Structural and functional analyses of mutations of the human phenylalanine hydroxylase gene

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Abstract

Background: Phenylketonuria (PKU) is an inborn error of metabolism that results from a deficiency of phenylalanine hydroxylase (PAH). We demonstrated PAH mutational spectrum from patients with PKU, including 10 novel and 3 tetrahydrobiopterin (BH₄)-responsive mutations. In this study, 11 PAH missense mutations, including 6 novel mutations (P69S, G103S, L293M, G332V, S391I, A447P) found in our previous study, 2 mutations common in east Asian patients with PKU (R243Q, R413P), and 3 tetrahydrobiopterin (BH₄)-responsive mutations (R53H, R241C, R408Q) have been functionally and structurally analyzed.

Methods: A transient protein overexpression system and an in vitro BH₄-responsiveness study were used. The effects of PAH missense mutations on the PAH protein structure were also analyzed. To determine the conservation of 12 mutated residues, PAH was aligned using BLAST against full genomic sequences of 221 different species. Model structures of PAH protein and the composite tetramer were constructed using the software program, SHEBA.

Results: No PAH activity was detected for some mutants. However, the residual activities associated with other mutants ranged over a wide spectrum. The missense mutations responsive to BH₄ were not highly conserved throughout the 43 species in the multiple sequence alignment that encode PAH. The composite model structure of PAH revealed that dimer stability was reduced in the BH₄-responsive mutants, whereas tetramer stability remained normal.

Conclusion: This expression study analyzed PAH mutations and model structures of mutant PAH proteins are proposed. Correlation between the proposed mutant PAH structures and functions are suggested.

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1. Introduction

Phenylketonuria (PKU; MIM 261600) is an autosomal recessive metabolic disease caused by a deficiency of phenylalanine hydroxylase (PAH; EC 1.14.16.1). PAH is a hepatic enzyme that catalyses the hydroxylation of L-phenylalanine (L-Phe) to L-tyrosine using tetrahydrobiopterin (BH₄) as a cofactor [1]. This reaction is the rate-limiting step in the catabolic pathway of phenylalanine, which results in the complete degradation of the amino acid.

The PAH gene spans about 90 kb on chromosome 12q and comprises 13 exons. It has 3 structural domains consisting of an N-terminal regulatory domain (residues 1-142), a central catalytic domain (residues 143-410), and a C-
terminal tetramerization domain (residues 411-452) [2]. The active PAH enzyme is composed of 4 monomeric proteins. Recent studies of PAH crystal structure have provided information on the active site and the binding sites of its substrate and cofactor.

More than 490 different mutations of the PAH gene have been reported (PAH Mutation Analysis Consortium Database, http://www.mcgill.ca/pahdb). The mutation profile of the PAH gene is not restricted to any one region, but is spread throughout the structural domains. The mutations lead to a variety of clinical and biochemical phenotypes with different degrees of severity, from mild hyperphenylalaninemia to classical PKU [3]. Several studies have investigated the relationship between genotype and phenotype.

To investigate the effects of BH4 on mutant PAH enzyme activities on PubMed (http://www.ncbi.nlm.nih.gov/PubMed/). Mutations were defined by exclusion from the PAHdb in east Asian patients with PKU (R243Q, R413P), and 3 L293M, G332V, S391I, A447P), two mutations common [16], including six novel mutations (P69S, G103S, base dietary and therapeutic strategies.

We investigated the enzyme activities of 11 PAH missense mutations identified in Korean PKU patients [16], including six novel mutations (P69S, G103S, L293M, G332V, S391I, A447P), two mutations common in east Asian patients with PKU (R243Q, R413P), and 3 BH4-responsive mutations (R53H, R241C, R408Q), using transient protein overexpression in mammalian cells. Novel mutations were defined by exclusion from the PAHdb (http://www.pahdb.mcgill.ca) and previously reported mutations on PubMed (http://www.ncbi.nlm.nih.gov/PubMed/).

To investigate the effects of BH4 on mutant PAH enzyme expression and activity in mammalian cells, we analyzed BH4 responsiveness in vitro using the natural cofactor BH4 ([6R]-5,6,7,8-tetrahydro-L-bioterpin dihydrochloride). The effects of PAH missense mutations on the protein structure were also analyzed.

2. Materials and methods

2.1. Construction of hPAH expression plasmids and mutagenesis

The full-length human PAH cDNA, phPAH247 (American Type Culture Collection, Manassas, VA) was digested with the restriction endonucleases EcoRI and NheI and ligated into pcDNA3.1(+) vector digested with EcoRI and XbaI. For mutagenesis, PAH cDNA was subcloned into the EcoRI and ApaI sites of the pBlueScript II SK(−) phagemid vector and mutagenized with the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). After DNA sequence verification of the desired mutations, all mutant PAH cDNAs were subcloned from pBlueScript II SK(−) into pcDNA3.1(+).

2.2. Transient eukaryotic expression of PAH

To achieve 60–80% confluence at the time of transfection, COS-7 monkey kidney cells were plated 1 day before transfection in Dulbecco’s Modified Eagle’s Medium (Invitrogen, San Diego, CA) supplemented with 10% heat-inactivated fetal bovine serum. Cells were transfected in 60 mm plates with 4 µg of wild-type or mutant pcDNA3.1(+)–PAH, using Lipofectamine Plus Reagent (Invitrogen). For the negative control, COS-7 cells were transfected with the pcDNA3.1(+) vector, the parent plasmid lacking the PAH cDNA insert. All transfections included 1 µg of the pcDNA3.1/His/LacZ vector (Invitrogen) as a control for transfection efficiency. Cells were harvested for RNA and protein extraction 48 h after transfection. A portion of the cells was lysed in Reporter Gene Assay Lysis Buffer as described in the β-Gal Reporter Gene Assay (Roche Applied Science, Mannheim, Germany), and the total protein concentration was determined with the Bradford assay (Bio-Rad, Munich, Germany). β-Galactosidase activity was measured using the β-Gal Assay Kit (Invitrogen) [17].

2.3. PAH activity assays

Enzyme activity was assayed by measuring the conversion of L-[14C]phenylalanine to L-[14C] tyrosine at 37 °C in a volume of 50 µl. The crude extract (100 µg) was incubated for 5 min in the presence of 0.2 µCi of L-[14C]Phe (460 µCi/mmol; Amersham, Buckinghamshire, UK), 0.25 mM L-phenylalanine, 40 units of beef liver catalase, and 250 mmol/l Tris-HCl (pH 7.8). All products were from Sigma (Sigma-Aldrich, Milano, Italy). After 5 min, the reaction was initiated by the addition of BH4 (Schircks, Jona, Switzerland) to a final concentration of 0.8 mmol/l. The reaction was stopped after 60 min by the addition of 5 µl of carrier phenylalanine/tyrosine (0.1 mol/l each) and incubation in boiling water for 5 min. Samples were then centrifuged at 12,000 ×g for 5 min and 15 µl of the supernatant was spotted onto a thin-layer chromatography (TLC) plate (TLC Silica Gel 60 plates, Merck, Darmstadt, Germany). TLC was performed using a mixture of chloroform: methanol: ammonia (55:35:10) for 60 min and the plate was dried for 10 min. The radioactivity of L-[14C]phenylalanine and converted L-[14C]tyrosine was quantified using ImageQuant after visualization with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The residual activities of mutant PAH enzymes were expressed as a percentage of the wild-type PAH enzyme activity and normalized to transfection efficiencies based on
β-galactosidase activities. All the PAH mutants were transfected 3 times, enzyme activities were also measured 3 times, and the mean enzyme activities were calculated.

2.4. In vitro BH₄-responsiveness assay

To test the in vitro BH₄ responsiveness of the wild-type and mutant PAH, we simultaneously transfected COS-7 cells with 4 μg of wild-type or mutant pcDNA3.1(+)–PAH in 2 60 mm dishes. All transfections included 1 μg of pcDNA3.1/His/LacZ vector as a control for transfection efficiency. Five hours after transfection, the culture media were replaced with fresh DMEM with or without 1 mmol/l BH₄. The cells were harvested 48 h after transfection and PAH enzyme activities were measured as described above.

2.5. Western blot

Fifty micrograms of the same crude extracts analyzed for both PAH and β-galactosidase activities were resolved electrophoretically on 10% polyacrylamide gel and blotted onto polyvinylidene difluoride membrane (Invitrogen). The membrane was hybridized with a 1:200 dilution of murine anti-PAH antibody (PH8; Immunocor; San Diego, CA), and subsequently with a horseradish-peroxidase-conjugated anti-mouse-IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The signals were then visualized using ECL Plus enhanced chemiluminescence (Santa Cruz Biotechnology).

2.6. RT–PCR analysis

Total RNAs were isolated using an RNeasy Mini kit (Qiagen GmbH, Hilden, Germany). RT reactions were conducted using 100 pmol/μl random primer (Takara Shuzo, Kyoto, Japan). The PCR amplification of cDNAs was performed on each RT sample and a no-template control using the GeneAmp PCR System 9700 (Applied Biosystems). To specifically detect PAH transcripts, the following primers were used at 10 pmol: forward primer, 5′-CTTGTA-TAAAACCCATGCTTGCT-3′; reverse primer, 5′-GTAA-TTCACCAAAGGATGACAGG-3′. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was amplified as an internal control: forward primer, 5′-AGACAA-GATGGTGAAAGTCG-3′; reverse primer, 5′-TCATG-AGCCCTTCCACGATG-3′. After PCR amplification, samples were resolved electrophoretically on 1.5% agarose gels and visualized with ethidium bromide.

2.7. Composite model structure

No crystal structure of PAH with 3 key domains (regulatory, catalytic, and tetramerization) had been deposited in the PDB database as of January 2005. Therefore, initially, a model structure of the PAH protein was derived from the 3-dimensional structures of a rat tyrosine hydroxylase (2toh) and a rat phenylalanine hydroxylase (2phm); 2toh contains catalytic and tetramerization domains and 2phm contains catalytic and regulatory domains. The 2toh protein was structurally aligned to 2phm and the coordinates of 2toh were transformed with the structural alignment program, SHEBA [18]. The sequence identity and C-α-atom root–mean–square deviation (cRMS value) after their superposition were 59% and 0.9 Å, respectively. A PAH composite model structure with 3 key domains was then constructed, and the mutation-affected residues were refined using the modeling package GEMM [19]. The composite model structure of the tetramer was then constructed to estimate the functional consequences of the mutations.

2.8. Conserved residues

To determine the conservation of 12 mutated residues, PAH was aligned by BLAST against the full genomic sequences of 221 different species [20]. Only the alignment with the highest score was selected for each genomic sequence. Only 43 of the 221 different genome sequences examined encoded PAH-like proteins on the basis of an E-value cutoff of 10⁻⁵.

3. Results and discussion

PAH enzyme activities encoded by the 11 PAH mutants ranged from 0% to 118% (Table 1, Fig. 1). The expression levels of the mutant enzymes R53H, P69S, G103S, R408Q, and R413P, detected by Western blot, were similar to the wild-type expression, at 79%, 52%, 39%, 118%, and 66%, respectively. In the mutants R241C, R243Q, L293M, and

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<th>Mutations</th>
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<td>A447P</td>
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*expressed as a percentage of wild-type PAH activity; **expressed as a percentage of BH₄-untreated wild-type PAH activity; WT, wild-type PAH control; NC, negative control; BH₄(−), No BH₄ in culture medium; BH₄(+), 1 mmol/l BH₄ in culture medium 5 h after transfection. †All the PAH mutants were transfected 3 times, enzyme activities were also measured 3 times, and mean enzyme activities were calculated.
A447P, the expression of mutant enzymes was reduced relative to that of the wild-type, with enzyme activities of 28%, 32%, 41%, and 8%, respectively. The mutant enzymes G332V and S391I were not detected by western blot, and consequently no residual enzyme activities could be measured. The enzyme activities of the BH₄-responsive PAH mutants were 28–118% (79% in R53H, 28% in R241C, 118% in R408Q).

Compared with cells expressing the wild-type PAH protein, enzyme assay revealed decreased PAH activities in the lysates of cells in which various missense mutant proteins were expressed. The activity of some mutants was undetectable, meaning that they were effectively <1% of wild-type activity. In contrast, the residual activities associated with other mutants ranged over a wide spectrum. Measurement of immunoreactive PAH protein almost always indicated a corresponding decrease in the protein level (Fig. 1A). Therefore, the specific activity of most mutant PAH proteins did not differ from that of the wild-type. Quantitation of PAH mRNA levels in the cell lysates revealed no differences between the wild-type and mutant forms (Fig. 1B). Therefore, mutation effects at the RNA level do not explain the decreased levels of PAH protein. This triad of characteristics has typified increasing numbers of missense mutations for over a decade and it has usually been ascribed to the instability of the mutant protein. However, “instability” is a loose term that begs several questions.

Wild-type PAH treated with 1 mmol/l BH₄ showed increased enzyme activity (161%) with no increase in PAH protein (Table 1, Fig. 2). COS-7 cells showed neither endogenous PAH protein expression nor enzyme activity. When treated with 1 mmol/l BH₄, clinically proven BH₄-responsive PAH mutants showed increased PAH enzyme activity (R53H, 63% → 161%; R241C, 26% → 38%; R408Q, 129% → 306%). Mutants P69S (45% → 60%), G103S (16% → 24%), R243Q (17% → 36%), L293M (55% → 57%), R413P (51% → 95%), and A447P (6% → 24%) also showed increased PAH enzyme activity.
Mutant G332V treated with 1 mM BH4 showed neither PAH protein expression nor enzyme activity. However, mutant S391I showed increased PAH protein expression and enzyme activity when treated with 1 mM BH4. For R413P mutation, Shintaku et al. described that it is one of severe PAH mutations detected in classical PKU, result in non-functional PAH allele [21], however PAH enzyme activity was increased when treated with BH4 in our study. It suggests that even patients with classical PKU can be treated with BH4 supplementation.

Several studies have investigated the molecular mechanisms underlying BH4 responsiveness and several mechanisms have been proposed: (i) decreased affinity for BH4 binding, (ii) stabilization of the active tetrameric or dimeric forms of the mutant proteins and protection from proteolytic cleavage, (iii) BH4-induced changes in BH4 biosynthesis, and (iv) PAH mRNA stabilization [11]. Thöny et al. reported that BH4 has a chaperon-like effect on PAH synthesis and/or is a protecting cofactor against enzyme auto-inactivation and degradation without affecting gene expression or PAH mRNA stability [22]. Particular amino acid residues may have key roles in the correct folding of transient intermediates en route.

The functional effects of 12 mutations found in the PAH gene in the Korean population [16] were determined and explored by multiple sequence alignments and the construction of a composite model of PAH. In the sequence analysis, all the eukaryotic PAH-like proteins contained regulatory, catalytic, and tetramerization domains, whereas all prokaryotic proteins contained only one catalytic domain (Fig. 3). This suggests an enhanced functional complexity during the evolution of these proteins from prokaryotes to eukaryotes. Of the 12 missense mutations we describe here, we previously found 3 (R53H, R241C, R408Q) to be responsive to BH4 [16]. Interestingly, these 3 mutations are not highly conserved throughout the 43 species that have at least one PAH protein (Fig. 3). Moreover, they are located at opposite sides of the BH4-binding pocket. Specifically, the composite model structure of the PAH tetramer reveals that these 3 mutations are in close proximity to the dimer interface (Fig. 4). Each of the 4 tetramerization domains (residues 428-425) forms a coiled-coil helical complex, and


\[ rH = \sum_{i=1}^{k} \sum_{j=1}^{j} q_{ij} s_{ij} \]

where RH is the relative entropy [27], k is the number of proteins in the multiple alignment, q_{ij} and s_{ij} are normalized frequency and BLOSUM62 scores, respectively, of amino acid pair types, i and j, found at each residue position in the multiple alignment. With an RH value of less than 1, the amino acid is not conserved; with an RH value of 1–4, it is conserved; and with an RH value of 4 or higher it is highly conserved.
once these complexes are formed, the 4 monomers adopt a two-fold symmetry whereby two regulatory domains face each other in a dimer and the two dimers form a tetramer.

The R53H, R241C, and R408Q mutations occur along the interface region of the regulatory domain (Fig. 4). This observation suggests that upon mutations of R53H, R241C
or R408Q, dimer stability is reduced. Increased BH4 levels rescue dimer stability and, consequently, enzyme activity also recovers to normal.

Other mutations, P69S, R243Q, S391I, R413P, and A447P, are also located at the interface region of the 2 dimers (Fig. 4). Of these, the S391I mutant showed no activity in an in vitro experiment. However, the introduction of the cofactor BH4 induced the recovery of the activity of some proteins (2%) that had been undetectable by immunoassay in the absence of BH4. The enzyme tyrosine hydroxylase (TyrOH) is very similar to PAH. The crystal structure of TyrOH has been reported to have chloride ions bound to Ser437 and Lys120 [23]. The corresponding residue positions in PAH are Ser391 and Lys74. When the PAH and TyrOH structures are superimposed with SHEBA [18], the cRMS value is about 0.9. In particular, the environment in which the chloride ion binds is very similar within the two structures, implying that they share a cofactor or have related chemical binding affinities. However, the substrate-binding affinities of PAH and TyrOH are reported to be quite different, thereby preventing unwanted diversions [23]. There are several similar examples of chloride ions that are coordinated by a polar group to stabilize coiled-coil trimers [24]. Considering these published data, it is plausible that mutations from polar to nonpolar amino acids at the surface (S391I) may disrupt the coordination of chloride ions as well as the tetramerization of PAH (Fig. 5).

The R243Q mutation is located in the surface region and affects a hydrogen bond between the amide nitrogen...
of Arg243 and the carbonyl oxygen of Asp129. The distance between the amide nitrogen of Arg243 and the carbonyl oxygen of Asp129 is normally 2.4 Å. Following substitution of Arg243 with Glu243, however, the minimum distance between Glu243 and Asp129, obtained by rotating the side chain of Glu243, is 4.5 Å. This distance is too wide to permit hydrogen-bond formation. The functional effect of the R243Q mutation at the protein surface is ambiguous, because some mutations are deleterious, whereas others are well tolerated [25]. However, when Arg243 is hydrogen bonded to other residues and the introduction of a mutation disrupts the side-chain hydrogen bonding, enzyme stability can be reduced (Fig. 6). In our study, the enzyme activity of the R243Q mutant was 30%, so our findings concur with general observations. Moreover, our multiple sequence alignment indicated that residue positions G332 and G344 are highly conserved in the 43 species that have at least one PAH protein (Fig. 3). Specifically, the novel mutation G332V, identified in our study, is located in a region within the secondary structure that corresponds to a direct transition from α-helix to β-strand without any coiled state, suggesting a limited degree of conformational freedom (Fig. 7). Therefore, if any residue other than the native Gly332 is located at this position, structural stability will be compromised.

Another 2 mutations, T278I and L293M, affect cofactor- and substrate-binding residues, respectively. A hydroxyl group hydrogen atom of Thr278 is bound to a carbonyl oxygen of the cofactor BH4 and this mutation from a polar to a nonpolar amino acid disrupts this hydrogen bonding, resulting in unfavorable ligand-binding affinity (Fig. 8). In the mutant carrying the T278I substitution, in vitro enzyme

**G332V**

![Fig. 7. View of G332V in a limited conformational space.](image)

**T278I**

![Fig. 8. View of cofactor analogue (THI) binding in T278I.](image)
activity was zero. With regard to mutation L293M, although Leu293 is located in the hydrophobic core of PAH near the substrate-binding site, the composite model structure indicates that it is not directly involved in substrate binding. Furthermore, PROSITE [26], a functional motif search tool, detected a motif with a strong hydroxylation signature between residues 281 and 292. Multiple alignments of all PAH-protein-containing genomes revealed that 3 residues, D282, H285, and H290, were 100% conserved within this motif (Fig. 3). Even though the L293M mutation is located just outside this motif signature region (281-292), it is evident that changing the hydrophobic core of Leu293 disrupts the substrate-binding affinity because enzyme activity was nearly zero in this mutant.

In summary, we investigated the enzyme activities of PAH missense mutants, including 3 BH4-responsive mutants. Although the activities of some mutants were undetectable in a transient protein expression system, the residual activities associated with other mutants ranged over a wide spectrum. The missense mutations responsive to BH4 are not strongly conserved throughout the 43 species that have at least 1 PAH protein identified in multiple sequence alignments. The composite model structure of PAH revealed that dimer stability of the BH4-responsive mutants is reduced, whereas tetramer stability remains normal. This result suggests that increased BH4 levels rescue dimer in stability and, consequently, enzyme activity is restored to normal. Further studies to validate these structural and functional analyses of BH4 responsiveness in PAH mutants should facilitate the development of new therapeutic strategies for patients with hyperphenylalaninemia.

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References


