Five human phenylalanine hydroxylase proteins identified in mild hyperphenylalaninemia patients are disease-causing variants
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Five human phenylalanine hydroxylase proteins identified in mild hyperphenylalaninemia patients are disease-causing variants

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Key words: PKU, PKU mutations, PKU mutations functional analysis, expression studies of PKU mutants, structural analysis.
ABSTRACT

Hyperphenylalaninemia is a group of autosomal recessive disorders caused by a wide range of phenylalanine hydroxylase (PAH) gene variants. To study the effects of mutations on PAH activity, we have reproduced five mutations (p.N223Y, p.R297L, p.F382L, p.K398N and p.Q419R) that we recently identified in a population of Southern Italy. Transient expression of mutant full-length cDNAs in human HEK293 cells yielded PAH variants whose L-phenylalanine hydroxylase activity was between 40% and 70% that of the wild-type enzyme. Moreover, Western blot analysis revealed a 50-kD monomer in all mutants thereby indicating normal synthesis of the mutant proteins. Because of the clinical mild nature of the phenotypes we performed an in vivo BH4 loading test. This was positive in all tested patients, which indicates that they are likely to respond to the coenzyme in vivo. We also analysed the environment of each mutation site in the available crystal structures of PAH by using molecular graphics tools. The structural alteration produced by each mutation was elucidated and correlated to the mutated properties of the mutant enzymes. All the data obtained demonstrate the disease-causing nature of the five novel variants.
1. INTRODUCTION

Hyperphenylalaninemia (HPA; OMIM 261600) is the most frequent disorder of amino acid metabolism and is mostly due to recessive mutations in the gene that encodes phenylalanine hydroxylase (PAH: EC 1.14.16.1) [1-3]. This enzyme, which is mainly expressed by the liver, converts L-phenylalanine to L-tyrosine in the presence of the essential cofactor tetrahydrobiopterin (BH4). Few HPA cases are due to defects in the biosynthesis or regeneration of BH4 [4].

HPA phenotypes are classified as follows: mild hyperphenylalaninemia (MHP or HPA III) when plasma Phe levels of untreated subjects are between 120 and 600 µM; mild phenylketonuria (MPKU or HPA II) when untreated plasma Phe levels are between 600 and 1200 µM; and classical phenylketonuria (PKU or HPA I) when untreated plasma Phe levels are >1200 µM (http://www.bh4.org/BH4_Deficiency_Nomenclature.asp). HPA patients with a Phe plasma level > 360 µM must adhere to a Phe-restricted diet throughout their lives to prevent the neurotoxic effects of Phe and its metabolites. An increasing number of PAH patients have been shown to benefit from BH4 supplementation, which in several cases can substitute dietary restrictions [5-7]. Patients who may benefit from BH4 supplementation can be identified with the BH4 loading test and by the presence of mutations found to be associated with the BH4 responsive phenotype [2, 8-10].

To date, more than 500 gene variants have been identified in the PAH gene (PAHdb Mutation Analysis Consortium database, at: www.pahdb.mcgill.ca) [11] that contains 13 exons spread over 80 kb. A number of PAH mutations are associated with a specific metabolic phenotype. For example, some "mild" PAH mutations reduce the affinity of the enzyme for BH4 and cause the BH4-responsive phenotype [12, 13]. Data regarding the phenotypic impact of other mutations are either lacking or controversial (http://www.pahdb.mcgill.ca). This is particularly the case of novel mutations, which are also difficult to identify as disease-causing mutations because of the lack of functional analyses [14].

PAH mutations are very heterogeneous in Italy. We recently identified 60 different gene variants in 126 PAH patients from Southern Italy. We described ten novel mutations, four of which are disease-causing (two nonsense, one splicing and one deletion). The others were missense mutations and we suggested they may be disease-causing [15].

Other investigators have elucidated the 3D structure of the PAH enzyme in the bound and unbound states [16-18]. Human PAH has a tetrameric architecture with each subunit consisting of three domains: an N-terminal regulatory domain (residues 1-142) containing an α/β sandwich, a large catalytic domain (residues 143-410 with an α/β fold), and a C-terminal
domain that is responsible for tetramerization (residues 411-452). The PAH tetramer is a dimer of dimers, with the tetramer core formed by four α-helices (one from each subunit) tightly packed in an antiparallel coiled-coil motif. Structural investigations of disease-causing mutant enzymes have often revealed correlations between the 3D structure and the impaired functioning of PAH in PKU [19-22].

In this study, we expressed five proteins, each containing one of the five novel mutations (p.N223Y, p.R297L, p.F382L, p.K398N, p.Q419R) previously identified by our group, to investigate the in vitro PAH activity of the mutant enzymes. We also studied the BH4 response in vivo using the BH4 loading test in patients bearing these mutations. In addition, we analyzed the structural perturbations induced by the mutations within the framework of PAH crystal structures.

2. MATERIALS AND METHODS

2.1 Mutagenesis
PAH mutant constructs were derived from the wild-type PAH expression plasmid pcDNA3, kindly provided by Prof. P. Knappskog (University of Bergen, Norway) and Prof. P. Waters (McGill University-Montreal Children’s Hospital Research Institute, Montreal, Canada). Each mutation was introduced into the wild-type expression plasmid using mutagenic primers and the Transformer II kit (Clontech, Palo Alto, CA). The resulting clones were sequenced to verify the introduction of each single mutation.

2.2 Expression studies
Ten micrograms of wild-type and mutant cDNA expression vectors were introduced into 1.6 x 10^6 of human HEK293 cells using calcium phosphate (ProFection® Mammalian Transfection System-Calcium Phosphate, Promega Italia, Milan, Italy). Forty-eight hours after transfection, the cells were harvested by trypsin treatment, washed twice with 150 mM NaCl, resuspended in the same buffer and frozen-thawed 6 times. All transfections were performed in triplicate. Each triplicate was assayed for total protein content using a protein assay kit (Biorad, Richmond, CA, USA). Parental vector pCDNA3, without insert, was transfected as negative control. We co-transfected 10 μg of a construct carrying a β-galactosidase reporter gene as a control for transfection efficiency. Forty-eight hours after transfection, total RNA was isolated using a standard protocol and RT-PCR analysis was performed using specific primers; the resulting cDNAs were sequenced.

2.3 Production of polyclonal antibody and Western blot analysis
A synthetic peptide, based on the human PAH amino acid region (NH$_2$-Phe-Gly-Leu-Gly-Arg-Lys-Leu-(Ser-Phospho)-Asp-Phe-Gly-Gln-Glu-Thr-Ser-Tyr-Ile-Glu-Asp-Asn-Cys-COOH), was used to immunize rabbits. PAH antiserum was precipitated with ammonium sulphate and the antibodies were purified on a protein A-Sepharose column. Immunoblotting experiments were performed using 10 µg of protein extracts electrophoresed on a 10% SDS-PAGE gel as previously reported [23].

2.4 Enzyme analysis
For each transfection, PAH activity was assayed on 50 µg of proteins, in duplicate, as previously reported [24]. This test measures the amount of $^{14}$C radiolabeled Phe converted to Tyr that were subsequently separated by thin-layer chromatography (TLC). Enzyme activity of the wild-type and mutant PAH constructs was measured; the mean PAH activities was calculated from the three sets of transfection data. The residual activities of mutant PAH enzymes were expressed as a percentage of wild-type enzyme activity and normalized to transfection efficiencies based on replicates β-galactosidase activities.

2.5 BH4 loading test
Patients (legal tutors for minors) provided informed consent to the BH4 loading test, which was performed according to international guidelines [12]. Briefly, a watery solution of 20 mg/body kg of (6R)-5,6,7,8-Tetrahydro-L-Biopterin (Schircks Laboratories, Jona, Switzerland) was given orally 30 min before feeding. Blood samples in EDTA were drawn 0, 2, 4, 6 and 8 hours later, and Phe serum concentration was measured. Patients ate as usual during the test. A patient is considered “responder” if the Phe serum concentration decreases more than 30% 8 hours after BH4 load, and “fast responder” if the decrease occurs after 2 hours.

2.6 Structural analysis
The effect of the mutations on the 3D-structure has been investigated by analysing the structural environment of each substituted residue in the PAH crystal structures deposited in the Protein Data Bank (PDB). Human PAH is a homotetramer with each subunit consisting of three domains. A dimeric form also exists in equilibrium with the tetrameric form. Since no crystal structure of any full-length tetrameric/dimeric PAH is available, different models have been used to study the isolated catalytic domain (human PAH; PDB code 1kw0), the contact interface between the catalytic and regulatory domain (rat PAH; PDB code 1phz) and the oligomeric interfaces (human PAH; PDB code 2pah). A composite full-length monomeric
model was built by superimposing the respective catalytic domain regions (Figure 1A). The notation for the secondary structure elements mentioned in the text refers to the assignment by Erlandsen and Stevens [19].

3. RESULTS
3.1 Mutation features and structural localization
Table 1 shows the PAH genotype, clinical and biochemical classification of the five HPA patients bearing on allele A2 the PAH missense mutations p.N223Y, p.R297L, p.F382L, p.K398N, and p.Q419R. All patients were classified as HPA III; their serum Phe levels at diagnosis ranged between 182 and 242 µM. Table 2 shows the main features of the five mutations. Some nucleotide changes involve a codon that contains other previously reported gene variants (http://www.pahdb.mcgill.ca).

As shown in Figure 1A, four of the five mutations are located in the catalytic domain (p.N223Y, Figure 1C; p.R297L, Figure 1D; p.F382L, Figure 1E; p.K398N, Figure 1F) and the remaining one in the tetramerization domain (p.Q419R, Figure 1G). To evaluate the structural implications of the missense mutations, we examined the 3D-localization of the substituted residues in detail, taking into account the full tetramer (Figure 1B). The structural alterations of the mutation sites are summarized in Table 2.

The c.667A>T nucleotide transversion changes the polar Asn residue at position 223 into the polar, aromatic Tyr residue (p.N223Y). Asn223 is a moderately conserved amino acid. Mutation p.R297L was identified in cis with mutation p.A403V (Table 1), which is widely found in PAH patients [15]. The c.890G>T nucleotide transversion changes the charged Arg residue at position 297 into the apolar, aliphatic Leu residue (p.R297L). Arg297 is a moderately conserved amino acid. It is located in the α-helix Cα8 at the dimer interface (Figure 1D).

The c.1146C>G nucleotide transversion changes the apolar, aromatic Phe residue at position 382 into the aliphatic Leu residue (p.F382L). Phe382 is not strictly conserved. In fact, it is frequently replaced by an aromatic Tyr residue. Phe382 is located at the end of the loop 378-382, near the entrance to the active site. The c.1194A>C nucleotide transition changes the polar, charged Lys residue at position 398 into the polar Asn residue (p.K398N). Lys398 is a conserved amino acid. The c.1256A>C nucleotide transition in exon 12 changes the polar Gln at position 419 into the polar, charged Arg residue (p.Q419R). Gln419 is a poorly conserved amino acid.

3.2 Functional analysis and expression studies by RT-PCR and Western blot
We expressed in vitro five proteins containing the missense mutations p.N223Y, p.R297L, p.F382L, p.K398N, p.Q419R. The p.R297L mutation, identified in cis together with the well characterized p.A403V mutation, was reproduced in a recombinant protein as the sole mutation (Table 1).

The RT-PCR (Figure 2A) showed a fragment, representing an 871-bp partial region of PAH mRNA, in the wild-type and in all mutant constructs examined (lanes 2–7), but not in the untransduced cells (lane 1) and in the RT-PCR negative control (water-lane 8). We identified PAH mutant polypeptides in transfected HEK293 cell extracts by immunoblot analysis using the anti-PAH serum (Figure 2B). The monomeric form of the enzyme appeared as a 50-kD band, with different intensities in cells transfected with the wild-type enzyme (lane 2) as compared with mutants (lanes 3–7), but was absent in the untransduced cells (lane 1).

PAH enzyme assay was performed, in three independent experiments, on total protein extracts harvested 48 hours after transfection. As shown in Figure 3, the residual enzyme activity in cells expressing mutant cDNAs was 70% (p.N223Y), 42% (p.R297L), 56% (p.F382L), 55% (p.K398N) and 71% (p.Q419R) that of the wild-type enzyme activity. There was no enzyme activity in the untransduced cells.

3.3 BH4 loading test

Figure 4 shows the results of the BH4 loading test in patients bearing the genotypes [p.N223Y]+[c.165delT] (magenta line and stars), [p.R297L+p.A403V]+[p.R243X] (cyan line and diamonds), [p.K398N]+[p.442-5C>G] (red line and triangles) and [p.Q419R]+[p.S231P] (black line and square). Patient [p.A403V]+[c.184delC] (blue line and circles) is a good responder who served as a positive control in the test because c.184delC is a null mutation and p.A403V is an in vivo responsive mutation [25]. The analysed patients proved to be “responders” and even “fast responders” since the decrease in serum Phe concentration (from 61% to 87%) occurred already 2 hours after BH4 load. The patient bearing the p.F382L mutation was not tested because his parents did not give consent.

4. DISCUSSION

Five new PAH mutations (p.N223Y, p.R297L, p.F382L, p.K398N, p.Q419R) were previously identified by our group in a population of HPA patients from Southern Italy (Table 1) [15]. These mutations can be considered “mild”, because they were found in compound heterozygote patients affected by a mild phenotype and bearing a “severe” mutation (www.pahdb.mcgill.ca) on the other allele (allele A1 in Table 1) [25]. In particular, the
missense mutations p.R252W and p.S231P were previously described as severe mutations [11]. The remaining ones are severe mutations leading to inactive PAH protein (c.165delT, p.R243X, c.442-5C>G) [11].

On a molecular level, in our patients, the PAH tetramer may be formed from various combinations of mutated alleles (Table 1). The two homotetramers (A1)₄ and (A2)₄, and the heterotetramers (A1)(A2)₃, (A1)₂(A2)₂, and (A1)₁(A2)₁ can be formed at variable ratios, depending on the effects generated by mutations (i.e. folding defects, reduced stability or low amount of expression) [26]. In fact, co-expression of both mutants from two mutated alleles resulted in activity values in between those of single mutants [26]. In our patients bearing a severe mutation in allele A1, the functionality of the PAH tetrameric enzyme would mainly result from the novel mutations in the allele A2. The resulting enzymes, being embodied in homo- or heterotetrameric proteins, may influence the overall in vivo activity [26].

In this work, we reproduced in vitro the five mutant enzymes and measured the PAH catalytic activity. The assays showed an activity decrease to between 42% and 71% that of the wild-type protein. Western blot analysis showed that the five mutated constructs yielded a relatively normal amount of regular-sized PAH polypeptides. This indicates that these mutations do not induce changes that lead to increased degradation of the mutants nor to alteration of transcription and translation processes. Therefore, the tested mutations are disease-causing and the observed decreased enzymatic activity well correlates with the mild patient phenotype.

Some residual PAH activity is a prerequisite for BH4 responsiveness [13] but BH4 responsiveness can only be established with an in vivo test. We therefore verified BH4 responsiveness for the studied HPA patients, some of them (ID 12, 74, 82, Table 1) may be considered functionally hemizygous (coupled to a null mutation). In all cases, BH4 supplementation resulted in a relevant decrease (>30%) in serum Phe concentrations, which remained low up to 8 hours after BH4 challenge. The BH4 response might be the result of a correction by BH4 of the kinetics of the PAH mutant and/or of stabilization defects, because the mutant activity is decreased and the mutations might affect the structural stability [18, 22].

HPA is responsive to BH4 supplementation in vivo in more than 70% HPA phenotypes [2]. Infants undergoing BH4 treatment may be fed a less rigid PKU-restricted diet, with consequent positive effects on weight gain and quality of life [5-7]. Moreover, this treatment improves intellectual development especially in patients who have difficulty in adhering to a semisynthetic Phe-restricted diet [6]. As the BH4 positive response requires some residual PAH activity, BH4 is believed to activate the PAH enzyme [27]. Indeed,
classification of BH4-responsive patients, based on genotype and biochemical phenotype, and
elucidation of the molecular mechanism underlying BH4 responsiveness are crucial for PKU
treatment. Various mechanisms have been implicated in BH4 responsiveness [22, 28]:
increased enzyme activity [2, 12, 22, 27] correction or compensation of the BH4 decreased
affinity, protection towards catalytic inactivation and chaperone-like activity that, by
stabilizing the protein, protects it from proteolytic degradation [2, 3, 22]. In addition, BH4
supplementation may restore the optimal concentration of BH4 cofactor in hepatocytes [29,
30]. In all cases, BH4 responsiveness is dependent on the genotype, even though in some
cases the genotype is not a reliable predictor of phenotype [9]. The latter authors reported a
case of a patient bearing two null mutations on both alleles who was responsive to BH4
treatment. Generally, there is no single nor simple explanation for each patient who presents
an own complex phenotype that requires an ad hoc therapeutical protocol. However, new
therapeutic strategies that monitor the safety and the response to long-term treatment have
recently been reported [9, 10, 31].

Previous studies on structure/function correlations showed that the disease-causing
mutations are spread throughout the protein structure, with a certain number localised at
dimer/tetramer interfaces. This finding highlights the relevance of interactions among
subunits for the correct functioning of PAH [16, 17]. The structural examination of the five
PAH mutants, which we describe, revealed that the substituted residues are not directly
involved in the active site or in the cofactor binding site. Mutations at positions 223 and 382
perturb the local structure of regions that are involved in substrate-induced molecular
motions; whereas mutations at position 297, 398 and 419 disturb the dimer/tetramer
interfaces. All the substitutions resulted in an enzyme with decreased residual catalytic
activity compared to the wild-type enzyme.

The p.N223Y substitution is found in a region that may be critical for substrate-
triggered molecular motions [17, 18, 32]. Indeed, it has been proposed that this residue is the
centre of an intra-domain hinge-bending region associated with the conformational transition
of PAH induced by L-Phe substrate binding [18, 32]. In all available PDB structures, the
Asn223 side-chain nitrogen is H-bonded to the main-chain Gly218 oxygen. This interaction
adds further stabilization to the β-turn (Table 2, Figure 1C). The change to Tyr would result in
the loss of this H-bond, thereby probably destabilizing the local structure. A further
perturbation could be caused by the exposure of the Tyr aromatic ring to the surface.

The p.F382L replacement might affect the accessibility of the substrate in the active
site. Indeed, Phe382 is located at the end of the loop region 378-382, which covers the
entrance to the active site. This loop is quite flexible in the absence of substrate and becomes more ordered when bound to pterin cofactor and L-Phe substrate. In the latter case, Phe382 forms aromatic interactions (within 3.7 Å) with Tyr277 thereby protecting the substrate from the solvent. In both the substrate-free and the substrate-bound structure, Phe382 forms aromatic interactions with Tyr356, whereas Tyr277 forms van der Waals interactions with His146. Upon substrate binding, the relative approaching of Phe382 and Tyr277 results in a network of hydrophobic interactions (Figure 1E). Substitution of the aromatic Phe382 with a less bulky and aliphatic Leu residue would impair the efficiency and stabilization of the side-chain packing upon substrate binding.

The mutations (p.R297L, p.K398N and p.Q419R) perturb the dimer/tetramer architecture and hence may destabilize the enzyme. In the p.R297L enzyme, replacement of the Arg side chain by an apolar group would disrupt the dimer-stabilizing electrostatic and polar interactions. The Arg side chain is indeed close to acidic residues from the other subunit of the dimer. It can also engage a H-bond with a main-chain oxygen from residue 71 in the regulatory domain (Table 2). It is noteworthy that in our patients p.R297L was identified in cis with the p.A403V substitution on the same allele (Table 1). The p.R297L and p.A403V mutation sites are far apart (about 19 Å). Ala403 is located at the end of α-helix Cα12 (Figure 1A), close to other apolar residues, namely Ala306 and Ala309 belonging to Cα8. The p.A403V substitution might affect the close packing of Cα8 and Cα12 helices by introducing a larger, β-branched, Val residue at the helix-helix interface. This results in a further reduction of dimer stability. Two other mutations at position 297 are reported in the PAH database, p.R297H and p.R297C. The database also includes a unique case of a patient with the [p.R297C]+[p.Y356X] genotype and a moderate phenotype (HPA II). Because mutation p.Y356X is a null mutation, it is conceivable that mutation p.R297C induces a moderate reduction of enzyme activity, consistent with dimer destabilization.

In the p.K398N enzyme, substitution of Lys with a smaller and uncharged Asn would perturb the charge distribution at the tetramer interface. Indeed Lys398 protrudes toward a dimer hydrated interface in a negatively-charged surrounding, being the acidic residues Asp394 and Glu390 within 6 Å (Figure 1F). Lys398 belongs to the Cα12 helix (392-403) and contributes to helix stabilization through ionic interactions with residues Glu390 and Asp394. It is noteworthy that the Cα12 helix is a key secondary structure element that undergoes significant motion upon substrate binding [17, 32]. Furthermore, in the tetramer, the Cα12 helix is directly connected to Ser439 of the Ta1 helix (430-449) of another subunit, via a H-bond formed by Glu397, the residue preceding K398 (Table 2, Figure 1F). The substitution
may therefore also interfere with correct positioning of the Cα12 helix, which is involved in interactions with the tetramerization domain, and in molecular motions [14].

In the p.Q419R enzyme, the replacement of Gln with a charged Arg would increase the charge repulsion between basic residues of the same subunit (Table 2). In the homotetramer (A2)_4, the replacement p.Q419R would also result in a close localization of two corresponding Arg419 residues on different subunits of the dimer (Figure 1G). The replacement of Gln with a charged Arg residue may hamper oligomerization.

In conclusion, all the data reported herein support the pathologic nature of the five studied variants. In fact, the enzyme activity exerted by the recombinant protein variants was from 40% to 70% that of the wild-type enzyme, which well correlates with the mild hyperphenylalaninemia found in our patients. Accordingly, the analysis carried out in silico shows that the structural alterations do not directly affect the binding site of either the substrate or the coenzyme — rather they interfere with favourable interactions at the molecular surface or at its quaternary structure, and hence may destabilize structural integrity. Finally, the beneficial effect of BH4 supplementation in patients indicates that altered activity/stability could be partially reversed, thus supporting the not severe nature of HPA in patients bearing the variant proteins studied in this report.

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REFERENCES


Figure legends

Figure 1. Schematic representation of PAH structure.

A: The localization of the five mutations is represented by red spheres in the composite monomeric model (see text); the position of residue 403 is also shown (mutation p.R297L is in cis with p.A403V). The catalytic domain, the regulatory domain, and the tetramerization domain are shown in blue, magenta, and green, respectively. BH4 cofactor is drawn in yellow, thienylalanine in cyan, Fe ion as a grey sphere.

B: The tetrameric model of human PAH containing the catalytic and tetramerization domains (PDB code 2pah). Dimers are coloured blue/cyan and red/orange. C: Asn223 site. Segment 217-224 is drawn in ball-and-stick form. The stabilizing H-bonds displayed by Asn223 (magenta) in the β-turn 220-223 are represented by dotted lines. D: Arg297 site. The negatively-charged side-chains (Glu422 and Asp415) close to Arg297 (magenta) are shown. The Arg71 residue is also shown, with its main-chain oxygen within H-bond distance of Arg297.

E: Phe382 site. Phe382 (magenta) and nearby residues (Tyr356, Tyr277, His146) are highlighted in the substrate-bound structure (PDB code 1kw0). The substrate analogue, thienylalanine (cyan), and the BH4 cofactor (yellow) are also shown. F: Lys398 site. The negatively-charged side-chains close to Lys398 (magenta) are shown. In the dimer, the H-bond between Glu397 (Cα12) and Ser439 (Tα1) is also drawn. G: Gln419 site. Two Gln419 (magenta) residues at the dimer interface are shown with the positively-charged Arg241 residues nearby.

Figure 2. RT-PCR (panel A) and Western blot analysis (panel B) in transfected human HEK293 cells. A: Forty-eight hours after transfection, total RNA was extracted for RT-PCR analysis; an 871-bp region of PAH cDNA was present in all samples (2 to 7) examined but not in untransduced cells (lane 1), or in an RT-PCR negative control (water-

Figure 3. Residual enzymatic activity of PAH mutants expressed as percent of wild-type PAH activity. The columns show the mean value and the standard deviation derived from three different transfections, for each construct.

Figure 4. Temporal decrease of serum Phe concentrations after 20 mg/kg BH4 loading test.
Table 1. Biochemical phenotype and PAH genotype of HPA III patients.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Serum Phe (µmol/L)</th>
<th>Allele A1</th>
<th>Allele A2*</th>
</tr>
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<tbody>
<tr>
<td>12</td>
<td>242</td>
<td>c.165delT</td>
<td>p.N223Y</td>
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<tr>
<td>96</td>
<td>182</td>
<td>p.R252W</td>
<td>p.F382L</td>
</tr>
<tr>
<td>82</td>
<td>242</td>
<td>c.442-5C&gt;G</td>
<td>p.K398N</td>
</tr>
</tbody>
</table>

*Mutations studied
Table 2. Features of the studied PAH mutations.

<table>
<thead>
<tr>
<th>Exon</th>
<th>Nucleotide</th>
<th>Amino acid</th>
<th>Other mutations described for the codon</th>
<th>Enzyme domain/Secondary structure position*</th>
<th>Amino acid conservation score§</th>
<th>Structural contacts in WT / Consequent alterations in mutated enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>667A&gt;T</td>
<td>N223Y</td>
<td>None</td>
<td>Catalytic/β-turn (220-223)</td>
<td>5</td>
<td>N223 is located in a solvent-exposed β-turn (residues 220-223) connecting α-helices Cα4 and Cα5 (Fig. 1C). N223 H-bonds to G218 oxygen / Loss of a H-bond; Tyr exposure</td>
</tr>
<tr>
<td>7</td>
<td>890G&gt;T</td>
<td>R297L</td>
<td>R297C and R297H</td>
<td>Catalytic/α-helix Cα8 (293-310)</td>
<td>5</td>
<td>R297 points toward (~4 Å) negatively-charged residues (E422 and D415) of the other subunit of the dimer (Fig. 1D). In the structure (PDB code 1phz), R297 forms a salt bridge with E422 and a H-bond with R71 oxygen in the regulatory domain of the other subunit of the dimer / Loss of polar interactions at the dimer interface</td>
</tr>
<tr>
<td>11</td>
<td>1146C&gt;G</td>
<td>F382L</td>
<td>None</td>
<td>Catalytic / Long loop preceding Cα12 (392-403)</td>
<td>4</td>
<td>F382 forms aromatic interactions (~3.7 Å) with Y356. In the substrate-bound state, it is close to Y277 that in turn displays contacts with H146 (Fig. 1E) / Loss of aromatic interactions</td>
</tr>
<tr>
<td>11</td>
<td>1194A&gt;C</td>
<td>K398N</td>
<td>K398K (c.1194A&gt;G)</td>
<td>Catalytic/α-helix Cα12 (392-403)</td>
<td>6</td>
<td>K398 points toward a hydrated interface in a negatively-charged surrounding and makes ionic interactions with D394 and E390 within 6 Å (Fig. 1F). In the tetramer, the Cα12 helix is connected to the Ta1 helix (430-449) of another subunit via a H-bond (E397”-S439) / Perturbation of charge distribution at the subunit interface and possible interference with the correct positioning of the Cα12 helix</td>
</tr>
<tr>
<td>12</td>
<td>1256A&gt;C</td>
<td>Q419R</td>
<td>None</td>
<td>Tetramerization / Loop (415-420) connecting two β-strands</td>
<td>1</td>
<td>Q419 lies in a rather hydrated region on the dimer interface, close to R241 on the same subunit (Fig. 1G). In the substrate-bound structure, it is close to Q419 on the other subunit of the dimer / Unfavourable ionic interactions at the dimer interface</td>
</tr>
</tbody>
</table>

* According to the nomenclature of the composite model [17]
§ Deduced from data deposited in the PDBsum database (ID: 2pah) in the range from 1 (variable) to 9 (conserved)
Figure 1
Figure 2
Figure 3
Figure 4