–6]).

In humans, the plasma level of the diet-derived PA is normally low, but is elevated in patients with ARD (OMIM 266500), a peroxisomal disorder, and to a lesser extent in patients with other peroxisomal disorders. Approx. 45% of reported cases of ARD in the U.K. are associated with defects in the function of the peroxisomal oxygenase PhyH (phytanoyl-CoA 2-hydroxylase, also known as PAHX), which catalyses an essential step in the α-oxidation pathway (for a review, see [2]). In addition to point mutants, frameshift, truncation and deletion mutations of PhyH have been identified. Other ARD cases are associated with defects in the PTS2 (peroxisomal-targeting sequence type 2) receptor protein Pex7 [7,8], and some probably remain unidentified.

ARD symptoms are normally diagnosed later in life and include cerebral ataxia, retinitis pigmentosa, anosmia, deafness, peripheral polyneuropathy and ichthyosis [9]. The symptoms can be subtle in the early stages, hence diagnosis can be difficult, and it has been proposed that ARD is more widespread than current clinical data suggest. Some symptoms, including skeletal abnormalities [10], occur during embryonic development, since they are present at birth. PA levels would not normally be elevated at this stage, suggesting an additional role for PhyH during development. Murine PhyH is identical with the murine lupus nephritis-associated protein (LN1) [11], suggesting a role for PhyH in kidney disease as observed in human ARD patients [2].

PA oxidation by α- and ω-pathways
In contrast with earlier reports, reporting evidence that the PA ω-oxidation does not occur in vivo, Wanders and colleagues demonstrated that cytochrome P450 class 4 oxidases from microsomes catalyse the ω-oxidation of PA [12,13]. The ω-oxidation pathway for the oxidation of β-substituted fatty acids involves the terminal (ω-) methyl...
group to a carboxylic acid, i.e. to produce a diacid derivative 3-methyladipic acid, which is excreted in the urine. Although the ω-oxidation pathway is up-regulated in ARD patients, the α-oxidation pathway, in which PhyH plays an essential role, is the major PA-degradation pathway (Figure 1).

**The role of PhyH**

PhyH, first identified in 1997 [14,15], is initially produced as a proprotein with an N-terminal PTS2 that enables its import into peroxisomes via Pex7. The targeting sequence is cleaved in the peroxisome, between Thr30 and Ser31, to produce mature PhyH (the numbering scheme uses that of 338-residue pro-PhyH; gi:6093646).

Potential protein–PhyH interaction partners, including blood coagulation Factor VIII [16], PhyH-associated protein 1 (PhyH-AP1) [17] and brain-specific angiogenesis inhibitor 1 [18] have been reported, raising the possibility that PhyH has other substrates. The physiological significance of these PhyH interactions and how they occur given the apparently predominant peroxisomal localization of PhyH is unclear.

PhyH is a member of the ubiquitous Fe(II) and 2OG (2-oxoglutarate) oxygenase superfamily (for a review, see [19]) (Figure 2). The first 2OG oxygenases to be identified were prolyl and lysyl hydroxylases involved in collagen biosynthesis, but 2OG oxygenases have been shown to catalyse oxidative reactions with a range of important functional roles in...
almost all types of organism. In addition to collagen biosynthesis, biological roles for the family in humans include transcriptional regulation, DNA repair and fatty acid metabolism. To date, in metazoans, these biological roles all involve the catalysis of hydroxylation reactions such as that catalysed by PhyH. However, in plants and microorganisms, the 2OG oxygenases catalyse a very wide range of oxidative reactions, leading to the proposal that they may be the most versatile of all oxidizing biological catalysts [20]. This versatility is manifested in the selectivity of PhyH.

Following formation of phytanoyl-CoA as a mixture of C-2 epimers (Figure 1), PhyH catalyses α-hydroxylation to give the two threo-stereoisomers, (2S,3R) and (2R,3S), of hydroxyphytanoyl-CoA; there is no evidence for formation...
of the two possible erythro-stereoisomers [21,22]. This type of stereoselectivity, i.e. different epimers giving different products, is unusual in enzymes, and the structural basis for it is of interest. Both processed and unprocessed forms of recombinant PhyH catalyse a range of CoA derivatives, including straight-chain derivatives and the short-chain homologue isovaleryl-CoA [23,24]. However, the catalytic efficiency of these substrates is reduced relative to phytanoyl-CoA, and the physiological relevance of these hydroxylations with alternative substrates is unclear. Optimization of in vitro assays with PhyH is complicated by the number of reactive components required; in addition to Fe(II), 2OG and a substrate CoA derivative, ascorbate and dithiothreitol are added as reducing agents, and a solubilizing agent, such as β-cyclodextrin, may be used for hydrophobic CoA derivative. In vivo, it has been proposed that sterol carrier protein 2 may fulfil the latter role [24].

Structural studies have revealed a common structural platform for the 2OG oxygenases, the DSBH (double-stranded β-helix) or jelly-roll motif, that supports iron-binding residues [19]. In nearly all studies, these residues comprise one aspartyl/glutamyl residue and two histidinyl residues that form a conserved triad as observed in the reported PhyH structure. Site-directed mutagenesis of PhyH has been used to support proposals for the identity of the iron-binding ligands (His175, Asp177 and His264) of the 2-His-1-carboxylate motif of PhyH [24,25]. Mutation of other histidine residues (His213, His259 and His281) suggested that these residues were not involved in Fe(II) binding. These predictions have been verified by the report of a crystal structure of PhyH to 2.5 Å (1 Å = 0.1 nm) resolution [26].

In combination with kinetic analyses [25,27], the crystal structure has been used to predict the molecular consequences of mutations of PhyH in causing ARD. As with other 2OG oxygenases, the DSBH core of PhyH supports the assignment of the three iron-binding ligands; the 2-oxoacid group of 2OG was observed to bind to the Fe(II) in a bidentate manner with the guanidino side chain of Arg275 forming an interaction with the 2OG 5-carboxylate. The way in which PhyH binds to Fe(II) and 2OG together with the presence of a cysteine residue (Cys191) 6.7 Å from the Fe(II) and two further histidine residues (His155 and His281) at its active site distinguishes it from other human 2OG oxygenases with reported structures.

Analysis of the PhyH crystal structure reveals that most of the clinical mutations [8,25,28] cluster in two regions: one around the Fe(II) (five mutants) and the other around the 2OG-binding pocket (six mutants) (Figure 3). The catalytic activities of the P29S, Q176K, G204S, N269H, R275W and R275Q mutations have been reported using recombinant PhyH [25]. Clinical mutations that affect Fe(II) binding include His175 (H175R) and Asp177 (D177G), whichcomplex the iron, and Gln176 (Q176K), located between the two Fe(II)-binding residues. Mutation of His175 or Asp177 to alanine ablates activity and impairs Fe(II) binding in vitro [23]; the H175R and D177G clinical mutants are likely to have a similar effect. The Gln176 side chain is positioned to make hydrogen bonds with Ser216 and Lys218, both located on random coil leading to a disordered loop (residues 223–233), which is probably involved in substrate binding. The Q176K mutant probably interrupts this hydrogen-bond network and probably causes changes in the backbone conformation for both the Fe(II)-binding residues and the potential substrate-binding loop (residues 223–233). In vitro, both Q176K and Q176A mutations uncouple 2OG oxidation and hydroxylation of phytanoyl-CoA [23,25]. Two other clinical mutations, H220Y and P173S probably also affect Fe(II) binding. His220 is located on the sequence approaching the disordered loop (residues 223–233), predicted to be involved in substrate binding, and is positioned to hydrogen bond with Gln176. Mutation of His220 to a bulky hydrophobic tyrosine residue may ablate the hydrogen bond and destabilize or modify the conformation of the Fe(II)-binding residues on either side of Gln176. Pro217 is apparently introduced in a conformationally rigid anchor point that prevents the flexibility of the sequence just before being transferred to the subsequent Fe(II)-binding residues His213 and Asp217. The clinical mutation, P173S, may remove this ‘anchor’ and destabilize Fe(II) binding.

Clinical mutations that affect, or probably affect, 2OG binding include Arg275 (R275W and R275Q), Trp193 (W193R), Ile199 (I199F), Glu197 (E197Q), Gly204 (G204S)
and Asn\textsuperscript{269} (N269H). Arg\textsuperscript{275} and Trp\textsuperscript{193} interact directly with 2OG via electrostatic and hydrophobic interactions respectively. Consistent with their role in binding 2OG, analyses on R275W and R275Q demonstrate very low catalytic activities [29]. Other mutations involving Glu\textsuperscript{197}, Ile\textsuperscript{199}, Gly\textsuperscript{204} and Asn\textsuperscript{269} are clustered in the region around Arg\textsuperscript{275} and are predicted to affect the 2OG-binding pocket.

Four reported clinical mutations (including one in the PTS2) are not obviously involved in Fe(II) or 2OG binding. Phe\textsuperscript{257} is located on the outer face of the major β-sheet contacting helix α-4 and is buried in a hydrophobic pocket. The F275S mutation may unfavourably place a polar side chain in the hydrophobic pocket and possibly interfere with the overall structure or impair protein folding. Two clinical mutations, R245Q and N83Y, are located on the surface of the enzyme and far from the active site. These mutations may result in disruption of protein–protein interactions proposed to involve PhyH, such as that with sterol carrier protein-A [24].

### Therapeutic possibilities for ARD

The only current ARD treatment involves application of a diet with low intake of PA or its precursors, which may be combined with plasmapheresis (for reviews, see [2–4]). Coupled to advances in diagnosis and PA detection, diet therapy is at least partially successful, but can be difficult to manage and alternatives are being considered. Some of these are very speculative, especially given the efficacy of diet therapy for ARD, but methodologies pioneered with ARD may lead to new approaches for other metabolic disorders.

The discovery that PA is degraded by the ω-oxidation pathway [12,13] raises the possibility that knowledge of this pathway could be exploited for therapeutic purposes. Cytochrome P450 oxidases involved in metabolism are well-known to be induced by many pharmaceuticals, and it is reasonable to propose that such induction occurs in response to the elevated levels of PA in ARD patients. A question arising from the ω-oxidation work is why the endogenous human ω-oxidation pathway does not compensate for the lack of the α-oxidation pathway in ARD patients. The ω-oxidation pathway is approximately doubled in ARD patients compared with unaffected individuals [2]. However, if such a response is insufficient to cope with the PA levels observed in ARD patients, this implies that the ω-oxidation pathway is saturated. Data on the reasons for the apparent saturation, particularly in animals, and the selectivity of the ω-oxidation pathway with respect to β-substitution may help in identifying new therapies. It also cannot be ruled out that development of ARD requires defects in both α-oxidation and ω-oxidation pathways. One therapeutic possibility is to up-regulate the ω-oxidation pathway by diet or pharmaceutical therapy [12,13].

The ω-oxidation work also leads to the question of whether there are other pathways for PA degradation. One possibility is that (an)other 2OG oxygenases can also metabolize PA; recent structural studies on human 2OG oxygenases have enabled estimates of human 2OG oxygenases to be in the range of 50–80 enzymes with folds of the canonical

### Conclusions

There are clear clinical problems with tailoring therapies to fit specific mutations occurring in individual patients or small patient groups within a diseased cohort. Nonetheless, advances in genomics are likely to mean that there is an increasing demand for such tailored therapies. The partially
successful treatment of ARD by diet therapy means that the impetus for new therapeutic advances is not as great as for genetic lesions associated with, e.g., cardiovascular disease or cancer. However, the detailed extent of accumulating detailed molecular knowledge on ARD coupled to clinical and animal data may enable it to act as a model for other diseases in which an enzyme-catalysed reaction is partially disabled, leading to a subtle phenotype that may take decades to present.

The structural and mechanistic knowledge gained of PhyH may also assist in understanding other genetic diseases associated with mutations of 2OG oxygenases or their regulation. Ehlers–Danlos syndrome is caused by mutations to procollagen lysyl hydroxylase and PHD2 (prolyl hydroxylase domain 2), the principal hydroxylase that regulates the activity of the hypoxia-inducible transcription factor under normoxic conditions, has been shown to be mutated in familial erythropoiesis. As with some of the clinical PhyH mutations, the PHD2 mutation, P317R, is close to the iron-binding site and reduces, but does not ablate, hydroxylation activity [32].

Finally, the 2OG-dependent enzymes such as the HIF prolyl hydroxylases are current targets for the development of anaemia and ischaemic disease; inhibition of these targets will probably be best achieved without inhibiting PhyH with the concomitant potential for inducing ARD symptoms. Knowledge of the structure and mechanism of PhyH may thus help to enable the development of selective and safe inhibitors of human 2OG oxygenases.

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