Studies on the specificity of unprocessed and mature forms of phytanoyl-CoA 2-hydroxylase and mutation of the iron binding ligands

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Abstract **The mature form of phytanoyl-coenzyme A 2-hydroxylase (PAHX), a nonheme Fe(II)- and 2-oxoglutarate-dependent oxygenase, catalyzes the α-hydroxylation of phytanoyl-CoA within peroxisomes. Mutations in PAHX result in some forms of adult Refsum's disease. Unprocessed PAHX (pro-PAHX) contains an N-terminal peroxisomal targeting sequence that is cleaved to give mature PAHX (mat-PAHX). Previous studies have implied a difference in the substrate specificity of the unprocessed and mature forms of PAHX. We demonstrate that both forms are able to hydroxylate a range of CoA derivatives, but under the same assay conditions, the N-terminal hexa-His-tagged unprocessed form is less active than the nontagged mature form. Analyses of the assay conditions suggest a rationale for the lack of activity previously reported for some substrates (e.g. isovaleryl-CoA) for the $(His)_6$pro-PAHX. Site-directed mutagenesis was used to support proposals for the identity of the iron binding ligands (His-175, Asp-177, His-264) of the 2-His-1-carboxylate motif of PAHX. Mutation of other histidine residues (His-213, His-220, His-259) suggested that these residues were not involved in Fe(II) binding.**—Searls, T., D. Butler, W. Chien, M. Mukherji, M. D. Lloyd, and C. J. Schofield. **Studies on the specificity of unprocessed and mature forms of phytanoyl-CoA 2-hydroxylase and mutation of the iron binding ligands.** ***J. Lipid Res.*** **2005.** 46: **1660–1667.**

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Phytol, the hydrophobic side chain of chlorophyll commonly found in dairy products and ruminant fat, is metabolized in animals to phytanic acid. As a result of the presence of a β-methyl group, phytanic acid cannot be degraded via the β-oxidation pathway for fatty acid degradation. Instead, an α-oxidation pathway located in peroxisomes excises a methylene group from phytanic acid (1–3) to give pristanic acid, which can be further metabolized via β-oxidation first in peroxisomes and subsequently in mitochondria (4). Phytanoyl-CoA 2-hydroxylase, an Fe(II) and 2-oxoglutarate (2OG)-dependent oxygenase, catalyzes the α-hydroxylation of phytanoyl-CoA to α-hydroxyphytanoyl-CoA, the first step in α-oxidation (**Fig. 1**). Defects in the α-oxidation pathway result in neurological syndromes in humans, including adult Refsum's disease (ARD) (2, 5, 6). Approximately 45% of cases of ARD in the United Kingdom are associated with defects in phytanoyl-coenzyme A 2-hydroxylase (PAHX) (7–9), some other cases are associated with defects in the Pex7 transporter protein responsible for the importation of PAHX into peroxisomes (10), and some remain unidentified.

PAHX exists in two forms, the translated unprocessed protein (pro-PAHX), which contains an N-terminal peroxisomal targeting signal [peroxisomal targeting sequence-2 (PTS2)] required for peroxisomal localization, and the processed mature protein (mat-PAHX) found within peroxisomes, which lacks PTS2 (6). It has been reported that mat-PAHX catalyzes the hydroxylation of a range of acyl-CoA substrates, including straight-chain (i.e. lacking the β-methyl group of phytanoyl-CoA) fatty acyl-CoA esters with lengths longer than four carbons as well as the branched short-chain acyl-CoA ester of isovaleryl-CoA, but less effectively than the β-methylated branched-chain substrates (11). However, Foulon et al. (12) have reported a study of PAHX substrate specificity using a hexa-His-tagged pro-PAHX enzyme that catalyzed the hydroxylation of neither straight-chain acyl-CoA moieties nor isovaleryl-CoA.

The only reported crystal structures for a mammalian 2OG-dependent oxygenase is that of factor-inhibiting hyp-

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Abbreviations: ARD, adult Refsum's disease; mat-PAHX, mature phytanoyl-coenzyme A 2-hydroxylase; 2OG, 2-oxoglutarate; PAHX, phytanoyl-coenzyme A 2-hydroxylase; pro-PAHX, unprocessed phytanoyl-coenzyme A 2-hydroxylase; PTS2, peroxisomal targeting sequence-2; TCEP, tris(carboxyethyl)phosphine.

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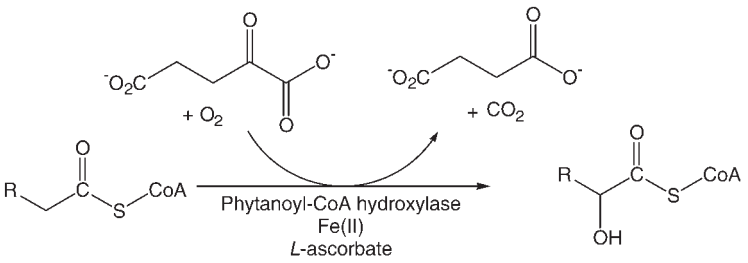

Fig. 1. Peroxisomally localized conversion of acyl-CoA to α-hydroxyacyl-CoA by the Fe(II)- and 2-oxoglutarate-dependent enzyme phytanoyl-coenzyme A α-hydroxylase (PAHX), with coevolution of carbon dioxide and succinic acid. Substrates used were phytanoyl-CoA (R = 1*RS*,5*R*,9*R*, 13-tetramethylbutyldecanoic acid), isovaleryl-CoA (R = isopropyl), octanoyl-CoA (R = *n*-heptane; C6), decanoyl-CoA (R = *n*-octane; C8), and arachidoyl-CoA (R = *n*-octadecane; C18).

oxia-inducible factor (13–15), which shows a double-stranded β-helix ("jellyroll") motif also observed in the structures of microbial (16–20) and plant (21) 2OG-dependent oxygenases. Sequence and secondary structure predictions (22) imply that PAHX also contains a double-stranded β-helix fold. Many of the conserved residues (23, 24) important for 2OG oxygenase catalysis are located in this conserved core. In the enzymes for which crystal structures are available, a conserved HXD/E . . . H motif is responsible for binding the iron in a tridentate manner. Clinically observed mutations in PAHX (9) and activity analyses (22) suggest that His-175 and Asp-177 are two of the likely three residues involved in iron binding, but the identity of the C-terminal histidine is undefined.

Using both unprocessed and mature forms of recombinant PAHX, we report that both forms of the enzyme have similar substrate specificities and that the differences in the previous reports probably result from the use of non-optimal assay conditions relating to the iron cofactors and/or to the method of $(His)_6$pro-PAHX purification. We also describe site-directed mutagenesis studies that define the iron binding ligands of PAHX.

EXPERIMENTAL PROCEDURES

Materials

All chemicals were supplied by Sigma-Aldrich Chemical Co. (Poole, Dorset, UK), unless stated otherwise, and were of analytical grade or higher. (3*R*,*S*,7*R*,11*R*)-Phytanoyl-CoA was synthesized as reported (25). Chromatography systems and columns were obtained from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK) or Bio-Rad (Hemel Hempstead, Hertshire, UK). Protein purification was performed on a fast-protein liquid chromatography Bio-pilot system. HPLC columns were obtained from Phenomenex (Macclesfield, Cheshire, UK). Oligonucleotides were obtained from Sigma-Genosys. Other molecular biology reagents were obtained from Stratagene (Europe) (Amsterdam, The Netherlands) or New England Biolabs Ltd. (Hitchen, Hertshire, UK).

PCR-mediated site-directed mutagenesis

Mutagenesis was performed using a Biometra Tpersonal Thermocycler. The following were mixed in a 0.5 ml Eppendorf tube: template (pGEMT + mat-PAHX; 50 ng), 5′ and 3′ primers (0.1 nM), deoxynucleoside triphosphates (0.625 mM each), $MgCl_2$ (10 mM), 10× buffer (2 μl), *pfu* turbo DNA polymerase (0.5 μl, 2.5 units), 80% (v/v) glycerol (2 μl), and water to 20 μl. The program was as follows: 95°C for 4 min; then 16 cycles of 95°C for 1 min, 40°C for 1 min, and 72°C for 8 min; 0°C thereafter. The products were analyzed by 0.8% (w/v) agarose gel electrophoresis. DNA products were digested by *Dpn*I to remove residual template, and the linear mutant plasmid was transformed into competent *Escherichia coli* XL1-Blue. Automated DNA sequencing (Department of Biochemistry, University of Oxford) confirmed the presence of the required mutations in the products.

Mutant *pahx* was purified using the Promega Wizard® System and digested with *Nde*I and *Hind*III, except in the cases of H220A and H259A, which were cleaved using *Nde*I and *Eco*RI, producing mutants longer by 17 residues at the N terminus. The required fragments were purified by gel electrophoresis, ligated into the pET24a vector using the same restriction sites, and transformed into competent *E. coli* XL1-Blue cells. Recombinant plasmids were then transformed into competent *E. coli* BL21 (DE3) for expression.

Purification of mat-PAHX and mat-PAHX mutants

Recombinant mat-PAHX [from *E. coli* BL21 (DE3)] was purified by cation-exchange chromatography as described for native mat-PAHX (22), with some modifications. To minimize the degradation of PAHX, cells were sonicated in a solution lacking DTT but containing 1.0 mM EDTA. Some mutants were purified using a RESOURCE-S (6 ml) column, with a 0–700 mM NaCl gradient over 900 ml (25 ml fractions were collected). The fractions containing mutant PAHX were pooled and concentrated in 300 ml of Amicon stirred cells with a YM-30 membrane to 10 mg/ml and stored at −80°C. All proteins were analyzed by electrospray ionization mass spectrometry, except for the H213A mutant, which was not analyzed because of insoluble expression, and had masses within the experimental error of the calculated mass. Purified mutants were analyzed by circular dichroism spectroscopy, as described (22).

Purification of hexa-His-tagged pro-PAHX

Recombinant PAHX was generated and purified as described (12, 26). All versions of the PAHX protein were exchanged into 50 mM Tris, pH 7.5, and 10% glycerol and stored at −80°C. Protein concentrations were determined by the method of Bradford (27).

Enzyme assays and chemical rescue experiments

Assays for 2OG conversion were performed using the reported [^{14}C]CO_2 turnover assay with the following modifications. Reactions took place in a final volume of 150 μl with final concentrations of 150 μM acyl-CoA substrate, 50 mM Tris-HCl, pH 7.5, 2.5 mM 2OG, 10 mM L-ascorbate, 0.1 mM tris(carboxyethyl)phosphine (TCEP), 0.1 mM $FeSO_4$ and 30 μg PAHX protein (condition 1), unless specified otherwise. For (3*RS*,7*R*,11*R*)-phytanoyl-CoA and other substrates with acyl chain lengths of >10, the reaction also contained 0.44 mM β-cyclodextrin as a solubilizing agent to hinder micelle formation. Reactions were also carried out in a final volume of 250 μl with final concentrations of 150 mM acyl-CoA, 50 mM Tris, pH 7.5, 3 mM 2OG, 10 mM L-ascorbate, 0.1 mM $FeCl_2$, 4 mM sodium acetate, 50 mM imidazole, 100 mM NaCl, 100 mM KCl, 12.5 μM BSA, 4 mM ATP, 2.4 mM $MgCl_2$, 0.2 mM CoA, and 30 μg of

pro-PAHX [condition 2 (12)], unless specified otherwise. Reactions were quenched by the addition of EDTA to a final concentration of 250 mM and an equivalent volume of methanol. Hydroxylation of substrates was evaluated by HPLC, after samples were centrifuged and the methanol was removed by rotary evaporation, with a Hypersil ODS C-18 column (4.6 × 250 mm) at 254 nm.

Assays for the effects of iron and for histidine rescue were performed using the reported HPLC assay (28) with the following modifications. Reactions took place with final concentrations of 50 μM synthetic (3*RS*,7*R*,11*R*)-phytanoyl-CoA, 50 M Tris-HCl, pH 7.5, 2.5 mM 2OG, 10 mM L-ascorbate, 100 μM TCEP, 1.0 mM $FeSO_4$, 4 mM ATP, and 20 μg of enzyme. Reactions were quenched after incubation for 5 min and analyzed using a Hypersil column (4.6 × 250 mm) at 254 nm with a gradient of 10–75% acetonitrile in a gradient of 30–15 mM NH_4PO_4, pH 5.5, at a flow rate of 1 ml/min.

LC-MS analyses were carried out using a Waters 600 HPLC system with a Waters 2700 autosampler and a Jupiter C4 column (150 × 4.6 mm; Phenomenex) with a gradient of 5–95% acetonitrile in 0.1% formic acid at a flow rate of 1 ml/min. The flow was split postcolumn via a flow-splitter into a Micromass ZMD quadrupole mass spectrometer using the electrospray probe in negative ion mode. The data were analyzed using Micromass Masslynx 3.5 software.

For iron binding analysis, equimolar protein samples were incubated in a 10-fold molar excess of $FeSO_4$ for 20 min on ice, and nonbound iron was removed using size-exclusion NAP® columns (Amersham Biosciences). The 1 ml protein samples were mixed with 500 μl of reagent A (1:1 4.5% $KMnO_4$ to 1.2 N HCl) and incubated at 60°C for 2 h. After cooling to room temperature, 100 μl of reagent B (8.8 g of ascorbic acid, 9.7 g of ammonium acetate, 80 mg of ferrozine, 80 mg of neocuproine, and double-distilled water to 25 ml total volume) was added followed by immediate vortexing. The samples were read at 562 nm after the color was fully developed by a 1 h incubation at room temperature.

RESULTS

Substrate specificities of pro- and mat-PAHX

We have reported that, in addition to phytanoyl-CoA, straight-chain acyl-CoA substrates and short branched-chain acyl-CoAs are in vitro substrates for recombinant mat-PAHX (11). In contrast, another group has reported that for N-terminal $(His)_6$pro-PAHX for substrates lacking a β-methyl group on the acyl chain, hydroxylation does not occur (12); pro-PAHX produced without an N-terminal His tag is produced in an insoluble form in *E. coli* (11). To address this discrepancy, the activities of highly purified (by SDS-PAGE analysis) samples of $(His)_6$pro-PAHX and mat-PAHX were assayed for turnover of 2OG to CO_2 under our reported conditions (condition 1) (11). For isovaleryl-CoA, octanoyl (C8) and decanoyl-CoA (C10) turnover of 2OG was clearly exhibited for both forms of PAHX, but at equimolar concentrations, the mat-PAHX exhibited consistently higher activity (for mat-PAHX, 28.0 vs. 9.3 nmol/min/mg for pro-PAHX with isovaleryl-CoA and 15.2 vs. 7.9 nmol/min/mg with decanoyl-CoA, respectively).

With some 2OG oxygenases, it has been shown that the use of substrate analogs can result in uncoupling of 2OG and substrate oxidation (29, 30). Assays for substrate hydroxylation by $(His)_6$pro- and mat-PAHX using HPLC were thus carried out to demonstrate the production of hydroxylated products for a variety of straight-chain acyl-CoAs [e.g. arachidoyl-CoA (C20), decanoyl-CoA, and octanoyl-CoA] as well as the short branched-chained isovaleryl-CoA. For isovaleryl-CoA, octanoyl-CoA and decanoyl-CoA LC-MS indicated a mass increase of 16 in the proposed product. Thus, we conclude that under the (partially) optimized condition 1, both pro- and mat-PAHX have similar substrate specificities; however, at least with an N-terminal His tag, the pro-PAHX is less active. Because divalent metals have been shown to inhibit 2OG oxygenases (31) and a pro-PAHX mutant (P29S) lacking a His tag had full in vitro activity compared with wild-type mature PAHX as measured by both 2OG and phytanoyl-CoA conversion (22), it was considered possible that the presence of Ni(II) used in protein purification results in reduced activity. Indeed, 2OG consumption assays using isovaleryl-CoA as a substrate showed that increasing concentrations of Ni(II) similarly inhibited mat- and $(His)_6$pro-PAHX (IC_{50} = 50 μM).

Previous work has shown that small changes in reaction conditions can have large effects on the in vitro activity of PAHX (22). A direct comparison of condition 1 with condition 2 using isovaleryl-CoA as a substrate found that condition 1 consistently gave higher levels of activity than condition 2, by a factor of ~2.5 for $(His)_6$pro-PAHX and 3.5 for mat-PAHX. The lower levels of activity for condition 2 versus condition 1, combined with the use of a (partially purified) His-tagged protein, likely accounts for the lack of observation of hydroxylation observed with nonoptimal PAHX substrates (e.g., straight-chain acyl-CoAs) by Foulon et al. (12).

Factors that may have been responsible for the different levels of activity in the two assay conditions were then examined. The importance of iron, 2OG, and ascorbate for in vitro PAHX catalysis has been established (11, 12, 22, 26), and these reagents were used consistently in the two contrasting previous studies. Differences between the two sets of conditions included substrate concentration, the presence of ATP, and the lack of a reducing agent in condition 2. The possibility that different acyl-CoA substrate concentrations used [50 μM (12, 22), 100 μM (26), or higher (11)] gave rise to the differing results was eliminated by experiment. The possibility that the different results may have resulted in part from different procedures for solubilizing the acyl-CoAs [i.e., the use of β-cyclodextrin or sterol carrier protein-2 (11) versus albumin (12)] was then ruled out. Furthermore, although solubilization is an issue for long-chain aliphatic-CoA moieties, it cannot be a major factor in rationalizing the differences because isovaleryl-CoA, which was not observed by Foulon et al. (12) to be a substrate, does not need a solubilizing factor.

The effects of ATP and Mg(II) on activity were then examined for mat-PAHX. In the absence of ATP, mat-PAHX had maximum activity in the presence of 0.05–0.1 mM Fe(II), with the other components of condition 1 fixed. The addition of 4 mM ATP inhibited PAHX activity under

TABLE 1. Effect of mutations at the HXD . . . H catalytic triad on the activity of mat-PAHX

Variable	Enzymes				
	mat-PAHX	H175A	Q176A	D177A	H264A
			%		
Activity	100	<0.1	16.3	<0.1	7.5
3.0 mM Fe	84.1	<0.1	N/D	<0.1	22.4
10 mM Fe	30.6	<0.1	N/D	<0.1	<0.1

mat-PAHX, mature phytanoyl-coenzyme A 2-hydroxylase; N/D, not determined. The effect of iron concentration on the activities of mutants to the iron binding catalytic triad of mat-PAHX in catalyzing the α-oxidation of phytanoyl-CoA substrate, relative to wild-type, as determined by HPLC. The wild-type mat-PAHX enzymes had a specific activity of 329 nmol/min/mg at 1 mM Fe(II). The activity of Q176K was similar to that shown for Q176A; the activity of D177S was similar to that shown for D177A.

conditions of 0.1 mM Fe(II). At increased Fe(II) concentrations, mat-PAHX activity was stimulated by ATP, with a maximum at 64 mM ATP for 1.0 mM Fe(II). Analogous results were observed with GTP, but no stimulation was observed for CTP, in support of previous observations (26). At 1.0 mM Fe(II), 0–20 mM $MgCl_2$ had a negligible effect on PAHX activity in both the absence and presence of 4 mM ATP (22). In the absence of ATP, increases in Fe(II) concentration of >1.0 mM showed reduced activity (**Table 1**). These results do not absolutely exclude the possibility of ATP binding directly to PAHX, but they show that the optimal ATP concentration is dependent on the Fe(II) concentration and is not essential for in vitro acyl-CoA hydroxylation by PAHX, suggesting that it is unlikely.

Imidazole exerted a similar effect to ATP at higher iron concentration (**Table 2**). In the absence of ATP and with 1.0 mM Fe(II), mat-PAHX activity was increased by ~50% upon addition of 50 mM imidazole. The apparent stimulatory effects of ATP, GTP, and imidazole in the presence of "excess" iron may be caused by the liberation of a nonproductive acyl-CoA-iron complex. Evidence for the ability of acyl-CoA substrates to bind iron came from the complete disappearance of the ultraviolet peak at 254 nm for phytanoyl-CoA when 1.0 mM Fe(II) was added (data not shown). That GTP and ATP, but not CTP or UTP, have a stimulatory effect is consistent with studies that have found that purines complex transition metal better than pyrimidines (32).

Although condition 1 used TCEP as a reducing agent, condition 2 did not use a reducing agent. Certain reducing agents generally enhance the in vitro activity of 2OG-dependent oxygenases, and titration of mat-PAHX with

TABLE 2. Effect of imidazole on the activity of histidine mutants

Variable	Enzymes				
	mat-PAHX	H175A	H220A	H259A	H264A
Activity	329	<1.0	30	68	25
50 mM imidazole	520	20	30	88	13
100 mM imidazole	569	15	28	129	8

The effect of imidazole on the activities of histidine mutants of mat-PAHX in catalyzing the α-oxidation of phytanoyl-CoA substrate at 1.0 mM Fe(II), relative to wild-type, as determined by HPLC.

di(thio-2-nitro-5-benzoate) has shown that six of the seven cysteine residues are relatively exposed to solvent and therefore prone to potentially faulty disulfide bridges (22). The specific activity of mat-PAHX with TCEP was more than two times greater than that with no reducing agent (28.0 and 11.5 nmol/min/mg, respectively). The soluble reducing agent TCEP does not interact strongly with iron in solution (33), is significantly more stable than DTT to oxidation in a solution containing iron ions (34), and is a better reducing agent than DTT at pH ⩽ 7.5 (35, 36). The absence of an optimal reducing agent in previous studies with PAHX (12, 26) might have been a problem, particularly when examining nonoptimal substrates.

Mutations of PAHX to identify iron binding residues

The presence of the HXD . . . H sequence motif in PAHX suggested that His-175 and Asp-177 were Fe(II) binding ligands (**Fig. 2**). This motif is conserved throughout known 2OG oxygenases (in some cases, a glutamate is present instead of aspartate), including the PAHX subfamily. In all 2OG-dependent oxygenases for which crystal structures are available (16–18, 21), the HXD sequence is located at or immediately before the second β-strand, and the second histidine residue of the 2-His-1-carboxylate motif (20) is located on the seventh strand of the jellyroll core. Structures of the 2OG oxygenases show that these residues ligate to one face of the octahedral Fe(II) coordination site, with the remaining sites in the enzyme-Fe(II)-2OG complex occupied by 2OG and water or vacant (**Fig. 3**). In PAHX, His-264 was considered to be the most likely candidate for the second histidine residue, based upon primary sequence homologies and secondary structure predictions. Other histidine residues in PAHX were selected for mutation based on sequence and structural analyses and known clinical mutations.

Construction and purification of mutants

The following mat-PAHX mutants were produced in *E. coli*: H175A, Q176A, D177A, D177S, H213A, H220A, H259A, and H264A. A plasmid containing the mutation Q176K was obtained, and the protein was purified as described (22, 24). No unwanted additional mutations were detected by DNA sequencing of the mutant clones or by electrospray ionization mass spectrometry analyses of the corresponding purified proteins.

All proteins were expressed at ~15% of the total soluble protein and purified to >95% (as judged by SDS-PAGE analysis) except for the H259A mutant, which was only partially soluble in *E. coli* (2–5% of the total expressed protein), and the H213A mutant, which was produced in an insoluble form and not analyzed further. Circular dichroism analyses of the soluble mutants suggested that they all had a similar overall three-dimensional structure to the wild-type enzyme (results not shown), although local changes in secondary structure cannot be ruled out.

Treatment of mat-PAHX with 0.25–1.0 mM diethyl pyrocarbonate on ice for 10 or 30 min abolished activity, indicating an important role for histidine residues in the hydroxylation of phytanoyl-CoA. Previous studies with enzymes (albeit involving residues that do not coordinate to

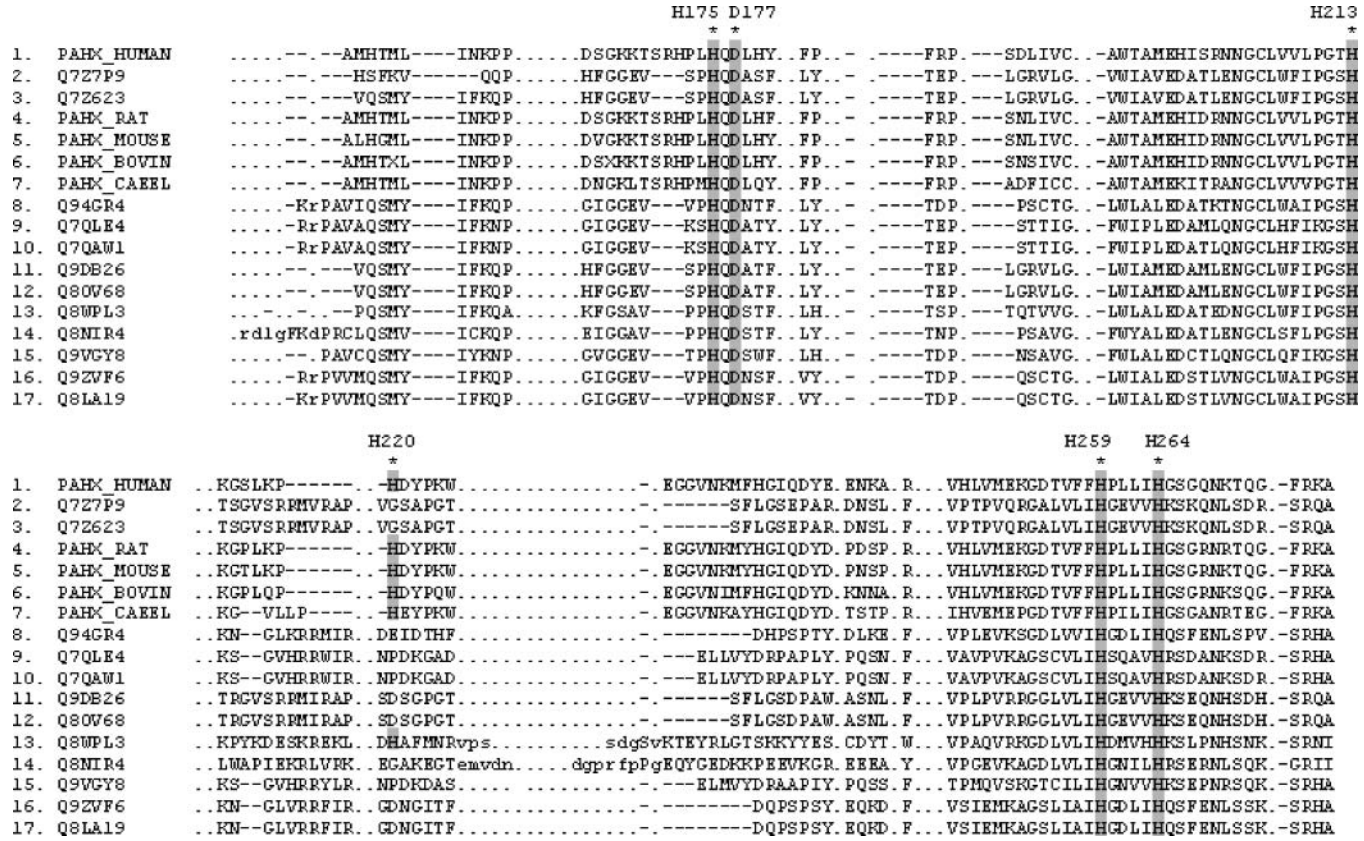

Fig. 2. Partial sequence alignment of human PAHX with homologs generated using Protein Families Database (PFAM; http: www.sanger.ac.uk/Software/Pfam/) with multiple sequence alignment (22). Proteins aligned are as follows: line 1, PAHX (*Homo sapiens*) (GenBank accession number AF023462) (8, 47); line 2, PHYHD1 protein (*H. sapiens*) (AAH51300.1); line 3, PHYHD1 protein (*H. sapiens*) (AAH53853.1); line 4, phytanoyl-CoA oxygenase, peroxisomal precursor [*Rattus norvegicus* (rat)] (AAF15971.1); line 5, phytanoyl-CoA oxygenase, peroxisomal precursor [*Mus musculus* (mouse)] (AAB81835.1); line 6, phytanoyl-CoA oxygenase, peroxisomal precursor [*Bos taurus* (bovine)] (AAB65800.1); line 7, probable phytanoyl-CoA oxygenase (*C. elegans*) (CAB05318.1); line 8, putative LN1 protein [*Oryza sativa* (rice)] (AAK50124.1); line 9, AgCP11691 (fragment) (*Anopheles gambiae str.* PEST) (EAA02969.1); line 10, AgCP7742 (fragment) (*Anopheles gambiae str.* PEST) (EAA08759.1); line 11, hypothetical protein (*M. musculus*) (BAB23937.1); line 12, Lrrc8-pending protein (*M. musculus*) (AAH39982.1); line 13, probable L-proline 4-hydroxylase (*Oikpleura dioica*) (AAL56447.1); line 14, conserved hypothetical protein (*Neurospora crassa*) (CAD37000.1); line 15, expressed protein [*Arabidopsis thaliana* (mouse-ear cress)] (AAC67325.2); line 16, hypothetical protein (*A. thaliana*) (AAM65626.1). Asterisks denote the positions of mutations in human PAHX investigated in this study.

metal ions) have shown that histidine-to-alanine mutations can be "rescued" using imidazole (37–42). Thus, all PAHX histidine mutants (except for the insoluble H213A mutant) were assayed for phytanoyl-CoA conversion in the absence and presence of imidazole (Table 2).

Activity of mutants at the HXD triad

The H175A, D177A, and D177S mutations resulted in total loss of activity (i.e., no conversion of 2OG or phytanoyl-CoA was observed), and attempts to rescue the activity of these mutants using higher concentrations of Fe(II) were unsuccessful (Table 1). These observations are consistent with His-175 and Asp-177 being Fe(II) binding residues.

Activity was observed for the H175A mutant in the presence of 50 or 100 mM imidazole (20 and 15 nmol/min/mg, respectively, vs. wild-type activity of 520 and 569 nmol/min/mg, respectively) (Table 2, Fig. 3B). Decreased levels of rescued activity were observed at imidazole con-

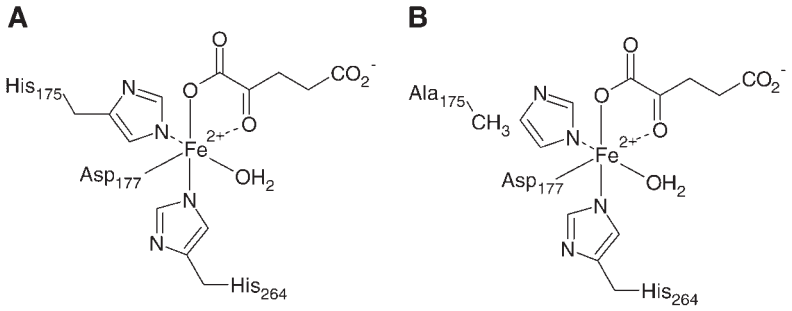

Fig. 3. A: Proposed Fe(II) binding ligands in wild-type PAHX, based on the arrangement of iron binding ligands observed in crystallographic work on deacetoxycephalosporin C synthase (19, 20). B: Proposed Fe(II) ligands for H175A PAHX in the presence of imidazole. In the absence of imidazole, Fe(II) binding is reduced significantly.

centrations of >100 mM. These observations probably reflect a balance between substitution of the missing imidazole ring of the histidine at the active site by exogenous imidazole and complexation of Fe(II) in solution by imidazole.

The structures of 2OG-dependent oxygenases [e.g., deacetoxycephalosporin C synthase (19, 20) and clavaminic acid synthase (18)] suggest that the side chain of the intermediate X residue of the iron binding HXD triad (Q176 in the case of PAHX) is not involved directly in Fe(II) binding and faces away from the iron. The Q176K mutant, which has been observed in patients with ARD (9), exhibited diminished but not eliminated activity and has been shown to "uncouple" the two oxidative reactions. Consumption of 2OG was found to occur at nearly 60% of the level of the wild-type PAHX, whereas oxidation of phytanoyl-CoA occurred at only 16% of wild-type PAHX level (22), a less than stoichiometric conversion of phytanoyl-CoA relative to 2OG. The Q176A mutant exhibited similar reduced activity by the 2OG turnover assay (Table 1) and a similar level of uncoupled turnover.

Identification of the second histidine in the HXD . . . H motif

The H220A mutant retained full activity (30 nmol/min/mg) under the optimized assay conditions, implying that it is not involved in iron binding. Moreover, a histidine residue in this position is conserved only in enzymes from *Caenorhabditis elegans* and mammals and does not occur in a conserved region of the β-strand core (22). Assays of the H259A mutant under optimized conditions showed that it also retained significant activity (68 nmol/min/mg). Although the activity of the H259A mutant was nearly doubled in the presence of 100 mM imidazole, a similar increase of activity was observed for the wild-type enzyme in 100 mM imidazole. These results imply that His-259 is also not involved in iron binding. In contrast, the H264A mat-PAHX mutant retained only a low level of residual activity (7.5% of wild-type activity) under the optimized assay conditions and showed a nearly 3-fold increase in activity when Fe(II) concentration was increased from 1.0 to 3.0 mM Fe(II) (Table 1). This was unexpected compared with the results for the His-175 and Asp-177 mutants, but in an analogous experiment with 2OG oxygenase clavaminic acid synthase, a mutation of the second histidine residue also did not completely abolish catalytic activity (43). The activity of H264A also did not increase in the presence of imidazole, in contrast to the wild type and other mutants (Table 2). Crystal structures for related 2OG oxygenases (16–20) suggest that the side chain of His-264 may be less accessible than that of His-175, particularly once Fe(II), 2OG, and substrate are bound, possibly making it more difficult for the imidazole to enter the active site and effect rescue.

To directly assess the relative iron binding affinity of the H264A mutant, the ability of mat-PAHX and the mutants containing residues potentially involved in Fe(II) binding were incubated with a 10-fold excess of iron followed by size-exclusion chromatography and analysis by the ferrozine method of Fish (44). These assays revealed the following Fe/PAHX molar ratios: mat-PAHX (0.89), H175A (0.36), Q176A (0.50), D177S (0.66), and H264A (0.35). These results clearly support the assignment of His-175, Asp-177, and His-264 as iron binding residues.

DISCUSSION

These results show that both mature and unprocessed forms of PAHX catalyze the hydroxylation of straight-chain as well as β-methylated acyl-CoAs but that under the same assay conditions, the unprocessed form is less active than the mature form. They also reveal that differing in vitro assay conditions likely led to the apparent differences in substrate specificity in the previous reports (11, 12). Although the precise reasons for the lack of activity with straight-chain CoA contained in one report are unclear, they likely arose because of nonoptimal assay conditions coupled to, possibly, contaminating Ni(II), which is a PAHX inhibitor. As noted previously (11), when mixtures of straight- and branched-chain (i.e., β-methyl) substrates are incubated with PAHX, the β-methylated substrates are preferentially hydroxylated. However, the results do not imply any evidence that PAHX plays any role in the metabolism of straight-chain acyl-CoAs, although neither can it be ruled out. The danger of inferring in vivo roles for enzymes based upon their in vitro specificities toward different substrates has been highlighted by a recent study on fatty acid amide hydrolase activity, in which it was shown that the relative in vitro activities of the enzymes with lipid metabolites was not predictive of substrates in vivo (45). There is also evidence that PAHX is not the only enzyme catalyzing the α-hydroxylation of fatty acids in mammals; Alderson et al. (46) have reported on the α-hydroxylation of long-chain fatty acids as catalyzed by a microsomal fatty acid 2-hydroylase.

The results of this study clarify the residues of the PAHX enzyme that bind to the ferrous iron cofactor as His-175, Asp-177, and His-264. It is of interest that the observed levels of oxidation for the Q176K mutant are higher than those reported for other PAHX mutations giving rise to ARD tested to date, all of which possess very low level activity (<1.0 nmol/min/mg). This demonstrates that in vitro enzyme activity assays alone are not definitive as a diagnostic method for ARD. The proposed binding residues His-175, Asp-177, and His-264 are present in a range of predicted proteins related to PAHX, including two human homologs (Fig. 2, lines 2 and 3). The substrates for these homologs are of interest because if either possesses PAHX activity, it may help to rationalize the pathologies of certain Refsum's disease patients.

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REFERENCES

1. Verhoeven, N. M., R. J. A. Wanders, B. T. Poll-The, J. M. Saudubray, and C. Jakobs. 1998. The metabolism of phytanic acid and pristanic acid in man: a review. *J. Inherit. Metab. Dis.* **21:** 697–728.
2. Wanders, R. J. A., C. Jakobs, and O. H. Skjeldal. 2001. Refsum disease. *In* The Metabolic and Molecular Bases of Inherited Disease. C. R. Scriver, A. L. Beaudet, W. S. Sly, D. Valle, B. Childs, K. W. Kinzler, and B. Vogelstein, editors. McGraw-Hill, Inc., New York. 3303–3321.
3. Wanders, R. J. A., G. A. Jansen, and O. H. Skjeldal. 2001. Refsum disease, peroxisomes and phytanic acid oxidation: a review. *J. Neuropathol. Exp. Neurol.* **60:** 1021–1031.
4. Verhoeven, N. M., D. S. Roe, R. M. Kok, R. J. A. Wanders, C. Jakobs, and C. R. Roe. 1998. Phytanic acid and pristanic acid are oxidised by sequential peroxisomal and mitochondrial reactions in cultured fibroblasts. *J. Lipid Res.* **39:** 66–74.
5. Gibberd, F. B., and A. S. Wierzbicki. 2000. Heredopathia atactica polyneuritiformis: Refsum's disease. *In* Handbook of Ataxia Disorders. R. Klockgether, editor. Marcel Dekker, New York. 235–256.
6. Wierzbicki, A. S., M. D. Lloyd, C. J. Schofield, M. D. Feher, and F. B. Gibberd. 2002. Refsum's disease: a peroxisomal disorder affecting phytanic acid metabolism α-oxidation. *J. Neurochem.* **80:** 727–735.
7. Jansen, G. A., R. J. A. Wanders, P. A. Watkins, and S. J. Mihalik. 1997. Phytanoyl-coenzyme A hydroxylase deficiency—the enzyme defect in Refsum's disease. *N. Engl. J. Med.* **337:** 133–134.
8. Mihalik, S. J., J. C. Morrell, D. Kim, K. A. Stackster, P. A. Watkins, and S. J. Gould. 1997. Identification of PAHX, a Refsum disease gene. *Nat. Genet.* **17:** 185–189.
9. Jansen, G. A., E. M. Hogenhout, S. Ferdinandusse, H. R. Waterham, R. Ofman, C. Jakobs, O. H. Skjeldal, and R. J. A. Wanders. 2000. Human phytanoyl-CoA hydroxylase: resolution of the gene structure and the molecular basis of Refsum's disease. *Hum. Mol. Genet.* **9:** 1195–1200.
10. Wierzbicki, A. S., J. Mitchell, M. Lambert-Hamill, J. Hancock, J. Greenwood, M. C. Sidey, J. de Belleroche, and F. B. Gibberd. 2000. Identification of genetic heterogeneity in Refsum's disease. *Eur. J. Biochem.* **8:** 649–651.
11. Mukherji, M., N. J. Kershaw, C. J. Schofield, A. S. Wierzbicki, and M. D. Lloyd. 2002. Utilization of sterol carrier protein-2 by phytanoyl-CoA 2 hydroxylase in the peroxisomal α-oxidation of phytanic acid. *Chem. Biol.* **9:** 597–605.
12. Foulon, V., S. Asselberghs, W. Geens, G. P. Mannaerts, M. Casteels, and P. P. van Veldhoven. 2003. Further studies on the substrate spectrum of phytanoyl-CoA hydroxylase: implications for Refsum disease? *J. Lipid Res.* **44:** 2349–2355.
13. Lee, C., S. J. Kim, D. G. Jeong, S. M. Lee, and S. E. Ryu. 2003. Structure of human FIH-1 reveals a unique active site pocket and interaction sites for HIF-1 and von Hippel-Lindau. *J. Biol. Chem.* **278:** 7558–7563.
14. Elkins, J. M., K. S. Hewitson, L. A. McNeil, J. F. Seibel, I. Schlemminger, C. W. Pugh, P. J. Ratcliffe, and C. J. Schofield. 2003. Structure of factor-inhibiting hypoxia-inducible factor (HIF) reveals mechanism of oxidative modification of HIF-1. *J. Biol. Chem.* **278:** 1802–1806.
15. Dann, C. E., R. K. Bruick, and J. Deisenhofer. 2002. Structure of factor-inhibiting hypoxia-inducible factor 1: an asparaginyl hydroxylase involved in the hypoxic response pathway. *Proc. Natl. Acad. Sci. USA.* **99:** 15351–15356.
16. Elkins, J. M., M. J. Ryle, I. J. Clifton, J. C. D. Hotopp, J. S. Lloyd, N. I. Burzlaff, J. E. Baldwin, R. P. Hausinger, and P. L. Roach. 2002. X-ray crystal structure of *Escherichia coli* taurine/α-ketoglutarate dioxygenase complexed to ferrous iron and substrates. *Biochemistry.* **41:** 5185–5192.
17. Clifton, I. J., L. C. Hsueh, J. E. Baldwin, K. Harlos, and C. J. Schofield. 2001. Structure of proline 3-hydroxylase: evolution of the family of 2-oxoglutarate dependent oxygenases. *Eur. J. Biochem.* **268:** 6625–6636.
18. Zhang, Z-H., J. S. Ren, D. K. Stammers, J. E. Baldwin, K. Harlos, and C. J. Schofield. 2000. Structural origins of the selectivity of the trifunctional oxygenase clavaminic acid synthase. *Nat. Struct. Biol.* **7:** 127–133.
19. Lloyd, M. D., H-J. Lee, K. Harlos, Z. H. Zhang, J. E. Baldwin, C. J. Schofield, J. M. Charnock, C. D. Garner, T. Hara, A. C. Terwisscha van Scheltinga, et al. 1999. Studies on the active site of deacetoxycephalosporin C synthase. *J. Mol. Biol.* **287:** 943–960.
20. Valegard, K., A. C. Terwisscha van Scheltinga, M. D. Lloyd, T. Hara, S. Ramaswamy, A. Perrakis, A. Thompson, H-J. Lee, J. E. Baldwin, C. J. Schofield, et al. 1998. Structure of a cephalosporin synthase. *Nature.* **394:** 805–809.
21. Wilmouth, R. C., J. J. Turnbull, R. W. D. Welford, I. J. Clifton, A. G. Prescott, and C. J. Schofield. 2002. Structure and mechanism of anthocyanidin synthase from *Arabidopsis thaliana*. *Structure.* **10:** 93–103.
22. Mukherji, M., W. Chien, N. J. Kershaw, I. J. Clifton, C. J. Schofield, A. S. Wierzbicki, and M. D. Lloyd. 2001. Structure-function analysis of phytanoyl-CoA 2-hydroxylase mutations causing Refsum's disease. *Hum. Mol. Genet.* **10:** 1971–1982.
23. Prescott, A. G., and M. D. Lloyd. 2000. The iron(II) and 2-oxoacid-dependent dioxygenases and their role in metabolism. *Nat. Prod. Rep.* **17:** 367–383.
24. Mukherji, M., N. J. Kershaw, C. H. MacKinnon, I. J. Clifton, C. J. Schofield, A. S. Wierzbicki, and M. D. Lloyd. 2001. 'Chemical cosubstrate rescue' of phytanoyl-CoA 2-hydroxylase mutants causing Refsum's disease. *Chem. Commun.* **11:** 972–973.
25. Kershaw, N. J., M. Mukherji, C. H. MacKinnon, T. D. W. Claridge, B. Odell, A. S. Wierzbicki, M. D. Lloyd, and C. J. Schofield. 2001. Studies on phytanoyl-CoA 2-hydroxylase and synthesis of phytanoyl-coenzyme A. *Bioorg. Med. Chem. Lett.* **11:** 2545–2548.
26. Croes, K., V. Foulon, M. Casteels, P. P. van Veldhoven, and G. P. Mannaerts. 2000. Phytanoyl-CoA hydroxylase: recognition of 3-methyl-branched acyl-CoAs and requirement for GTP or ATP and Mg^{2+} in addition to its known hydroxylation cofactors. *J. Lipid Res.* **41:** 629–636.
27. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.
28. Mihalik, S. J., A. M. Rainville, and P. A. Watkins. 1995. Phytanic acid α-oxidation in rat liver peroxisomes. Production of α-hydroxyphytanoyl-CoA and formate is enhanced by dioxygenase cofactors. *Eur. J. Biochem.* **232:** 545–551.
29. Welford, R. W. D., I. Schlemminger, L. A. McNeil, K. S. Hewitson, and C. J. Schofield. 2003. The selectivity and inhibition of AlkB. *J. Biol. Chem.* **278:** 10157–10161.
30. Hausinger, R. P., J. McCracken, and T. F. Henshaw. 2003. Mettalocenter characterization of AlkB, a mononuclear non-heme Fe(II) DNA repair enzyme. *J. Inorg. Biochem.* **96:** 72.
31. Hewitson, K. S., L. A. McNeil, M. V. Riordan, Y. M. Tian, A. N. Bullock, R. W. D. Welford, N. J. Oldham, S. Bhattacharya, J. M. Gleadle, P. J. Ratcliffe, et al. 2002. Hypoxia-inducible factor (HIF) asparagine hydroxylase is identical to factor inhibiting HIF (FIH) and is related to the cupin structural family. *J. Biol. Chem.* **277:** 26351–26355.
32. Sigel, H., R. Tribolet, R. Malini-Balakrishnan, and R. B. Martin. 1987. Comparison of the stabilities of monomeric metal ion complexes formed with adenosine 5′-triphosphates (ATP) and pyrimidine-nucleoside 5′-triphosphates (CTP, UTP, TTP) and evaluation of the isomeric equilibria in the complexes of ATP and CTP. *Inorg. Chem.* **26:** 2149–2157.
33. Dubus, A., M. D. Lloyd, H-J. Lee, C. J. Schofield, J. E. Baldwin, and J-M. Frere. 2001. Substrate selectivity studies on deacetoxycephalosporin C synthase using a direct spectrophotometric assay. *Cell. Mol. Life Sci.* **58:** 835–843.
34. Getz, E. B., M. Ziao, T. Chakrabarty, R. Cooke, and P. R. Selvin. 1999. A comparison between the sulfhydryl reductants tris(2-carboxyethyl)phosphine and dithiothreitol for use in protein biochemistry. *Anal. Biochem.* **273:** 73–80.
35. Burns, J. A., J. C. Butler, J. Moran, and G. M. Whitesides. 1991. Selective reduction of disulphides by tris(2-carboxyenthyl)phosphine. *J. Org. Chem.* **56:** 2648–2650.
36. Han, J. C., and G. Y. Han. 1994. A procedure for quantitative determination of tris(2-carboxyethyl)phosphine, an odourless reducing agent more stable and effective than dithiothreitol. *Anal. Biochem.* **220:** 5–10.
37. Lehoux, I. E., and B. Mitra. 1999. (*S*)-Mandelate dehydrogenase from *Pseudomonas putida*: mutations of the catalytic base histidine-274 and the chemical rescue of activity. *Biochemistry.* **38:** 9948–9955.
38. Tu, C. K., D. N. Silverman, C. Forsman, B. H. Jonsson, and S. Lindskog. 1989. Role of histidine 64 in the catalytic mechanism of human carbonic anhydrase II studied with a site-specific mutant. *Biochemistry.* **28:** 7913–7918.

39. Taoka, S., C. Tu, K. A. Kistler, and D. N. Silverman. 1994. Comparison of intra- and intermolecular proton transfer in human carbonic anhydrase II. *J. Biol. Chem.* **269:** 17988–17992.
40. Silverman, D. N. 2000. Marcus rate theory applied to enzymatic proton transfer. *Biochim. Biophys. Acta.* **1458:** 88–103.
41. Newmyer, S. L., J. Sun, T. M. Loehr, and P. R. Ortiz de Montellano. 1996. Rescue of the horseradish peroxidase His-170 to Ala mutant activity by imidazole: importance of proximal ligand tethering. *Biochemistry.* **35:** 12788–12795.
42. Newmyer, S. L., and P. R. Ortiz de Montellano. 1996. Rescue of the catalytic activity of an H42A mutant of horseradish peroxidase by exogenous imidazoles. *J. Biol. Chem.* **271:** 14891–14896.
43. Doan, L. X., A. Hassan, S. J. Lipscomb, A. Dhanda, Z. H. Zhang, and C. J. Schofield. 2001. Mutagenesis studies on the iron binding ligand of clavaminic acid synthase. *Biochem. Biophys. Res. Commun.* **279:** 240–244.
44. Fish, W. W. 1988. Rapid colorimetric micromethod for the quantitation of complexed iron in biological samples. *Methods Enzymol.* **158:** 357–364.
45. Saghatelian, A., S. A. Trauger, E. J. Want, E. G. Hawkins, G. Siuzdak, and B. F. Cravatt. 2004. Assignment of endogenous substrates to enzymes by global metabolite profiling. *Biochemistry.* **43:** 14332–14339.
46. Alderson, N. L., B. M. Rembiesa, M. D. Walla, A. Bielawska, J. Bielawski, and H. Hama. 2004. The human FA2H gene encodes a fatty acid 2-hydroxylase. *J. Biol. Chem.* **279:** 48562–48568.
47. Jansen, G. A., R. Ofman, S. Ferdinandusse, L. I. Ijlst, A. O. Muisers, O. H. Skjeldal, O. Stokke, C. Jakobs, G. T. N. Besley, J. E. Wraith, et al. 1997. Refsum disease is caused by mutations in the phytanoyl-CoA hydroxylase gene. *Nat. Genet.* **17:** 190–193.