Structure of Human Phytanoyl-CoA 2-Hydroxylase Identifies Molecular Mechanisms of Refsum Disease

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Refsum disease (RD), a neurological syndrome characterized by adult onset retinitis pigmentosa, anosmia, sensory neuropathy, and phytanic acidemia, is caused by elevated levels of phytanic acid. Many cases of RD are associated with mutations in phytanoyl-CoA 2-hydroxylase (PAHX), an Fe(II) and 2-oxoglutarate (2OG)-dependent oxygenase that catalyzes the initial α-oxidation step in the degradation of phytanic acid in peroxisomes. We describe the x-ray crystallographic structure of PAHX to 2.5 Å resolution complexed with Fe(II) and 2OG and predict the molecular consequences of mutations causing RD. Like other 2OG oxygenases, PAHX possesses a double-stranded β-helix core, which supports three iron binding ligands (His175, Asp177, and His264); the 2-oxoacid group of 2OG binds to the Fe(II) in a bidentate manner. The manner in which PAHX 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 His284) at its active site distinguishes it from that of the other human 2OG oxygenase for which structures are available, factor inhibiting hypoxia-inducible factor. Of the 15 PAHX residues observed to be mutated in RD patients, 11 cluster in two distinct groups around the Fe(II) (Pro173, His175, Gln176, Asp177, and His220) and 2OG binding sites (Trp193, Glu197, Ile199, Gly204, Asn269, and Arg275). PAHX may be the first of a new subfamily of coenzyme A-binding 2OG oxygenases.

In humans, the plasma level of the diet-derived isoprenoid, phytanic acid, is normally low (<30 μM) (1). Significantly elevated levels of phytanic acid are found in patients with Refsum disease (RD) (OMIM 285002) and to a lesser extent in patients with other peroxisomal disorders (2). Approximately 45% of reported cases of RD in the United Kingdom have been associated with defects in the function of the peroxisomal enzyme phytanoyl-CoA 2-hydroxylase (PAHX) (3), which is initially produced as a proprotein with an N-terminal type-2 peroxisomal targeting signal (PTS2) (4). The targeting sequence is cleaved in the peroxisome, between Thr30 and Ser31, to produce mature PAHX, used ubiquitously in this study and referred to hereafter as PAHX (the numbering scheme used follows that for the 338-residue pro-PAHX; gi:6093646) (5, 6). Some observed cases of RD are associated with a second genetic locus recently identified as the PTS2 receptor protein, PEX7 (7, 8). Symptoms of RD, for which the only current treatment is diet therapy, usually arise later in life and include retinitis pigmentosa, anosmia, deafness, peripheral polyneuropathy, cerebellar ataxia, and ichthyosis (9). Symptoms of RD can be subtle in the early stages, making diagnosis difficult, and it has been proposed that the disease is more widespread than clinical data suggest. Most symptoms of RD are thought to develop from toxic accumulation of phytanic acid. Some symptoms, including skeletal abnormalities (10), occur during embryonic development, since they are present at birth. Phytanic acid levels would not normally be elevated at this stage, suggesting an additional role for PAHX during development. The murine form of PAHX is identical to the murine lupus nephritis-associated protein (LN1) (11), suggesting a possible role for PAHX in kidney disease that has also been observed in human RD patients (3).

Various proteins have been identified as potential PAHX interaction partners, raising the possibility that phytanoyl-CoA may not be the only substrate and that PAHX may have unidentified functions. PAHX has been reported to interact with the blood coagulation factor VIII; it has been proposed that PAHX regulates the expression of this coagulation factor (12), potentially in an oxygen-dependent manner. A further PAHX interaction partner, PAHX-associated protein 1 (PAHX-AP1), identified by a yeast two-hybrid screen (13), interacts with the brain-specific angiogenesis inhibitor 1 (10, 14), suggesting a mechanism for some of the neurological symptoms of RD. The physiological significance of these PAHX interactions has not been determined, and how they occur given the apparent peroxisomal localization of PAHX is unclear.

The presence of a 3-methyl group in phytol prevents its degradation by the normal β-oxidation pathway for saturated fatty acids. Instead, a preliminary α-oxidation pathway occurs, first in the endoplasmic reticulum and subsequently in peroxisomes, to shorten the chain length of 2-hydroxyphytanoyl-CoA by one carbon, thereby enabling β-oxidation to proceed (Fig. 1). It has recently been shown that phytic acid is first condensed to its coenzyme A ester prior to reduction to phytanoyl-CoA (15), which is subsequently hydroxylated to 2-hydroxyphytanoyl-CoA in the PAHX-catalyzed reaction (Fig. 2) (4–6). The stereospecificity of PAHX is interesting, since it accepts both C-3 epimers as substrates but produces only the threo products (25R or 2R,3S) (18, 19). Thiamine pyrophosphate-dependent lyase-catalyzed fission of the...
C1-C2 bond of 2-hydroxyphytanoyl-CoA then occurs to produce formyl-CoA and pristanal (20–22), the latter of which is oxidized in an NAD⁺-dependent reaction to pristanic acid (23). After re-esterification with coenzyme A and epimerization (24–26), metabolism via “normal” β-oxidation pathway, occurs, initially in peroxisomes and later in mitochondria.
Recent studies have investigated the substrate specificities and activities of both pro-PAHX and mature PAHX as well as clinically observed mutants (19, 27). The pro and mature forms of PAHX have a similar substrate specificity, and both accept isovaleryl-CoA as a substrate (27, 28), demonstrating that in vitro a long chain fatty acid side chain is not required for binding and activity of PAHX (4). Site-directed mutagenesis studies have led to proposals for the Fe(II)-binding residues of PAHX and identified other residues likely to be involved in catalysis (27, 29).

The PAHX-catalyzed conversion of phytanoyl-CoA to 2-hydroxyptanoyl-CoA with co-evolution of carbon dioxide and succinic acid.

**EXPERIMENTAL PROCEDURES**

Protein Expression and Purification—Purification of PAHX was carried out as reported (29). Briefly, *Escherichia coli* BL21(DE3) were transformed by pET24a/mat-pahx and grown at 37 °C in 2TY medium containing 30 μg/ml kanamycin. Following reduction of the temperature to 25 °C, protein production was induced at an A600 of 0.7 by the addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 1.0 mM. In previous studies, we found that PAHX is unusually prone to site-specific Fe(II) and oxygen-mediated fragmentation. For this reason, dithiothreitol was omitted, and 1 mM EDTA was added to all buffers to prevent fragmentation during purification. PAHX was purified by carboxymethyl-Sepharose cation exchange, followed by size exclusion chromatography (Superdex S75) to yield PAHX of >95% purity by SDS-PAGE analysis. Electrospray ionization mass spectrometry revealed that the mass of the purified PAHX was consistent with loss of the N-terminal methionine from the predicted amino acid sequence (observed, 35,436 Da; calculated without N-terminal methionine, 35,435 Da).

Amino acid sequence analysis by Edman degradation confirmed the identity of the protein and loss of the N-terminal methionine (observed, STGISS). The activity of purified PAHX was confirmed for 2OG turnover using the reported assay monitoring release of 14CO2 (32).

**Selenomethionine-substituted Protein Expression—**Selenomethionine (SeMet)-substituted PAHX was produced using a metabolic inhibition protocol and LeMaster medium supplemented with 50 mg/liter l-selenomethionine. Selenomethionine incorporation was >95% by electrospray ionization mass spectrometry (observed, 35,858 Da; calculated, 35,859 Da).

**Crystallization**—Crystallization conditions were initially sought using high throughput robotic screening methods at the Oxford Protein Production Facility (33). Optimization of the initial crystallization condition was performed using the hanging drop vapor diffusion method in VDX™ plates (Hampton Research, Aliso Viejo, CA). Hanging drops containing 2 μl of 5.2 mg/ml PAHX and 2 μl of well solution were suspended over 500 μl of well solution containing 21% polyethylene glycol 3350, 0.3 M triammonium citrate, pH 7.1, at 18 °C. Crystallization with 1 mM iron(II) sulfate and 2 mM 2OG in an anaerobic environment (Bell Technologies glove box under an argon atmosphere) produced square, plate-shaped crystals over a period of 3 weeks to a maximum size of 200 μm × 100 μm × 50 μm. SeMet PAHX crystals were grown aerobically using the same conditions, except that iron(II) sulfate and 2OG were substituted with zinc(II) chloride and N-oxalylglycine.

**Crystalllographic Data Collection and Structure Solution**—A single crystal, anaerobically grown (200 × 100 × 50 μm) was transferred to cryoprotectant (1:7 glycerol/well solution) and immediately cryocooled in liquid nitrogen. Native data were collected at 100 K using beamline 10.1 (34) of the Synchrotron Radiation Source (SRS, Daresbury, UK) equipped with a MAR 225CCD detector. The native data were processed with MOSFLM and SCALA of the CCP4 suite version 5.0.2 (35, 36) with an i222 lattice (TABLE ONE). Calculation of a Matthews coefficient of 2.1 Å3/dalton implied a single monomer in the asymmetric unit. Attempts at molecular replacement and isomorphous replacement were unsuccessful, so crystals were produced from SeMet-substi-
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TABLE ONE

Crystallographic data and structure statistics

|                     | SeMet (SLS beamline X10A) | Native anaerobic (SRS Daresbury beamline 10.1) |
|---------------------|---------------------------|-----------------------------------------------|
| Data collection     |                           |                                               |
| Wavelength (Å)      | 0.9790                    | 0.9800                                        |
| Resolution (Å)      | 50–2.6 (2.69–2.6)          | 30–2.5 (2.64–2.50)                            |
| Space group         | F222                      | F222                                          |
| Unit cell (Å)       | a = 67.6, b = 85.3, c = 97.2 | a = 67.9, b = 86.7, c = 97.5                  |
| Reflections observed/unique | 31,391/7003            | 51,212/9583                                   |
| Mean I/σ(I)         | 22.9 (7.9)                | 13.5 (2.0)                                    |
| Rmerge               | 0.078 (0.428)             | 0.119 (0.841)                                 |
| Completeness (%)    | 78.5 (17.7)               | 94.2 (97.2)                                   |
| Redundancy           | 4.5 (1.7)                 | 5.3 (5.2)                                     |
| Refinement          |                           |                                               |
| Resolution (Å)      | 40–2.5                    |                                               |
| Completeness (%)    | 93.05                     |                                               |
| Rmerge/Rfree        | 0.20/0.27                 |                                               |
| Root mean square deviation bond length (Å) | 0.012                  |                                               |
| Root mean square deviation bond angle (degrees) | 1.360                |                                               |
| No. of atoms        |                           | 1961 (37.7)                                   |
| Protein (average Bfactor Å²) |                      | 1 (40.1)                                      |
| Iron (average Bfactor Å²) |                         | 10 (54.9)                                     |
| 2OG (average Bfactor Å²) |                         | 15 (44.4)                                     |
| Waters (average Bfactor Å²) |                   |                                               |
| Ramachandran statistics |                       |                                               |
| Core 85.0%          |                           |                                               |
| Allowed 11.6%       |                           |                                               |
| Generous 0.5%       |                           |                                               |
| Disallowed 0.0%     |                           |                                               |

RESULTS AND DISCUSSION

The PAHX Structure—PAHX crystallizes as a monomer in space group F222 with one molecule per asymmetric unit. The solvent content of the crystals is 40%, and the crystals contain large solvent channels that coincide with access to the active site. Three disordered loops that border the active site face this solvent channel and include residues 165–172, 223–233, and 303–318. Other disordered regions are near the N terminus and comprise residues 31–42 and 51 and 52. The most extensive contact between monomers in the lattice, 841 Å² per monomer, is a ∼18-Å-long extended pair of antiparallel β-strands (β-3 and β-4) in which β-4 is antiparallel to β-4 of a symmetry-related molecule along a crystallographic 2-fold axis. Another significant lattice contact involves loop 240–244, which packs against Lys821 of the C-terminal helix, and residues 288–291 of one symmetry-related molecule and residue 94 of another.

The PAHX fold is a mixed α-β structure composed of a major and a minor β-sheet surrounded by five α-helices and four 3_10 helices (Fig. 3). As in other 2OG oxidases for which structures are available (30, 31, 44–49), the core of the protein consists of a DSBH fold composed of eight β-strands (Fig. 4 and supplemental Fig. 2S). However, in PAHX, only seven β-strands of the DSBH are apparent: β-6, β-8, β-9, β-10, β-11, β-12, and β-13, here defined as β-strands I, III, IV, V, VI, VII, and VIII, respectively. In PAHX, the residues that correspond to DSBH β-strand II (β-7) (residues 165–172) in other 2OG oxidases are disordered and left out of the model. This strand is probably involved in substrate binding (see below). Four additional β-strands (β-1, β-2, β-5, and β-15) hydrogen-bond antiparallel to the core DSBH, resulting in the eight-stranded major β-sheet (Fig. 3). It is interesting to note that both

tuted protein. A single SeMet crystal (120 μm × 70 μm × 5 μm) was transferred to cryoprotectant (1:9 glycerol/well solution) before cryocooling in liquid nitrogen. Single wavelength anomalous dispersion (SAD) data were collected at 100 K and at the selenium peak wavelength at beamline X10A (Swiss Light Source, Villigen, Switzerland). Data were integrated with HKL2000 version 1.98.0 (37) and merged, and anomalous differences were analyzed with XPREP (38). The substructure was solved with SHEXLXD of the SHELEX-97 suite (39) using anomalous differences to 3.5 Å in space group P222, and the sites and chirality were confirmed with SHELXE. The substructure (top seven selenium sites) was refined with SHARP version 2.0.4 (40) against the SAD data, and two additional selenium sites were identified. Phases were calculated to 3 Å using SHARP, and the combined anaerobic native and SAD data sets result in a figure of merit of 0.33. Phases were improved by density modification using RESOLVE version 2.08 (41) to 2.7 Å with a final figure of merit of 0.85, resulting in core secondary structure elements and a 7σ peak in the core region attributable to the iron atom clearly visible in the electron density.

The initial model was built using the program COOT version 0.0.31 (42) into composite omit maps calculated with RESOLVE. Before refinement commenced, 5% of the data were flagged for calculation of a free R-factor (Rfree). Initially, simulated annealing was performed in CNS version 1.1 using combined phases calculated from the model and phase probabilities from SHARP. Iterative refinement using CNS version 1.1 and model building using COOT continued until Rfree was below 30%. At this stage, restrained TLS refinement was performed using REFMACS (43) with only the model phases. Iterative rounds of manual reﬁtting and crystallographic refinement using the programs COOT and REFMACS continued until Rfree was no longer improved. Statistics are given in TABLE ONE.
The conserved eight stranded DSBH core found in all Fe(II) and 2OG-dependent oxygenases is colored yellow. Only seven strands (β-6, β-13, β-8, and β-11 of the major sheet and β-9, β-10, and β-12 of the minor sheet) are observed in PAHX, since the residues making up the DSBH β-strand II are disordered. Additional β-strands attached to the major β-sheet are colored slate blue. The Fe(II)-binding residues and 2OG C5-carboxylate interacting arginine of PAHX along with the 2OG co-substrate and Fe(II) cofactor are shown as a stick representation. α- and 310-helices are shown in red.

β-strands β-5 and β-15 are paired antiparallel to DSBH β-strand I (β-6), a unique feature among the 2OG oxygenase family.

Fe(II) Binding Site—The Fe(II)-containing active site is located between one end of the β-sheets that form the DSBH core in a manner similar to other 2OG oxygenases (30, 31, 44–49) (Fig. 4). The experimental electron density clearly shows the Fe(II) coordinated in an approximately octahedral manner by two oxygens from 2OG, a water molecule (B-factor 44.6 Å²), and the side chains of His175, Asp177, and His264 (Fig. 5, A–C). The HXD motif is located just after the sequence that is typically DSBH β-strand II (residues 165–172), and the second Fe(II)-binding histidine (His264) is located on DSBH β-strand VII (β-12). The observation of Fe(II) coordination by these three residues confirms their provisional assignment by mutation analyses coupled to iron-binding assays (27). The two Fe(II)-binding histidines adopt similar conformations to those observed in other 2OG oxygenases. The aspartate is in a similar conformation to that observed in deacetoxycephalosporin synthase (DAOCS) and proline-3-hydroxylase (30, 48) but differs from that observed in FIH and clavaminic acid synthase (CAS) (Fig. 4) (31, 46). However, the structures of FIH and CAS may reflect unusual cases. In addition to binding Fe(II), the aspartate of FIH forms a hydrogen bond to a backbone amide of the peptide substrate, and in the trifunctional CAS the Fe(II) binding carboxylate is derived from a glutamate rather than an aspartate residue.

2OG Binding—The 2OG co-substrate coordinates the Fe(II) in a bidentate manner with its 2-keto group trans to Asp177 O6 and the oxygen of the 1-carboxylate trans to His264 Nε2 (Fig. 5B). The position of the 2OG 2-keto oxygen relative to the three Fe(II)-binding residues is observed to be conserved in all relevant crystal structures for 2OG oxygenases. However, the relative position of the 1-carboxylate of 2OG varies between being trans to His264 (or its equivalent), as observed in PAHX, or trans to His175 (or its equivalent) (Fig. 5B). In the case of CAS, the relative position of the 1-carboxylate was observed to change upon binding the dioxygen analogue, nitric oxide (50), from being trans to His175 in CAS to being trans to His279 (His264) in PAHX). Binding of the substrate has been observed to displace a water molecule from the Fe(II), thus enabling binding of oxygen (51). Thus, the coordination position of the 2OG-1-carboxylate group in PAHX may vary from its observed position upon (co-)substrate binding or under non-crystalline conditions. It is possible that the observed coordination position of the C1′-carboxylate in PAHX reflects a protected form of the enzyme, preventing oxygen binding to avoid the generation of potentially damaging reactive oxidizing intermediates in the absence of substrate. This may be particularly important in the oxidizing environment of the peroxisomes.

The 1-carboxylate oxygen of 2OG not ligated to the Fe(II) is in position to form a hydrogen bond (3.2 Å) with Nε of Lys120 located on β-strand β-5 (Fig. 5A). For some 2OG oxygenases, the side chain of a basic or polar residue is also positioned close to the C1′-carboxylate of 2OG (e.g. Arg297 of CAS, Arg160/162 of DAOCS, and Arg95/97/122 of proline-3-hydroxylase). However, PAHX is the first case where the basic residue, Lys120, is actually close enough to hydrogen-bond (3.2 Å) with the 2OG C1′-carboxylate (Fig. 5A). It is possible either that substrate binding weakens the hydrogen bond, enabling a rearrangement of the...
FIGURE 5. A view of the active site of the PAHX-Fe(II)-2OG complex showing coordination of Fe(II) to 2OG, His177, Asp177, and His281. 2OG also ligates to Arg275, Ser266, and Lys120. Active site residues are also shown and include Cys191, His281, and His155. B, comparison of 2OG binding to iron in PAHX (yellow) and FIH (blue), also showing differences in conformation of the aspartate carboxylate. C, representative electron density maps, (2F_o – F_c) (blue) contoured to 1σ, OMIT (F_o – F_c) (green) contoured to 2.75σ (2OG and the Fe(II)-binding water omitted from the map calculation), showing the octahedral coordination of Fe(II) with bidentate coordination from 2OG. D, surface representation of PAHX showing the large substrate binding groove (black arrow), leading to the active site. A scale model of phytanoyl-CoA is shown in a sphere representation to emphasize the relative size of the substrate and the binding groove.
ligation position of the 2OG C1'-carboxylate, or that after oxidative decarboxylation of 2OG the side chain of Lys\(^{370}\) is involved in the release of carbon dioxide and/or succinate from the catalytic site.

In 2OG oxygenases, the C5'-carboxylate of 2OG is typically bound by an arginine or lysine and by a hydroxyl group (30, 31, 44 – 49). For the DAOCs subfamily, the basic arginine and hydroxyl originate from an RXS motif located on DSBH β-strand VIII (β-13) (30). In FIH, Lys\(^{314}\) (from DSBH β-strand IV) and Thr\(^{318}\) (from DSBH β-strand II) interact with the C5'-carboxylate of 2OG. In PAHX, Arg\(^{275}\), located on DSBH β-strand VIII (β-13) at the back of the 2OG binding pocket, is in position to form an electrostatic interaction with the C5'-carboxylate of 2OG similar to that observed in DAOCs. Based upon the weaker electron density and higher temperature factors, the C5'-carboxylate appears less ordered than the 2-oxoacid portion of 2OG. This is probably due to the disordered state of DSBH β-strand II discussed below.

There is no RXS motif in PAHX, since the serine is substituted by an alanine. However, Oy of Ser\(^{266}\), two residues from the His\(^{264}\) of the HXD . . . H Fe(II) binding triad on β-12 (DSBH β-strand VII), is in position to hydrogen-bond (2.7 Å) to the C5'-carboxylate of 2OG. It is notable that this serine is conserved in close homologues of PAHX from *Mus musculus*, *Xenopus laevis*, *Danio rerio*, *Caenorhabditis elegans*, the more distant human homologue PHYHD1, and two homologues from the thienaminycin antibiotic biosynthetic gene cluster encoded for by *thnG* and *thnQ* (see supplemental information for sequence alignment) (53, 54).

**Unusual Features of the PAHX Active Site and Substrate Binding**—In addition to the Fe(II)- and 2OG-binding residues, there are a number of other polar residues in the PAHX active site including two histidines, His\(^{155}\) and His\(^{381}\) (Fig. 5A). The side chain of His\(^{281}\) is close to Asp\(^{77}\) (which binds to Fe(II)) and is also conserved in the close homologues of PAHX from *M. musculus*, *X. laevis*, *D. rerio*, and *C. elegans*. These residues are not conserved in FIH, and their presence may explain why PAHX appears particularly susceptible to inhibition by Ni(II) (27, 29, 55), although competition at the Fe(II)-binding site is also important in this regard. Cys\(^{191}\) is conserved in the PAHX homologues from *M. musculus*, *X. laevis*, *D. rerio*, and *C. elegans*. The observation that the sulfydryl of Cys\(^{191}\) is 6.7 Å from the Fe(II) and only 4.6 Å from the water molecule ligated to Fe(II) suggests that it may be of mechanistic significance (Fig. 5A). Assuming that oxygen binds in the position occupied by the water molecule, the sulfur of Cys\(^{191}\) might be within interacting distance of the oxygen. Another mechanistic possibility is that Cys\(^{191}\) is reversibly acylated by the acyl-CoA substrates for PAHX. However, the relatively inaccessible position of Cys\(^{191}\) at least when 2OG is bound, suggests that acylation is unlikely.

Taking into account the size of the PAHX substrate, phytanoyl-CoA, it is likely that the disordered loops that surround the active site play a role in substrate binding and may only become ordered in the presence of phytanylo-CoA or in the presence of a protein that "presents" the substrate to the enzyme, such as sterol carrier protein-2 (28). As noted previously, residues equivalent to 165–172 have β-strand secondary structure and form DSBH β-strand II in other reported structures for 2OG oxygenases. The region between residues 223 and 233 appears to extend from a long loop between DSBH β-strands IV and V (β-9 and β-10) and probably serves to enclose the active site. The region between residues 303 and 318 is located prior to the C-terminal helix, which for some 2OG oxygenase family members has been shown to play a role in substrate recognition (56); in FIH, the C-terminal helices are involved in dimerization in addition to substrate recognition (57). Assuming the structure of PAHX as observed represents an "open" conformation with its disordered loops, a large groove leading to the active site is clearly visible on its surface. The groove has dimensions of \(\sim 10 \times 40\) Å, the appropriate size for phytanylo-CoA to bind (Fig. 5D). The co-crystallization of PAHX with substrate would provide a greater understanding of the substrate selectivity of PAHX. Soluble substrate analogues such as isovaleryl-CoA are an alternative for co-crystallization studies, since phytanylo-CoA is relatively insoluble. Co-crystallization attempts with isovaleryl-CoA have not yet been successful.

** Clinically Observed and Other Mutants**—A total of 16 clinically observed missense mutations in PAHX have been identified (TABLE TWO) (8, 58). Analysis of the PAHX structure reveals the majority of these mutations to be clustered in two regions: one around the Fe(II) (five mutants) and the other around the 2OG binding pocket (six mutants) (Fig. 6). The enzymatic activity of P29s, Q176K, G204S, N269H, R275W, and R275Q clinical mutations have been assayed using recombinant enzyme (29). The P29s mutation, located in the PTS2, has been proposed to effect targeting of pro-PAHX to the peroxisome (29, 59).

Clinical mutations that affect iron binding include those involving His\(^{75}\) (H175R) and Asp\(^{77}\) (D177G), which directly ligate the iron, and Gin\(^{176}\) (Q176K), located between the two Fe(II)-binding residues. Mutation of His\(^{175}\) or Asp\(^{177}\) to alanine ablates PAHX activity and causes impaired Fe(II) binding in vitro (27), and the H175R and D177G clinical mutants are likely to have a similar consequence. The side chain of Gin\(^{176}\) is in position to make two hydrogen bonds with the backbone atoms Ser\(^{216}\) oxygen and Lys\(^{218}\) nitrogen located on a stretch of random coil leading to a disordered loop (residues 223–233), which is probably involved in substrate binding as mentioned above. The Q176K clinical mutation is likely to interrupt this hydrogen bond network and cause changes in the backbone conformation for both the Fe(II)-binding residues and the potential substrate binding loop 223–233. In vitro, both the Q176K and Q176A mutations show uncoupling of 2OG oxidation and hydroxylation of phytanoyl-CoA (27, 29).

Two other clinical mutations, H220Y and P173S, are also predicted to affect Fe(II) binding. His\(^{220}\) is located on the sequence approaching the disordered loop (223–233), predicted to be involved in substrate binding, and its Nε1 atom is in position to hydrogen-bond with the backbone amide of Gin\(^{176}\). Mutation of His\(^{220}\) to a bulky hydrophobic tyrosine may ablate the hydrogen bond and destabilize or modify the conformation of the Fe(II)-binding residues on either side of Gin\(^{176}\). Region 165–172 is disordered, representing a high level of conformational flexibility. Pro\(^{173}\) introduces a conformationally rigid anchor point that prevents the flexibility of the sequence just prior from being transferred to the subsequent Fe(II)-binding residues His\(^{175}\) and Asp\(^{77}\). The clinical mutation, P173S, removes this rigid anchor and probably destabilizes Fe(II) binding.

Clinical mutations that affect, or probably affect, 2OG binding include Arg\(^{275}\) (R275W and R275Q), Trp\(^{193}\) (W193R), Ile\(^{199}\) (I199F), Glu\(^{197}\) (E197Q), Gln\(^{204}\) (G204S), and Asn\(^{269}\) (N269H). Arg\(^{275}\) and Trp\(^{193}\) interact directly with 2OG via electrostatic and hydrophobic interactions, respectively. Consistent with their apparent important role in binding 2OG, analyses on the recombinant versions of the clinically observed mutants R275W and R275Q demonstrate very low catalytic activities (<0.5%) (60). Other mutations involving Glu\(^{197}\), Ile\(^{199}\), Gln\(^{204}\), and Asn\(^{269}\) are clustered in the region around Arg\(^{275}\) and are predicted to affect the 2OG-binding pocket. The Oε2 of Glu\(^{197}\) is in position to form a hydrogen bond to the backbone nitrogen of Lys\(^{276}\) (2.9 Å), the residue adjacent to Arg\(^{275}\), and a long range electrostatic interaction with the side chain amine of Lys\(^{276}\) (5.5 Å). Therefore, E197Q probably disrupts the 2OG-binding pocket. Ile\(^{199}\) makes hydrophobic interactions with the methylenes of Arg\(^{275}\) and Trp\(^{193}\), and I199F probably...
| Mutation       | Homozygous/heterozygous | Location                                                                 | Predicted functional effect/available assay results for purified mutant PAHX                  |
|---------------|-------------------------|--------------------------------------------------------------------------|------------------------------------------------------------------------------------------------|
| P29S          | 1/3                     | N-terminal peroxisomal targeting sequence                                | Disrupts peroxisomal targeting or processing/Fully catalytically active (29)                  |
| N83Y          | NC/1                    | α-Helix 2, surface of protein                                           | May disrupt protein-protein interactions                                                     |
| P173S         | 1/-                     | Normally DSBH β-strand II in 2OG oxygenases, close to Fe(II)-binding residues | Disrupts Fe(II) binding                                                                       |
| H175R         | -/1                     | Between what is normally DSBH β-strand II in 2OG oxygenases and helix 3_10-1 to Fe(II) | Disrupts Fe(II) binding/Exhibits reduced Fe(II) binding, catalytically inactive (27, 29)     |
| Q176K         | -/1                     | Between two Fe(II)-binding residues                                     | Disrupts Fe(II) binding/Q176K and Q176A uncouple 2OG and phytanoyl-CoA oxidation (27, 29) |
| D177G         | 2/2                     | On helix 3_10-1, subsequent to what is normally DSBH β-strand II in 2OG oxygenases, binds to Fe(II) | Disrupts Fe(II) binding/D177A and D177S exhibit reduced Fe(II) binding and are inactive (27, 29) |
| W193R         | 1/-                     | DSBH β-strand III (β-8), interacts with 2OG                            | Disrupts 2OG binding pocket                                                                 |
| F197Q         | -/1                     | Between DSBH β-strand III (β-8) and DSBH β-strand IV (β-9), close to 2OG binding pocket | Disrupts 2OG binding pocket                                                                 |
| F204S         | 1/-                     | Between DSBH β-strand III (β-8) and DSBH β-strand IV (β-9), 2OG binding pocket | Disrupts 2OG binding pocket/Uncouples 2OG and phytanoyl-CoA oxidation (29)                   |
| H213A         | NC                      | Helix 3_10-3                                                            | Disrupts secondary structure                                                                |
| H220Y         | -/1                     | Close to Fe(II) binding residues and disordered loop (223–233) capping the active site | Disrupts Fe(II) binding                                                                     |
| R245Q         | 2/2                     | Loop after DSBH β-strand IV (β-9)                                       | May disrupt protein-protein interactions                                                     |
| H257S         | 2/1                     | DSBH β-strand VI (β-11), contacting α-helix 4                           | May disrupt protein folding or destabilize DSBH                                             |
| H259A         | NC                      | β-Turn between DSBH β-strands VI and VII (β-11 and β-12)                | Destabilization of core DSBH/Severely reduces activity (27)                                  |
| H260A         | NC                      | DSBH β-strand VII (β-12), binds to Fe(II)                               | Disrupts Fe(II) binding/Severely reduces activity (27)                                       |
| H269H         | -/1                     | Turn prior to DSBH β-strand VII (β-12), close to 2OG binding pocket      | Disrupts 2OG binding pocket/Uncouples 2OG and phytanoyl-CoA oxidation (29)                   |
| R275W/Q       | 2/2                     | DSBH β-strand VIII (β-13), binds to 2OG                                 | Disrupts 2OG binding/Inactive in vitro (29)                                                 |
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is located on a small stretch of helix (residues 212–214) and is positioned to interact with the C-terminal end of helix α-1 and probably stabilizes the helix dipole through electrostatic interactions. The H213A mutation results in insoluble protein during expression (27), consistent with the proposed structural role for this residue.

Medicinal and Biological Implications—Knowledge of the 2OG binding site of PAHX may help to develop new treatments for RD and in the design of selective inhibitors for human 2OG oxygenases. Mukherji et al. (60) have shown that it is possible to partially rescue the activity of a purified 2OG binding pocket mutant using complementary 2OG analogues. An R275Q mutant showed a 500-fold increase in activity using 2-oxobutyrate in place of 2OG as the co-substrate. Structural knowledge of the 2OG binding pocket of PAHX may enable the design of more efficient substituted 2-oxoacids, which are useful for “chemical co-substrate rescue” of mutant PAHX activity.

Human 2OG oxygenases involved in the hypoxic response, including FIH and the hypoaxia-inducible factor (HIF) prolyl-hydroxylases, are targets for inhibition with the medicinal objective of up-regulating HIF target genes (64). Inhibitors of 2OG oxygenases based upon analogues of 2OG (e.g. N-oxalylamino acids) have been shown to be active against the HIF hydroxylases (65), including FIH. N-oxalylglycine is also an inhibitor of PAHX and probably inhibits many human 2OG oxygenases and other 2OG-utilizing enzymes. Recently, it has been reported that N-oxalylamino acids with the D-stereocchemistry and hydrophobic side chains are selective for FIH over a HIF prolyl-hydroxylase (human prolyl-hydroxylase domain containing 2) (66). Given the differences in the 2OG binding pocket between FIH and PAHX, it might be possible to use the PAHX structure to design FIH inhibitors that do not significantly inhibit PAHX and possibly its nearest human relative, PHYD1.

The observation of homozygous patients possessing inactive mutant PAHX that can still metabolize phytanoyl-CoA, albeit at a very reduced rate (67), have led to proposals for an alternative pathway for phytanoyl-CoA metabolism via ω-oxidation (68), or the existence of other phytanoyl-CoA-metabolizing enzymes. The putative human 2OG oxygenase, PHYD1 (phytanoyl-CoA dioxygenase domain-containing 1) (52), is clearly related to PAHX, and both have additional homologues in a wide range of metazoans and bacteria. Although the Fe(II)- and 2OG-binding residues (including His175, Asp264, Arg275, Lys286, and Ser269), together with other active site residues (e.g. His261), are conserved among the homologues, the three disordered regions in PAHX proposed to be involved in substrate binding are very different. This suggests that phytanoyl-CoA is not a common substrate. However, the evolutionary relationship of PAHX to its homologues might indicate a new subfamily of coenzyme A-binding 2OG-dependent oxygenases.

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REFERENCES

1. Steinberg, D. (1995) in The Metabolic and Molecular Bases of Inherited Disease (Valle, D., ed) 7th Ed., pp. 2351–2369, McGraw-Hill Inc., New York.

2. Wanders, R. J. O. H. (2001) in The Metabolic and Molecular Bases of Inherited Disease (Valle, D., ed) Vol. 2, 8th Ed., pp. 3303–3321, McGraw-Hill Inc., New York.

3. Wierzbicki, A. S., Lloyd, M. D., Schofield, C. J., Feher, M. D., and Gibberd, F. B. (2002) J. Neurochem. 80, 725–735.

4. Mukherji, M., Schofield, C. J., Wierzchicki, A. S., Jansen, G. A., Wanders, R. J., and Lloyd, M. D. (2003) Prog. Lipid Res. 42, 359–376.

5. T. Searls and C. J. Schofield, unpublished data.
