ABSTRACT. Mutations in the gene encoding for phenylalanine hydroxylase (PAH) result in phenylketonuria (PKU) or hyperphenylalaninemia (HPA). Several 3-dimensional structures of truncated forms of PAH have been determined in our laboratory and by others, using x-ray crystallographic techniques. These structures have allowed for a detailed mapping of the >250 missense mutations known to cause PKU or HPA found throughout the 3 domains of PAH. This structural information has helped formulate rules that might aid in predicting the likely effects of unclassified or newly discovered PAH mutations. Also, with the aid of recent crystal structure determinations of co-factor and substrate analogs bound at the PAH active site, the recently discovered tetrahydrobiopterin-responsive PKU/HPA genotypes can be mapped onto the PAH structure, providing a molecular basis for this tetrahydrobiopterin response. Pediatrics 2003;112:1557–1565; phenylalanine hydroxylase, 3-dimensional structure, structural basis, phenylketonuria, BH₄-responsive hyperphenylalaninemia, co-factor therapy.

ABBRVEVIATIONS. PAH, phenylalanine hydroxylase; t-Phe, l-phenylalanine; BH₄, tetrahydrobiopterin; PKU, phenylketonuria; HPA, hyperphenylalaninemia; ARS, autoregulatory sequence; CBR, co-factor–binding region.

Human phenylalanine hydroxylase (PAH) converts the essential amino acid l-phenylalanine (l-Phe) into l-tyrosine using the co-factor (6R)-l-erythro-5,6,7,8-tetrahydrobiopterin (BH₄) and molecular oxygen. Catalysis by this iron-dependent enzyme is the major pathway for catabolic degradation of dietary l-Phe and accounts for approximately 75% of the l-Phe disposal. The autosomal recessive disorder phenylketonuria (PKU) is the result of a deficiency of PAH enzymatic activity or loss of enzyme expression as a result of mutations in the PAH gene. Because of extensive newborn screening for PKU and genotyping of the PAH alleles, >400 mutations in the PAH gene are known to cause PKU or the milder form referred to as hyperphenylalaninemia (HPA). The recently solved crystal structures of PAH¹–⁶ provide a structural scaffold to explain the effects of PAH mutations. This review covers the structural work done so far on PAH and discusses some of the structural effects of the currently known mutations in the PAH gene, including some of the BH₄-responsive PKU/HPA mutations (PKU database at www.pahdb.mcgill.ca and PAH Mutation Analysis Consortium Newsletter, December 2001).

Human (liver) PAH (EC. 1.14.16.1) exists in a pH-dependent equilibrium of homotetramers and homodimers,⁷ and, like the 2 other aromatic amino acid hydroxylases tyrosine hydroxylase (EC 1.14.16.2) and tryptophan hydroxylase (EC 1.14.16.4), consists of 3 domains: an N-terminal regulatory domain (residues 1–142), a catalytic domain (residues 143–410), and a C-terminal tetramerization domain (residues 411–452; Fig 1). Because of the difficulty of crystallizing full-length PAH, no full-length tetrameric structures exist for PAH. However, several truncated forms of PAH have been structurally characterized, including a dimeric form containing the regulatory and catalytic domains³ and a tetrameric form containing the catalytic and tetramerization domains.² On the basis of these structures and a higher-resolution dimeric double-truncated form of PAH,¹ a composite full-length structural model was constructed by superimposing the respective catalytic domain regions (Figs 2–4).⁸

The regulatory domain of PAH contains an α-β sandwich with an interlocking double βββ motif (βββββ topology)³ (Fig 4). The N-terminal autoregulatory sequence (ARS; residues 19–33) extends over the active site in the catalytic domain.

An N-terminal truncated form of PAH that includes the catalytic and tetramerization domains (residues 116–452) crystallized as a tetramer (dimer of dimers).² The tetramerization domain contains 2 β-strands, forming a β-ribbon, and a 40-Å-long α-helix.² The 4 α-helices (1 from each monomer) pack into a tight antiparallel coiled-coil motif in the center of the tetramer structure (Fig 4).

Recombinant double-truncated human PAH (ΔNH102–ΔCOOH428, hPAHcat) represents a fully activated form of the enzyme, without any cooperativity of l-Phe binding.¹⁰ Thus, this structure represents the activated, or R state, of PAH. The catalytic domain region of the PAH structure (hPAHcat) has a basket-like arrangement, with a total of 13 α-helices and 8 β-strands. Because part of the C-terminal tetramerization domain is missing, this double-truncated PAH construct forms a dimer in solution, as well as in the crystal.

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The active site, located in the center of the catalytic domain, consists of a 13-Å-deep and 10-Å-wide pocket (Fig 3). Adjacent to the active site is a 16-Å-long and 8-Å-wide channel that may provide substrate access to the active site. The majority of the 34 amino acids lining the active site are hydrophobic, but 3 charged glutamates, 2 histidines, and 1 tyrosine are also located in this region. Covering the entrance to the active site is a short loop (residues 378–381) that contains some of the highest B-factors in the structure (60–80 Å²), indicative of a flexible, or disordered, loop region.

As isolated, the PAH protein contains an active site Fe(III) atom. In the crystal structures, the iron atom is located 10 Å below the surface of the protein on the “floor” of the active site, at the intersection of the channel and the active site pocket. The Fe(III) atom is coordinated to His285, His290, and 1 oxygen atom in Glu330. Both His285 and His290 have been shown by site-directed mutagenesis to be required for iron binding. Well-defined electron density is also observed for 3 water molecules coordinated to the iron. The iron ligands are arranged in an octahedral geometry, making the iron 6-coordinate as previously suggested from spectroscopic studies.

The PAH hydroxylation reaction requires the binding of BH₄, dioxygen, and Fe(II) before hydroxylation of l-Phe can occur. A stretch of 27 PAH amino acids (His263 to His289), which is highly conserved in all 3 aromatic amino acid hydroxylases, was
thought to be responsible for tetrahydrobiopterin binding\(^{14,15}\) before any structures were known. In the PAH structures, 10 residues (Phe263, Cys265, Thr266, Thr278, Pro279, Glu280, Pro281, His285, Glu286, and Gly289) in this co-factor–binding motif are present in the active site.

Based on co-crystal structures of double-truncated Fe(III)-containing human PAH with oxidized co-factor (hPAH\(_{Cat}^{\cdot}7,8\text{-BH}_2\)) or Fe(II)-containing human PAH with reduced (natural) co-factor (hPAH\(_{Cat}^{\cdot}\text{BH}_4\)),\(^5\) structural details of co-factor binding have been determined. The pterin binds close to the catalytic iron and forms hydrogen bonding interactions with 2 of the water molecules coordinated to the iron, as well as to the main chain carbonyl oxygens of Ala322, Gly247, and Leu249; the main chain amide of Leu249; and the O\(_\gamma\) atom of Ser251.\(^4,5\) The pterin ring forms an aromatic \(\pi\)-stacking interaction with Phe254, and the pterin ring and dihydroxypropyl side-chain are positioned by a Tyr325 hydrophobic interaction. The BH\(_2\) dihydroxypropyl side chain O\(_2\) atom hydrogen bonds to the carbonyl oxygen of Ala322, and Glu286 hydrogen bonds to 1 of the water molecules coordinated to the iron and also hydrogen bonds to a water molecule that is hydrogen bonded to the N3 position of the pterin ring. In addition, a major conformational change occurs in the active site upon pterin binding; residues 245 to 250 move in the direction of the iron, allowing several protein hydrogen bonds to the pterin ring to be formed. Bound pterin co-factor is in an ideal location for dioxygen binding in a bridging position between the iron and the pterin, as is presumed to happen during the \(\text{L-Phe}\) hydroxylation reaction.

Although several alternative co-factor analogs are capable of being used in the PAH hydroxylation reaction, only the natural co-factor (BH\(_4\)) inhibits the \(\text{L-Phe}\) induced activation of the enzyme\(^{13}\); the molecular mechanism of this inhibition is not yet understood.\(^3,13,16\) BH\(_2\) inhibits the activation by \(\text{L-Phe}\) less than the inhibition observed with BH\(_4\).\(^7\) Hydrogen bonding between the pterin dihydroxypropyl side chain and the carbonyl oxygen of Ala322, in combination with the PAH loop 245 to 250 conformational changes observed on co-factor binding, could provide a specific regulatory function of the pterin upon binding at the active site. It is interesting that when the binary complex hPAH\(_{Cat}^{\cdot}7,8\text{-BH}_2\) structure is superimposed onto the structure of the ligand-free C-terminal truncated rat PAH (rPAH\(_{Reg}\) containing the regulatory and catalytic domains\(^3\)), BH\(_2\) interacts with the N-terminal ARS region.

This interaction of pterin with the ARS gives a plausible structural explanation for the inhibitory effect of BH\(_4\) on the rate of phosphorylation of Ser16 in rPAH by the cAMP-dependent protein kinase, which is specific for the \(R\)-isomer of the natural
co-factor (BH₄). Furthermore, phosphorylation of Ser16 in the mobile ARS region may facilitate access of L-Phe to the active site. These conclusions are consistent with the finding that the phosphorylated form of rPAH requires less L-Phe to be activated (S₀.₅ = 29 μM) than the nonphosphorylated form (S₀.₅ = 51 μM). Thus, the regulatory properties of BH₄ in full-length PAH does not require an additional binding site of the co-factor in the regulatory domain as previously postulated.

STRUCTURAL BASIS FOR HPA AND PKU

Currently, 269 missense point mutations are known for the PAH gene (PAH Mutation Analysis Consortium Newsletter, December 2001 issue; PKU database at www.pahdb.mcgill.ca), in addition to 23 nonsense mutations and 10 silent mutations. Most of the point mutations map onto the exon 5 (PAH residue 148) to exon 12 (PAH residue 438) region of the sequence. As shown in Figs 1 and 5, 57 PAH mutations are located in the regulatory domain sequence (residues 1–142), whereas 231 PAH mutations are located in the catalytic domain (residues 143–410) and 14 are located in the tetramerization domain sequence (residues 411–452). Previously, a summary of genotype/phenotype/structural interpretations for these mutations was published.

In vitro expression analyses have been used to characterize PAH gene mutations. In general, the PAH mutations that lead to PKU or HPA genotypes result in reduced enzyme activity and stability to varying extents, and some mutations also have been found to alter the oligomeric state of the protein. At least 3 groups of HPA/PKU mutations have been categorized, dependent on the kinetic behavior and/or stability that has been observed. The first category involves mutations that affect both PAH kinetics and stability. A second class describes structurally stable mutations with altered kinetic properties, whereas a third class encompasses PAH mutations displaying normal kinetics but with reduced stability in vitro and in vivo. In addition, on the basis of the 3-dimensional structure of PAH, mutations that cause PKU/HPA affect residues in 5 different categories: 1) residues located at the active site, 2) structural residues, 3) residues involving interdomain interactions in a monomer, 4) residues that interact with the N-terminal ARS, and 5) residues at the dimer or tetramer interface regions of the structure. The most prevalent single point mutations in the PAH gene are discussed according to these 5 different structural groups.

PAH Active Site Mutations

In total, 31 point mutations can be found in the residues lining the active site. Seven of these can be found in the putative pterin-binding motif, consisting of residues 264 to 290 (PKU database at www.pahdb.mcgill.ca).

The D143G mutation is associated with a severe PKU phenotype. Asp143 is located at the entrance to the active site and may be involved in controlling substrate/co-factor access to the active site. Another PKU/HPA mutation is Thr278, which is also located at the entrance to the active site, forming a hydrogen bond with Glu280. The mutations T278I and T278A substitute a polar amino acid into different hydrophobic amino acids, losing an important hydrogen bond that would perturb the structure around the entrance to the active site. The more...
electrostatically conserved mutation T278N would most likely result in a changed hydrogen bonding pattern that would have the same effect as the 2 other mutations, although presumably slightly smaller in magnitude. A frequent mutation among black Americans is the L255S mutation, which causes severe PKU. Leu255 is most likely involved in controlling the separation of helices Ca6 and Ca9, and in this active site mutation, a nonpolar group is substituted with a smaller polar side chain that would be expected to result in significant structural perturbation.

Another mutation in the active site is the E280K mutation, found within the pterin-binding motif and associated with mild to severe PKU phenotypes. Glu280 hydrogen bonds to His146 and also forms a salt bridge to Arg158. Also, in the active site of PAH, there are only 2 free, charged groups (both glutamic acids), and substitution of Glu280 into a lysine represents a dramatic change in the electrostatic potential of the active site. As expected, expression of E280K human PAH in Escherichia coli results in an enzyme with only approximately 1% of the specific activity of wild-type PAH. The R158Q mutation is a frequent mutation in patients with PKU. As mentioned above, Arg158 forms a salt bridge to Glu280, but also forms a hydrogen bond to Tyr268. Both of these interactions are important for conserving the shape of the active site, and substitution into a glutamine or the larger aromatic residue tryptophan (R158W) will alter the active site architecture and lower enzymatic activity.

Another active site residue that is involved in BH4 co-factor binding is Phe254, which π-stacks onto the pterin ring in hPAHcat, 7,8-BH2. Phe254 is located 5.9 Å away from the active site iron, and substitution into an isoleucine, as in PKU mutant F254I, would interfere with the proper binding of the pterin and possibly the Phe substrate.

One of the most frequent PAH mutations found in southeastern Europe is the P281L mutation that is associated with HPA to severe PKU phenotypes. Pro281 helps to define the shape of the active site very close to the iron. Therefore, substitution to a less rigid leucine will change the conformation of the active site by removing the conformational constraints imposed by the proline. As expected, in vitro expression of P281L PAH in E coli or African green monkey Cercopithecus aethiops kidney (COS) cells results in complete loss of PAH activity.

Three residues in the active site that are located near the (putative) location of substrate binding or in the region near the catalytic iron are Phe331, Ala345, and Gly346. Phe331 π-stacks onto Trp326, a residue that may be involved in determining substrate specificity in the aromatic amino acid hydroxylases. Two PKU mutations have been reported for Phe331, F331C and F331L, and both of these mutations will remove important π-stacking interactions of the active site. Both Ala345 and Gly346 are located close to the triad of residues that bind the iron (His285, His290, and Glu330). Ala345 has 2 reported PKU mutations, A345S and A345T, and Gly346 has 1 mutation, G346R. All 3 of these mutations into larger and more polar residues would interfere with the location and electrostatics of the residues that act as iron ligands, thus destabilizing the active site structure. One additional residue that is important in holding the catalytic iron in place is Ser349, which hydrogen bonds to His285, one of the iron ligands. Two PKU mutations have been reported for this residue, one that results in classical PKU (S349P). This substitution into a proline so close to the catalytic iron will change the shape of the active site. As expected, expression of S349P hPAH in E coli or COS cells results in only <0.2 to <1% residual activity. The second mutation of Ser349, S349L, also has minimal activity in E coli or COS cells. Both mutations will alter the hydrogen bond to His285, having severe detriment on the PAH catalytic activity.

**PAH Mutations in Structural Residues**

The G46S mutation in the regulatory domain is associated with classical PKU and is one of the most frequent mutations found clinically. Gly46 is located on the surface of the regulatory domain in a loop preceding helix Ra1. Substitution into a serine would generate potential side-chain hydrogen bonding to 1 or more residues in close proximity to Gly46, resulting in distortions of the regulatory domain secondary structure. This G46S mutation thus potentially results in the formation of inactive PAH aggregates, as previously reported.

Another PKU mutation found in the regulatory domain is I65T. This mutation is associated with non-PKU HPA to variant PKU. Ile65 is located in the hydrophobic core of the regulatory domain, and substitution into a polar threonine (or asparagine as in the I65N mutant) would result in a significant structural perturbation of the regulatory domain core. A frequent PAH mutation found in patients with PKU is A104D. This mutation is associated with variant PKU, and Ala104 is located in a loop between Ra2 and Rj4 in the regulatory domain. Substitution into a larger and charged residue may destabilize this loop structure. Consistent with this hypothesis, in vitro expression led to increased PAH aggregation and marginal activity.

Three residues in the active site that are located near the (putative) location of substrate binding or in the region near the catalytic iron are Phe331, Ala345, and Gly346. Phe331 π-stacks onto Trp326, a residue that may be involved in determining substrate specificity in the aromatic amino acid hydroxylases. Two PKU mutations have been reported for Phe331, F331C and F331L, and both of these mutations will remove important π-stacking interactions of the active site. Both Ala345 and Gly346 are located close to the triad of residues that bind the iron (His285, His290, and Glu330). Ala345 has 2 reported PKU mutations, A345S and A345T, and Gly346 has 1 mutation, G346R. All 3 of these mutations into larger and more polar residues would interfere with the location and electrostatics of the residues that act as iron ligands, thus destabilizing the active site structure. One additional residue that is important in holding the catalytic iron in place is Ser349, which hydrogen bonds to His285, one of the iron ligands. Two PKU mutations have been reported for this residue, one that results in classical PKU (S349P). This substitution into a proline so close to the catalytic iron will change the shape of the active site. As expected, expression of S349P hPAH in E coli or COS cells results in only <0.2 to <1% residual activity. The second mutation of Ser349, S349L, also has minimal activity in E coli or COS cells. Both mutations will alter the hydrogen bond to His285, having severe detriment on the PAH catalytic activity.

Arg252 forms a salt bridge to Asp129 in the Cα1 helix. In vitro expression of either of 2 PKU mutants (R243Q or R243X) in COS cells resulted in <10% residual activity.

Arg252 forms a salt bridge to Asp129 and hydrogen-bonds to the carbonyl oxygen of Ala131 as well as the side chain of Asp27 in the ARS. There are 3 PKU mutations associated with this residue: R252Q, R252G, and R252W. The R252W mutation results in classical PKU, and in vitro expression of the R252G and R252W mutations results in <1% residual activity. Recombinant expression of the R252Q mutation, however, results in somewhat larger residual activity (16% and 3%). Thus, any substitution at Arg252 results in disruption of stabilizing interactions in the catalytic domain.
imately 4 Å away from Leu311 and Leu308, which both hydrogen-bond to Arg408. These residues hold the tetramerization arm close to the catalytic domain, and thus substitution of Ala 259 into a larger or polar residue, as in A259T and A259V, cannot be accommodated. Both A259T and A259V hPAH when expressed in vitro result in 0.2% to 0.3% residual activity.27 One of the most frequent mutations in the PKU database is R261Q. This mutation results in phenotypes varying from variant PKU to classical PKU. Arg261 is located in a loop between helix Ca6 and Cβ2 in the catalytic domain, forming hydrogen bonds to Gln304 and Thr238, and substituting an arginine for proline (R261P) would destabilize the catalytic domain structure.

PAH Mutations Involved in Interdomain Interactions in a Monomer

The most important residues at the interface between the catalytic and tetramerization domains are Leu311, Leu308, and Arg408. Arg408, located in the loop between Ca12 and Tβ1, forms hydrogen bonds to the carbonyl oxygens of Leu308 (at the end of Ca8) and Leu311 (in the loop between Ca8 and Ca9). R408W is the single most frequent mutation in the PKU database, resulting in a severe PKU phenotype, with a low (<1% to <2.7%) recombinant PAH activity.41,42 Another mutation, R408Q, associated with an HPA phenotype, reportedly shows 55% residual PAH activity.43 Substitution of Arg408 into a larger and bulkier tryptophan would alter the hydrogen bonding network at the interface of the tetramerization and catalytic domains, interfering with the correct positioning of the β-ribbon (Tβ1 and Tβ2).

PAH Mutations in Residues That Interact With the N-Terminal ARS

Tyr377 is 1 of the residues in the catalytic domain that is in contact with the N-terminal ARS3 and hydrogen-bonds to Ser23 in the regulatory domain, along with stacking on top of Trp326 (a residue involved in determining substrate specificity for the aromatic amino acid hydroxylases). The proximity of Tyr377 to the ARS and the putative substrate binding site may regulate access to the active site. In the PAH PKU mutant Y377C, the hydrogen bond to Ser23 would be lost, altering access to the active site.

PAH Mutation in Residues at the Dimer or Tetramer Interfaces

Residues Ser67, Glu76, Arg297, Gln304, and Glu422 can be found at the dimer interface and have PKU mutations associated with them.13 Ser67 is located in β-strand Rβ2, forming a hydrogen bond to Tyr216 in another monomer; the PKU mutation S67P would alter the PAH structure at this interface. Glu76 is located in β-strand Rβ3 on the surface of the regulatory domain, hydrogen-bonding to His208 in another monomer, as well as Asn73 in the same monomer. The PKU mutations E76A and E76G are substitutions of a charged residue into surface-exposed hydrophobic residues, resulting in destabilization of the secondary structure in the regulatory domain. Arg297 is located in α-helix Ca8 and hydrogen-bonds to Arg71 as well as Glu422 in a second monomer. Arg297 has 2 PKU mutations, R297H and R297C,37 and both substitutions will result in disrupting the dimer-stabilizing hydrogen bonds, resulting in unstable PAH. Gln304 in α-helix Ca8 is also involved in dimer/tetramer interactions, forming hydrogen bonds to Tyr417 in a second monomer, and Arg261. Gln304 has 2 PKU mutations, a silent mutation (Q304Q)43 and Q304R; substitution of a polar glutamine to a charged arginine would alter both hydrogen bonds and destabilize PAH. Last, both Pro362 and Pro366 position Glu368, which mediate dimer contacts. Thus, the PKU mutations P362T44 and P366H45 will most likely prevent proper dimer formation.

POTENTIAL PKU/HPA THERAPEUTIC APPROACHES

In light of the difficulty of PKU/HPA patients in adhering to a restrictive diet for life,46,47 new and less restrictive therapies for PKU/HPA are needed. Because gene therapy solutions are many years off, alternative solutions such as enzyme replacement therapy using recombinantly produced PAH (in a truncated, monomeric, and constitutively active form) or the plant enzyme phenylalinine ammonia lyase may provide some relief to PKU/HPA Phenylalanine. For example, phenylalanine has been shown to lower l-Phe levels, as well as reduce the symptoms of HPA, in mice engineered with a model for HPA.48

BH4-RESPONSIVE HPA/PKU MUTATIONS

Recently, a number of patients harboring a subset of PAH mutations showed a marked lowering (normalization) of their blood l-Phe levels upon oral administration of the PAH co-factor BH4 (10–20 mg BH4/kg body weight).49,51 It is believed that this subset of mutations results in expressed mutant enzymes that are Km variants of PAH with an altered binding affinity for BH4. More and more PAH mutations are continuing to be reported as being responsive to BH4,50,51 and characterization of the biochemical, molecular, and physiologic bases for their BH4 responsiveness is required for a detailed understanding of this phenomenon.

Most of the BH4-responsive PKU mutations are in the PAH catalytic domain, either located in co-factor–binding regions (CBR 1 [residues 245–266], CBR 2 [residues 280–283], CBR 3 [residues 322–326], and CBR 4 [residues 377–379]) or in locations that directly interact with the CBR regions involved with co-factor binding. The following PAH genotypes were found to be responsive to BH4: P407S/R252W, IVS4–1g→a/A373T, R413P/R241C,52 IVS10nt-11g→a/E390G, A313T/1099insC, V190A/R243X, A300S/A403V, and R241C/A403V.54 These mutations are found in distinct regions of the PAH primary sequence (Fig 1) and tertiary structure of the composite model of hPAH (Fig 6).

Neither residue in the R252W/P407S genotype interacts directly with the co-factor, but Arg252 follows Ser251 and is located in CBR 1 (Figs 1 and 6). Ser251 is presumed to position BH4 by hydrogen bonding to the dihydroxypropyl side chain. As previously men-
tioned, Arg252 hydrogen bonds to residues Ala313, Asp315, and Asp27 in the ARS. The second allele residue in the R252W/P407S genotype, Pro407, precedes the start of the tetramerization domain. Mutation into a less rigid serine residue might inhibit PAH tetramerization; however, dimeric or monomeric P407S mutant PAH might still retain co-factor binding affinity and activity. Kure et al52 also reported a non–BH4-responsive patient, having a P407S/R111X genotype. The R111X mutation results in a truncated PAH lacking enzymatic activity. Therefore, enzymatic activity for this second genotype must be attributable to the P407S PAH. On the basis of these observations, BH4 responsiveness is dependent on the specific PAH genotype.

The R413P/R241C genotype was also responsive to co-factor loading.52 Arg241 is located on the surface of the catalytic domain preceding 1 of the CBRs, at the start of Cβ1. In the tetrameric structure of PAH, Arg241 is hydrogen-bonded to Gln419 of the tetramerization domain, participating in important intradomain interactions.2 Arg413 is located in the tetramerization domain and hydrogen-bonds to Glu422, forming 1 of a few hydrogen bonds that hold the β-ribbon formed by Tβ1 and Tβ2 together. This interaction ensures proper tetramer formation by positioning the 40-Å-long tetramerization domain α-helix for intermonomer coiled-coil interactions. Three PKU mutations have been found for Arg413: R413S, R413C, and R413P. The R241C mutation, as a result of the mild effect of this mutation on the PAH structure, as compared with the R413P mutation, must be responsible for the BH4 responsiveness of the R413P/R241C genotype.

Trefz et al53 reported a patient with PKU who was responsive to BH4 oral loading, having an IVS10nt-11g-->a/E390G genotype. The latter mutation, E390G, must be responsible for the BH4 responsiveness, because the other mutation leads to a splicing defect in intron 10 of the PAH gene, with no functional protein expressed. The E390G PAH mutation shows 70% residual activity when expressed in COS cells (PKU database). In the structure of tetrameric PAH, Glu390 is located on the surface in the catalytic domain, following 1 of the CBRs (CBR 4)2 (Figs 1 and 6). The Glu390 side chain points toward the catalytic domain of a second monomer and also toward the tetramerization interface. Because Glu390 is located on the surface, mutation into a flexible glycine may destabilize the PAH tetramer and reduce enzymatic activity somewhat. The E390G mutant PAH most likely retains co-factor binding ability but with an increased Km; however, this hypothesis must still be experimentally confirmed.

Another BH4-responsive genotype reported by Kure et al52 is IVS4–1g-->a/A373T, with a mild HPA phenotype. The first mutation affects intron 4, resulting in nonfunctional protein expression. Ala373 forms hydrophobic interactions with helix Ca9 containing Tyr325, Trp326, and Ala322 (located in CBR 3), which hydrogen-bonds to the dihydroxypropyl side chain of the co-factor. An A373T substitution might be relatively easily accommodated in this region of the structure and might result in minimal structural perturbation of the Ca9 helix and the active site. Thus, this A373T PAH retains some binding affinity for BH4, providing for the observed BH4 responsiveness.

The variant HPA genotype A313T/1099insC was also found to be responsive to BH4 loading.54 The
A313T allele is most likely BH₄ responsive, because the 1099insC mutation results in a frameshift after Leu367, but this frameshift mutation has all of the residues needed for catalysis and so might retain some enzymatic activity. Unfortunately, no expression information for either of these mutants exists. Ala313 hydrogen-bonds to Arg252 (in CBR 1) and is located in a loop between helices Ca8 and Ca9, at the interface with the regulatory domain (close to Pro119). The effect of the A313T substitution on co-factor binding might not be too large because the residue preceding Ala313 is a glycine, which might compensate for a distortion in the backbone imposed by a threonine mutation at Ala313.

Genotype V190A/R243X also displays variant HPA, and the BH₄-responsive mutation must be V190A, because the R243X mutation results in a truncated enzyme missing all of the catalytically required residues. Val190 is located in helix Ca3, behind His285, and close to Cys284 and Arg270. Substitution into an alanine at position 190 would create space where the valine side chain was originally located. The mutant enzyme might have Cys284 (located at the end of CBR 2 [residues 280–283]) and His285 rearranged into the empty space created by the V190A substitution. These alterations to the co-factor binding site might require higher concentrations of co-factor for productive catalysis, consistent with the BH₄ responsiveness observed.

Variant HPA genotypes A300S/A403V and R241C/A403V both were found responsive to BH₄ loading. Both genotypes contain the A403V allele. Ala403 is located at the end of helix Ca12, close to Ala309 in helix Ca8. Alanine or another smaller residue might be necessary for close packing of helices Ca8 and Ca12. Thus, substitution into a larger valine as in A403V PAH might result in a less stable protein. The R241C/A403V genotype also shares 1 allele with R413P/R241C, and in this genotype, the R241C mutation was presumed to be responsible for the BH₄ responsiveness, making the R241C allele the responsive mutation for the R241C/A403V genotype as well. In A300S/A403V, Ala300 is located in the middle of helix Ca8, lining the bottom of the active site. Ala300 does not form any hydrogen bonds, so substitution into a serine might be allowed, with minor structural perturbations. The closest residue to Ala300 is Arg261, located in CBR 1 (residues 245–266). Substitution of Ala300 into a slightly larger serine could alter the position of Arg261, altering the co-factor binding site. Thus, the BH₄ binding site in A403V PAH might be only slightly different as compared with the wild-type PAH structure, explaining the BH₄ responsiveness of this genotype.

BH₄ oral therapy has the potential to become more widely used, provided the cost of purchasing the necessary large amounts of the co-factor does not continue to be an issue. It remains to be seen exactly what percentage of patients with PKU/HPA will continue to be an issue. It remains to be seen exactly what percentage of patients with PKU/HPA will continue to be an issue. It remains to be seen exactly what percentage of patients with PKU/HPA will continue to be an issue.

**CONCLUSIONS**

Crystal structures of different truncated forms of human and rat PAH have allowed for the construction of a composite structural model for full-length, tetrameric PAH and provided a structural basis for the numerous mutations resulting in deficient PAH activity. In addition, PAH crystal structures with substrate analog, inhibitor, or co-factor bound at the active site have provided details of ligand binding. This structural information has helped formulate rules that may aid in predicting the likely effects of unclassified or newly discovered PAH mutations. Also, with the aid of recent crystal structure determinations, recently discovered BH₄-responsive PKU/HPA genotypes can be mapped onto PAH structure, providing a molecular basis for this BH₄-dependent response. However, more structural work is needed, such as determination of the structure of full-length PAH, as well as more site-directed mutagenesis studies of PAH, to understand completely the catalytic mechanism and substrate specificity of PAH and tyrosine hydroxylase/trpoyphan hydroxylase, the other members of the aromatic amino acid hydroxylases.

**ACKNOWLEDGMENTS**

This study was funded by National Institutes of Health grant no. RO1-HD38718 from the National Institute of Child Health and Human Development (Bethesda, MD).

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Structural Studies on Phenylalanine Hydroxylase and Implications Toward Understanding and Treating Phenylketonuria
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