Mini Review

The metabolic and molecular bases of tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency

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Abstract

About two-thirds of all mild phenylketonuria (PKU) patients are tetrahydrobiopterin (BH_4)-responsive and thus can be potentially treated with BH_4 instead of a low-phenylalanine diet. Although there has been an increase in the amount of information relating to the diagnosis and treatment of this new variant of PKU, very little is know about the mechanisms of BH_4-responsiveness. This review will focus on laboratory investigations and possible molecular and structural mechanisms involved in this process.
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Tetrahydrobiopterin (BH_4)-responsive phenylalanine hydroxylase (PAH) deficiency is a subgroup of hyperphenylalaninemia (HPA) caused by specific mutations in the *PAH* gene. It can be detected by a positive BH_4 loading test. HPAs can be divided into two groups: those due to deficiency of the apo enzyme PAH [1], and those due to a deficiency of its cofactor BH_4 (BH_4 deficiencies) [2]. The spectrum of HPAs caused by PAH deficiency ranges from the mild HPA (MHP), to mild phenylketonuria (mild PKU), and intermediate or classical PKU. Patients with BH_4-responsive PAH deficiency belong mostly to the groups of MHP and mild PKU.

BH_4-responsive PAH-deficient patients, in whom BH_4 deficiency was excluded, had been observed since 1985 by Niederwieser and Curtius [3] who suggested that the responses to BH_4 may be caused by a K_m mutant in the *PAH* gene. However, only recently Kure et al. [4] described four patients with HPA who responded to the loading test with 10 mg BH_4/kg body weight. BH_4 defects were excluded in all of them and mutations detected in the *PAH* gene suggested a novel subtype of PAH deficiency. In a similar patient with PAH deficiency (E390G/

IVS10nt-11g > a) who responded to 20 mg BH_4/kg by lowering plasma Phe concentrations from initially 885 to 67 μmol/L, 8 h post-BH_4-loading, Trefz et al. [5] introduced BH_4 therapy as an alternative approach to the low-phenylalanine diet. Under normal diet and on 10 mg/kg per day of BH_4, blood Phe levels remained between 84 and 222 μmol/L in this patient. Several other patients characterized as BH_4-responsive were described by Spaapen et al. [6] and Lindner et al. [7]. In these patients, the response to BH_4 was variable and one patient with the same genotype (R408W/Y414C) did not respond at all. This inconsistency within the same genotype group was due to a non-standardized BH_4 loading test used in the past. Some of these patients were tested with the older BH_4 product containing about 1/3 of the inactive isomer (6S-BH_4), thus with a ∼30% lesser amount of the active BH_4. During the last 2 years a number of additional cases have been published [8–17], and different mechanisms for the phenotypes proposed. Based on the location and proximity of particular mutations to the BH_4-binding site in the three-dimensional structure of PAH, a hypothesis of K_m variants (with a lower binding affinity for BH_4) were proposed [18,19]. However, some mutations found in patients with BH_4-responsive HPA/PKU are not located within the BH_4-binding site, and mechanisms such as stabilization of the protein or mRNA were suggested. BH_4 may act as a

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chaperon, protecting the enzyme from degradation by the ubiquitin-degradation pathway [20]. In some patients with homozygous mutations (L48S/L48S) BH_4 may increase enzyme activity by inducing PAH gene expression [21,22]. The fact that the BH_4-responsive phenotype is quite common among patients with HPA, as demonstrated by our group [23], and that more than 60% of patients with mild PKU may benefit from BH_4 substitution [24] warrants further careful investigations of the etiology of BH_4-responsiveness.

Tetrahydrobiopterin loading test

BH_4 is available as tablets (10 or 50 mg) from Schircks Laboratories, Jona, Switzerland (www.schircks.com) or from Daiichi Suntory, Japan as a granulate (Bioptern). A new product (Phenoptin) from BioMarin, Novato, CA (www.biomarinpharm.com) is currently under development.

All newborns detected in the screening for PKU (plasma Phe > 120 μmol/L) as well as older HPA/PKU children, previously not tested, need to be investigated for BH_4 disorders (analysis of urinary pterins and dihydropteridine reductase activity in blood spots) [25]. The BH_4 loading test has been used for many years as a practical additional tool to discriminate between cofactor defects (BH_4 deficiencies) and PKU [26]. However, this test discriminates only between BH_4-responders and non-responders, but cannot distinguish between BH_4 deficiencies and PAH deficiency alone. The following protocol proposed at the European Metabolic Group Workshop in Zürich [27] is recommended to detect patients with BH_4-responsive HPA/PKU:

Loading test with BH_4 (20 mg/kg body weight)

Initial plasma Phe levels should be > 400 μmol/L. Food (Phe) intake should be continuous during the test. Dissolve BH_4 tablets in 20 ml water, orange juice, or infantile formula by gently mixing in dim light (BH_4 is light- and oxygen-sensitive). Administer the suspension (20 mg/kg) within 30 min and after at least 3 h of fasting. Older children and adults may swallow the tablets undissolved.

The Phe and tyrosine should be measured in plasma or blood before, 4, 8, and 24 h after administration of BH_4. Urine should be collected before and 4–8 h after BH_4 administration to control intestinal BH_4 adsorption. The same urine sample is used to exclude BH_4 deficiency.

The BH_4 loading test is considered positive when initial plasma Phe concentrations decrease by at least 30% after 8 h [23] or by 50% after 24 h. When using the above protocol for the oral loading test, 60–70% of patients with MPH and mild PKU responded significantly (Fig. 1). Using an extended protocol over more than 24 h
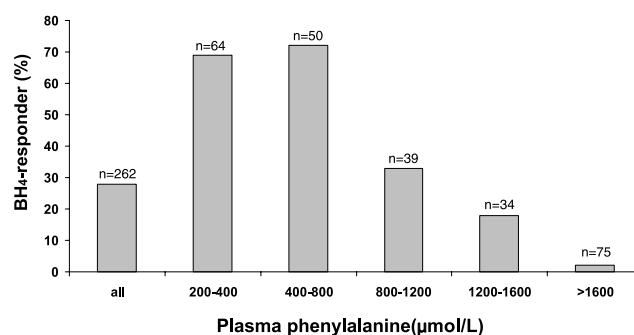

Fig. 1. Percentage of positive BH_4 loading tests (20 mg/kg) among PAH-deficient patients with different degrees of hyperphenylalaninemia.

with repeated administration of 10 mg BH_4/kg/day, Shintaku et al. [28] were able to detect additional patients with mild or moderate PKU (slow responders). Patients with mild PKU exhibiting decreases in blood phenylalanine concentrations of >20% in the single-dose test (10 mg/kg) also demonstrated decreases of >30% in the four-dose test (10 + 10 + 5 + 5 mg/kg) [28]. Thus, the optimal loading test may be a combination of the repeated administration of higher doses of BH_4 (20 mg/kg) over more than 1 day. Dhondt et al. [29] used multiple doses of BH_4 (2× 10 mg) and found 5 out of 17 patients with HPA or mild PKU (<800 μmol/L) to be responsive. A marked inter- and intra-individual variability in phenylalanine reduction 4 h after loading with 20 mg/kg was found in subjects with the same genotype (R408R/R408R and L48S/L48S): 5 and 21% and 17 and 41%, respectively [30]. Matalon et al. [31] reported BH_4-responsiveness in 21 of 26 patients (58%) with atypical and classical PKU when loaded with 10 mg/kg BH_4. Of the patients that responded, 12 were classical, 7 atypical (mild PKU), and 2 mild HPA, however, all these patients were on the low-phenylalanine or low-protein diet and thus cannot be compared with other loading tests data.

BH_4 is absorbed mostly in the duodenum and the jejunum and less in the stomach and adsorption may differ with age. Absorption from the digestive tract is not always favorable, and the rate of absorption is probably 5–6 times lower in older patients than in newborns. Furthermore, great inter- and intra-individual variations occur with regard to the maximal BH_4 plasma levels and the half-life time [32]. For some patients the 8-h protocol may be optimal, for others with a shorter BH_4 half-life time, longer protocols (24 h) or higher BH_4 doses (2× 10–20 mg/kg) may be necessary [29]. A typical plasma profile of BH_4 and total biopterin after oral administration of 10 mg BH_4 per kg body weight is shown in Fig. 2.

Combined Phe (100 mg/kg) and BH_4 (20 mg/kg) loading test

This protocol is used in patients with plasma Phe levels of <400 μmol/L or in patients who are on the diet. The procedure is the same as described for the single
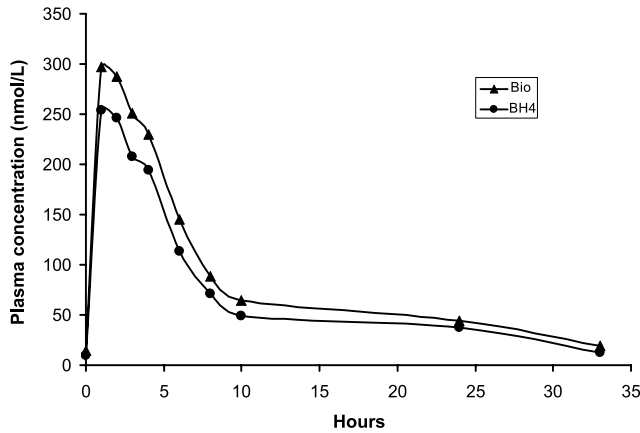
Fig. 2. Time course of plasma BH_4 and total biopterin after oral administration of 6R-BH_4 (10 mg/kg) in a control person.

BH_4 loading test except that Phe (100 mg/kg) is administered 3 h before BH_4 and there is one additional blood sampling [27]. Thus blood sampling is done at 0, 3, 7, 11, and 27 h. Muntau et al. [12] modified this test by administration of BH_4 1 h after Phe loading. Blood Phe and tyrosine were measured before Phe loading and 4, 8, and 15 h after BH_4 loading. Although this test is useful one should take into account that plasma Phe peaks 3 h after the challenge and that a portion of the administered Phe is not metabolized in the liver, but rather eliminated via other routes. Under ideal conditions one would need to perform both the combined Phe/BH_4 loading test as well as a single Phe (100 mg/kg) loading test. Unfortunately, there are no appropriate normal values for the combined

Phe/BH_4 loading test and in some instances the interpretation may be difficult.

PAH breath-test

More accurate is the in vivo analysis of Phe hydroxylation with and without BH_4 (10 mg/kg) after oral administration of [L-^{13}C]Phe (6 mg/kg) [33]. The recovery of carbon-13 in breath was measured and calculated for residual Phe hydroxylation. Using this method 87% of patients with MHP or mild PKU were found to respond to BH_4 by lowering Phe levels by at least 30% after 15 h [12]. Furthermore, this test confirms the hypothesis that impaired Phe hydroxylation can be corrected by BH_4.

Genotypes

Fig. 3 summarizes mutations detected in patients with BH_4-responsive HPA/PKU. A total of 75 mutations (BH_4-responsive and non-responsive), most of them in the compound heterozygous state, were described in 121 patients and about 50% of them were detected in more than one allele [34]. The R408W mutation is the most common one (25 alleles), followed by Y414C (23 alleles), A403V and R241C (14 alleles each), A300S and E390G (8 alleles each), IVS12nt + 1g > a (7 alleles), R413P (6 alleles) and I65T, R68S, and R158Q (5 alleles each). The complete list of mutations is available from the BIOPKU database [34]. Within the mutations described, some were expressed recombinantly in eukaryotic cell systems or *Escherichia coli* and found to have substantial residual activity
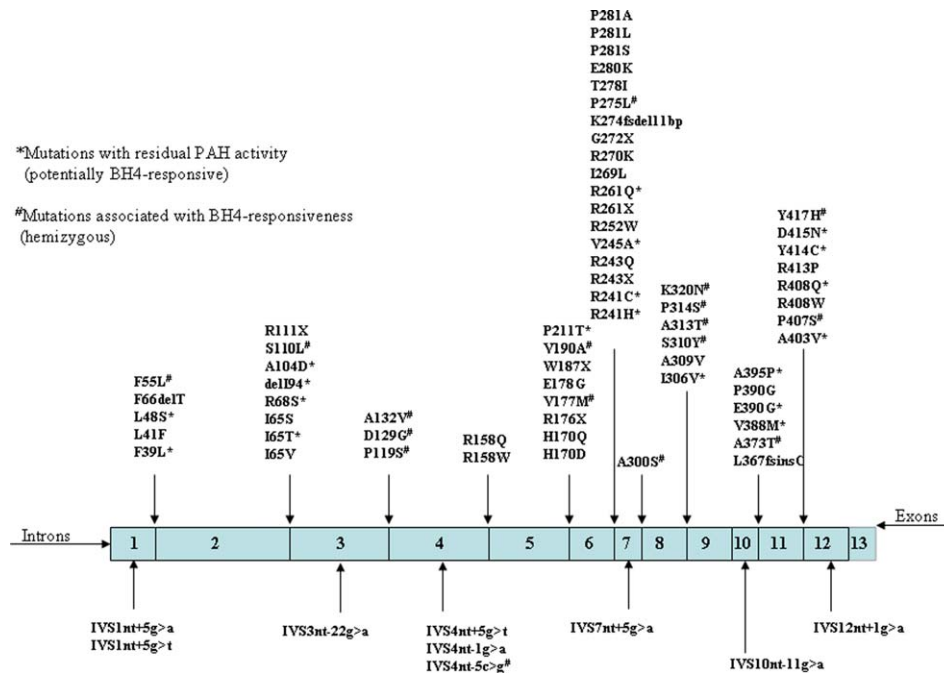

Fig. 3. Mutations detected in patients with BH_4-responsive HPA/PKU (source BIOPKU database; http://www.bh4.org/biopku.html and PAHdb; http://www.pahdb.mcgill.ca [61]).
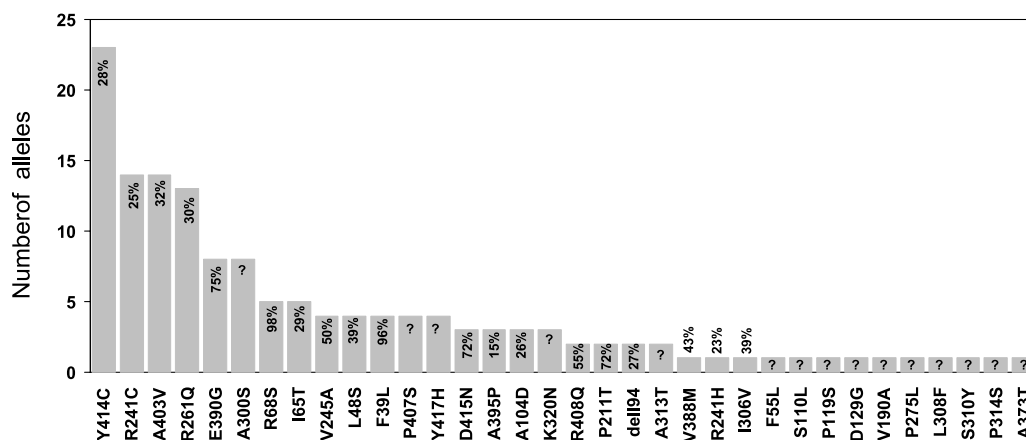
Fig. 4. Frequency of mutations associated with BH$_4$-responsive HPA/PKU and corresponding residual activity (%) of recombinantly expressed proteins.

(Fig. 4). About 62% of all mutations are located in the catalytic domain of PAH, 21% in the regulatory domain, 5% (4 mutations) are located in the tetramerization domain, and 19% (9 mutations) are intronic. Only very few of the described mutations are located within the two cofactor-binding regions CBR1 (V245A, R252W, R261X, and R261Q) and CBR2 (P281A, P281S, and P281L) and only two of them (V245A and R261Q) seem to be associated with BH$_4$-responsiveness. The sites of the proposed BH$_4$-responsive mutations, their amino acid interactions, and potential effect of mutation, along with known in vitro residual enzyme activities are listed in Table 1. The sites are also shown in Fig. 5 mapped on the structure of a monomer of the composite model of PAH [18].

Table 1
Structural consequences of mutations associated with BH$_4$-responsiveness

PAH mutation	Structural contacts/comments[a]	Residual activity[b]
F39L	In hydrophobic core of RD formed by Leu37, Leu41, Val51, Phe55, Ile65, Phe79, Leu98, Ile102, and Ala104, and Leu106. Substitution to a smaller Leu may change the core structure and destabilize RD	96% (TNT-T7)
L48S	Mutation to Ser can be accommodated, but adds charge to hydrophobic area between RD and CD of second molecule. Leu48 is 4.8 Å away from Pro211 of CD in other monomer	39% (A293)
F55L	In hydrophobic core of RD formed by Leu37, Phe39, Leu41, Val51, Ile65, Phe79, Leu98, Ile102, Ala104, and Leu106. Substitution to a smaller Leu may change the core structure and destabilize RD	N/A
I65T/V	In hydrophobic core of RD. Substitution to a more polar Thr may distort the hydrophobic packing in the RD core. Mutation to Val not as disruptive	I65T: 29% (TNT-T7); 26% (COS); 21% (A293)
R68S	H-bonds to Ser67, and thus stabilizes secondary structure of Rβ2 strand. In tetramer model Arg68 is close to Tyr216 from another monomer. Substitution to Ser may disrupt H-bond and dimer/tetramer interactions.	98% (COS); 76% (*E. coli*)
del94	In middle of helix Rα2 in RD, deletion of Ile94 disturb helix and packing of RD	27% (SW613-12A1)
A104D	On edge of hydrophobic core of RD formed by Leu37, Phe39, Leu41, Val51, Phe55, Ile65, Phe79, Leu98, Ile102, Ala104, and Leu106. Substitution to Asp adds charge to RD core. May destabilize loop between Rα2 and Rβ4	26% (A293)
S110L	Ser side chain hydrogen bonds with Lys85 side chain, Arg111 amide, Asp112 amide. Mutation to hydrophobic Leu may break up stabilizing hydrogen bonds at start of CD	N/A
P119S	Close to 311–313 region of CD. Mutation may destabilize RD	N/A
D129G	H-bond to Arg243 and His170. Mutation to Gly destabilizes start of CD	N/A
H170D	On surface of CD, close to TD and RD. Substitution into an Asp may disrupt a H-bond to Arg241 at the start of Cβ1	N/A
E178G	On surface of CD. Substitution to a small and flexible hydrophobic residue may be very unfavorable, as it can change fold of CD core, which is important for maintaining proper catalytic function	E178V; 18% (COS cells)
V190A	Close to L-Phe substrate binding site (7.1 Å). Important for proper substrate orientation for catalysis	N/A
P211T	At end of helix Cα4, close to Tyr77 of RD in other monomer. Mutation to Thr may destabilize helix	72% (COS-7)

Table 1 (*continued*)

PAH mutation	Structural contacts/comments[a]	Residual activity[b]
R241C/H	Arg241 is close to His170, Gln235, and Gln419. Mutation to Cys or His destabilize dimerization interface formed by residues 416–419	R241H, 23% (COS); R241C, 25% (COS)
V245A	Close to Leu249, which is involved in pterin binding. Mutation to Ala changes active site	~50% (TNT-T7)
R261Q	In loop between $C\alpha6$ and $C\beta2$. Interacts with Gln304 and Thr238 by H-bonds. Close to Tyr417 in tetrameric model. A substitution would disrupt H-bonds to Gln304 and Thr238 that stabilizes the secondary structure in the active site, and potentially interfere with proper dimer/tetramer formation	30% (COS); 47% (*E. coli*)
P275L	In region just before Pro279-Pro281 important for substrate binding. Mutation to Leu may destabilize/change substrate-binding site	N/A
A300S	Close to Thr238. Not enough room for larger side chain of Ser. Destabilization due to change of polarity in CD core	N/A
I306V	Towards end of helix $C\alpha8$. In hydrophobic core of CD. No predictable effect of mutation	39% (TNT-T7)
L308F	Close to TD Val412 and Tyr414, and CD Ala259 and Glu305. No room for Phe sidechain. Substitution would push TD away. Mutation may interfere with proper dimer/tetramer formation	N/A
S310Y	H-bonds to Gly307 (carbonyl oxygen) and Leu311 (amide). Mutation to a Tyr may push Leu255 towards BH_4-binding site. Destabilize BH_4-binding	N/A
A313T	Right behind Ser310. Close to TD Ile406 and Pro407. Mutation may interfere with proper dimer/tetramer formation, but also BH_4-binding	N/A
P314S	At start of helix $C\alpha9$. Mutation to Ser may destabilize helix that contains residues important for substrate binding/specificity	N/A
K320N	Lys320 in helix $C\alpha9$ towards surface. No predictable effect of mutation	N/A
A373T	Close to Phe402 and Lys320. Mutation may interfere with proper dimer/tetramer formation	N/A
V388M	Val388 located to hydrophobic patch on surface close to second monomer. May increase aggregation upon mutation to Met	43% (COS)
E390G	No contacts. On surface, close to second monomer. Substitution to Gly may induce local distortions in CD. Putative loss of dimer interactions	75% (*E. coli*); 70% (COS)
A395P	Mutation to Pro destabilize helix $C\alpha12$ located prior to β-strands involved in dimer contacts	15% (*E. coli*); 16% (TNT-T7)
A403V	In hydrophobic patch of CD at end of $C\alpha12$. May be necessary for starting loop before $T\beta1$ at start of TD	32% (COS)
P407S	Pro may be important for positioning TD helix. Mutation may interfere with proper dimer/tetramer formation	N/A
R408Q	Arg side chain H-bonds to Leu311 and Leu308 carbonyl oxygens. Mutation to Gln may preserve H-bonds but with slight distortions. Mutation to Trp, as in R408W much more disruptive than to Gln (R408W is not BH_4-responsive)	55% (COS); 9% (*E. coli*); 0% (TNT-T7)
Y414C	Stacks between Pro416 (TD) and Phe260 (CD). Important for keeping TD close to CD. Mutation may interfere with proper dimer/tetramer formation	28% (COS); 42% (TNT-T7); 38% (*E. coli*)
D415N	Mutations to Asn may change dimer interactions and destabilize loop between $T\beta1$ and $T\beta2$	72% (*E. coli*); 114% (TNT-T7)
Y417H	Close to other monomer. Mutation to His may change interactions of dimer	N/A

[a] *Abbreviations used:* RD, regulatory domain; CD, catalytic domain; TD, tetramerization domain.
[b] Compared with the wild-type enzyme.

Based on the presently detected genotypes of BH_4-responsive patients, it would appear that the allelic PAH mutation-combination is the most important indicator of BH_4-responsiveness. Two severe mutations found on the two alleles for PAH will very likely result in severe PKU, and thus little or no PAH enzymatic activity, and very likely no BH_4-responsive. It would be very difficult to propose a possible mechanism for BH4-responsiveness in patients with homozygosity for severe null mutations. Nevertheless, a few severe/classical PKU patients (with blood Phe levels $>1200\,\mu mol/L$) have been found to be BH_4-responsive [23,31], all of them with at least one partially active allele (as determined by enzymatic activity assays performed on expressed protein in vitro).

Similarly, patients with two mild mutations that show relatively high residual activity (i.e., for example $>30\%$ activity as compared to wild-type PAH) will likely display HPA or mild PKU, and possibly be BH_4-responsive. The combination of one mild mutation with one severe mutation will questionably be BH_4-responsive, and based on the currently known genotypes that are BH_4-responsive, this will depend upon the combination of the mutations present in the genotype. Thus, most of the genotypes found currently to be BH_4-responsive (see http://www.bh4.org/biopku.html for a complete listing) consist of one mild mutation and one severe mutation, or two relatively mild mutations, and they also display high residual enzymatic activity.
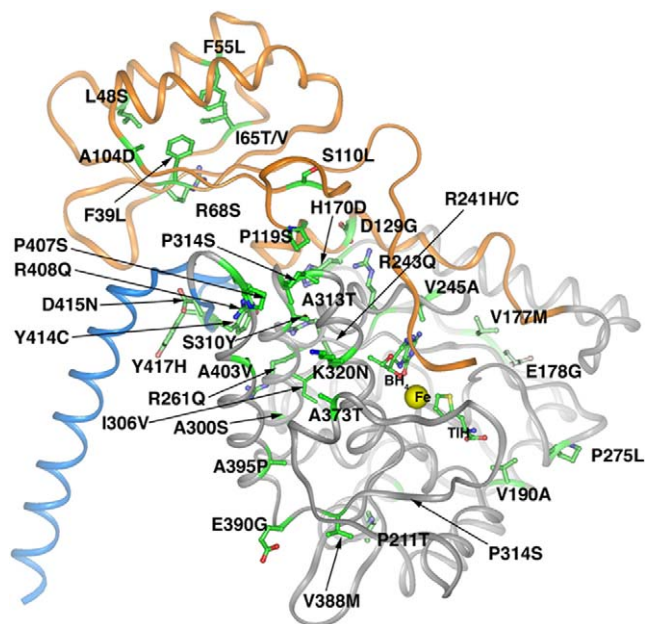
Fig. 5. C-α trace of a monomer of the composite model of phenylalanine hydroxylase [62]. The N-terminal regulatory domain is colored orange (residues 1–142), the central catalytic domain (residues 143–410) is colored gray and the C-terminal tetramerization domain (residues 411–452) is colored blue. The active site iron atom is shown as a yellow sphere, and the BH_4 cofactor and a substrate analog (3-2-thienylalanine) is shown for reference of the active site. Each amino acid that has been implicated in BH_4-responsiveness is shown in ball-and-stick mode (with carbon atoms green, oxygen atoms red, nitrogen atoms blue, and sulphur atoms yellow).

Mechanisms of BH_4-responsiveness

The phenylalanine hydroxylase enzyme is a homotetrameric enzyme where each monomer is composed of three domains: an N-terminal regulatory domain (residues 1–142), a central catalytic domain that contains the BH_4-binding site (at the active site) and a ferrous iron atom, necessary for the substrate L-Phe to tyrosine conversion, and finally a C-terminal oligomerization domain (residues 411–452). Both the substrate L-Phe and the cofactor BH_4 are found to regulate the activity of PAH.

Based on the present knowledge of the regulative properties of the cofactor BH_4 and substrate phenylalanine (reviewed in Erlandsen et al. [19]), the following mechanisms have been postulated as possible causes for BH_4-responsiveness: (1) K_m mutants of the PAH enzyme displaying reduced binding affinity for BH_4, (2) stabilization of PAH by the chaperon-like activity of BH_4, i.e., BH_4 may act as a chemical chaperon protecting the active tetramer/dimer forms from proteolytic cleavage or degradation by the ubiquitin-dependent proteasome pathway, (3) BH_4-driven change in regulation of BH_4 biosynthesis, (4) induction/up-regulation of PAH enzyme expression

by BH_4 [12,18,21], and (5) PAH mRNA stabilization, similar to the effect of BH_4 on inducible nitric oxide synthase mRNA. These hypotheses will be discussed in further detail below.

K_m mutants with reduced affinity for BH_4

The molecular basis of disease arising from as many as one-third of the mutations in a gene is an increased Michaelis constant (K_m), or decreased binding affinity of an enzyme for a vitamin-derived cofactor or substrate, which in turn lowers the rate of the enzymatically catalyzed reaction. The K_m is a measure of the binding affinity of an enzyme for its ligand (substrate or cofactor) and is defined as the concentration of ligand required to fill one-half of the ligand-binding sites. It is likely that therapeutic vitamin/cofactor regimens increase intracellular ligand (cofactor) concentrations, thus activating a defective/mutant enzyme; this alleviates the primary defect and remedies the disease [35]. Examples for K_m mutant enzymes have been found for many diseases, for example in homocystinuria (pyridoxine-responsive), maple-syrup urine disease (thiamine-responsive), methylmalonic aciduria (cobalamin-responsive), and hemophilia (vitamin K-responsive), to mention just a few. These are reviewed extensively in Ames et al. [35].

It is believed that some of the BH_4-responsive PAH mutations are K_m mutants, as described above. Thus BH_4 therapy for PKU or HPA would join the list of high-dose vitamin treatments that are available to correct mutation-induced decreased binding-affinity for certain cofactors. Some of the known BH_4-responsive mutations map onto the catalytic domain in regions that interact with secondary structural elements involved in cofactor-binding [18] (Fig. 5). However, many are also found in the regulatory domain or in the oligomerization domain, and thus are not directly involved in BH_4-binding. In vitro kinetic studies on recombinantly expressed mutant PAH will tell us whether or not the K_m of mutant enzymes have changed as compared to wild-type. Studies are currently underway to classify the effects of BH_4 on BH_4-responsive mutant PAH enzyme.

So far, the only BH_4-responsive allelic PAH mutation-combination characterized kinetically contains the V388M mutation [36]. The recombinant V388M mutant form exhibited a reduced specific activity equivalent to 30% of the wild-type hPAH enzyme when assayed using the synthetic cofactor (6-methyltetrahydropterin). Lower values were obtained (23 and 19%) when the mutant enzyme was assayed with the natural cofactor ($6R$-BH_4) and different concentrations of L-phenylalanine. The enzyme kinetic studies of the V388M mutant protein revealed that this enzyme is a kinetic variant form of hPAH with a reduced affinity for the natural cofactor BH_4 ($K_{m\,V388M} = 82\,\mu M$ versus $K_{m\,wt} = 22\,\mu M$).

Chaperon-like activity of BH4

PAH has been found to be a substrate (by poly-/multi-ubiquitination) for degradation by the ubiquitin (Ub)-proteasome-dependent pathway for protein degradation [20]. This mechanism has particular significance for the turnover of mutant forms of human PAH [37]. Many cases of PKU are proposed to be the result of mutations that interfere with normal folding and oligomerization of catalytically active forms of wild-type human PAH, leading to an increased rate of degradation of PAH.

There are two pools of BH4 in hepatocytes, one that is metabolically available (free BH4) and one that is not (bound BH4) (Fig. 6). The metabolic availability of BH4 is determined by whether or not it is sequestered in the form of a PAH·BH4-complex, which has much less activity and is less readily L-Phe-activated than the uncomplexed enzyme [38]. In addition, the inactive complex of BH4 and PAH may play an important physiological role in that it can stabilize PAH and thus prevent the Ub-dependent degradation. It potentially also protects against loss of the catalytic metal (Fe^{2+}) at the active site, and may "lock" the enzyme into a folded conformation that makes the enzyme a poorer substrate for Ub-degradation. This chaperon-like activity of BH4 on PAH also remains to be studied in vitro and studies are underway towards understanding it in more detail.

Change in regulation of BH4 biosynthesis

Originally, regulation of BH4 synthesis was ascribed to direct feedback inhibition by BH4 of GTP cyclohydrolase
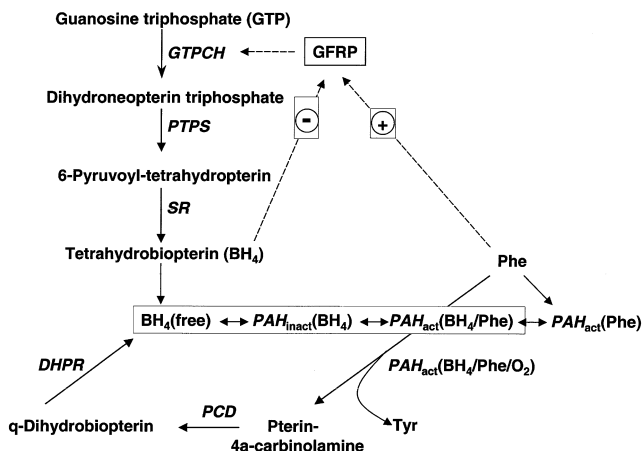

Fig. 6. Tetrahydrobiopterin pathway and phenylalanine hydroxylating system. Effect of BH4 and phenylalanine on the activity of PAH and regulation of BH4 biosynthesis. PAH is inactivated by BH4 in the absence of phenylalanine PAH$_{inact}$(BH4). Only the active complex PAH$_{act}$(BH4/Phe/O$_2$) can convert phenylalanine to tyrosine. GTPCH: GTP cyclohydrolase I, PTPS, 6-pyruvoyl-tetrahydropterin synthase; SR: sepiapterin reductase; PCD: pterin-4a-dehydratase; DHPR: dihydropteridine reductase; PAH: phenylalanine-4-hydroxylase; GFRP: GTPCH feed-back regulatory protein.

I (GTPCH) [39]. GTPCH catalyzes the first step in the de novo biosynthesis of BH4 [40] (Fig. 6). This inhibition by BH4 is found to be mediated by the GTP cyclohydrolase I feedback regulatory protein (GFRP) [41]. In the liver, L-Phe specifically stimulates de novo BH4 synthesis by displacing BH4 from the GTPCH–GFRP inhibitory complex [42]. A physiological consequence of GFRP action on GTPCH is the high plasma BH4 concentrations observed in patients with PKU or HPA [43,44].

As mentioned in the previous section, two pools of BH4 exist and the metabolic availability of BH4 depends on the amount that is sequestered in the low activity PAH·BH4-complex. It has also been suggested that the inter-conversion of L-Phe-activated and inactivated PAH, and bound and free BH4 is, like the de novo BH4-biosynthesis, driven by the level of L-Phe [38]. Thus, free BH4 concentration is determined by the state of activation and activity of PAH.

The only other known BH4-requiring enzymes in liver; glyceryl-ether mono-oxygenase and NOS, are present in relatively low amounts, and PAH (subunit) and BH4 concentrations in non-PKU liver have been found to be approximately equal (8–9 μM) [45]. As a consequence, formation of the PAH–BH4 complex will cause equal decreases in free enzyme and free BH4 concentrations, and thus L-Phe, by controlling the activation state of the PAH enzyme, will control both the metabolic availability of BH4 and the amount of active PAH in a normal cell [46]. Thus, one can speculate that in the BH4-responsive PKU patients, at high plasma and hepatocytic levels of L-Phe, an increase in plasma BH4-levels (by oral therapy) will further increase the levels of BH4, due to the fact that the already present high levels of L-Phe reverses the feedback inhibition of GTPCH. Furthermore, it is additionally also possible that BH4-responsive mutant PAHs have a dysfunctional or altered L-Phe-dependent activation mechanism, and so the mutant PAH enzyme would be already activated (i.e., no L-Phe-dependent activation mechanism), or have lost its cooperativity. This would be reflected in the Hill-coefficient for L-Phe binding/activation of PAH [47]. Preliminary data on in vitro enzyme kinetic studies of PAH mutants suggests that the Hill-coefficients are reduced from 2 (cooperative) as in wildtype PAH, to <2, and in some mutants are 1 (non-cooperative), but this could also be generally the case for all PKU mutations. Thus, an increase in BH4-concentration at high L-Phe levels (upon oral BH4 therapy) will under these conditions potentially result in a "bypass" of the PAH·BH4 inactivated state, which normally is reversed by the L-Phe-dependent activation mechanism. Subsequently there is an increase in the conversion of L-Phe to L-Tyr (due to the presence of more cofactor) in vivo, as compared to at lower BH4 concentrations (before oral BH4 therapy). Eventually, the effects of the BH4 oral therapy in the patient would be determined by the relative ratios of L-Phe versus

orally added BH_4 and the severity/effect of the mutation upon the activation mechanism in the mutant PAH enzyme.

Induction of PAH expression by BH_4

GTPCH/BH_4-deficient mice (*hph-1*) were used to test the effects of TH deficiency in L-dopa-responsive dystonia (an inherited disease caused by mutations in the GTPCH enzyme) [48]. TH activity and mRNA levels in the *hph-1* mouse were significantly decreased, compared to wild-type mice, but addition of BH_4 (200 mg/kg IP) increased both TH mRNA levels and activity *as well as* protein levels (increased by as much as 50%). Similar changes were seen for PAH mRNA activity and protein levels upon addition of BH_4. Although no original data are available, this communication suggests that BH_4 may regulate both TH and PAH gene expression and thereby plays a role in the control of the steady-state levels of the protein for which it acts as a cofactor. Under normal conditions, BH_4 has no significant effect on PAH activity in humans. In healthy persons, oral administration of BH_4 shows no change in blood phenylalanine levels [32]. The *pts* knockout mouse (BH_4 deficiency) shows very low BH_4 levels and low PAH activity in the liver when compared with heterozygote or wild-type animals (Beat Thöny, personal communication). Thus the wild-type and mutant PAH may be differently regulated by BH_4.

PAH mRNA stabilization

The biosynthesis of BH_4 is not only regulated on the substrate level but also through transcriptional regulation/control of the interacting proteins on the level of mRNA (as mentioned in the section above). L-Phe has been found to stimulate the biosynthesis of BH_4 not only by reversing the negative feedback inhibition of GTPCH, but also by increasing the mRNA level of GTPCH [49]. Immuno-stimulation was found to alter protein expression of GTPCH and its regulatory protein GFRP in a way that favors BH_4 synthesis. Similarly, cytokines can induce PTPS activity [50]. BH_4 was found also to regulate inducible nitric oxide synthase (iNOS) as a cofactor and allosteric effector [51]. By varying BH_4 levels with dicumarol (an inhibitor of BH_4 synthesis) and sepiapterin (an exogenous source of cofactor), iNOS expression was investigated in activated rat aortic smooth muscle cells (SMC). In sepiapterin-supplemented cells, iNOS protein levels were increased, while in dicumarol-treated cells iNOS levels were diminished. Time-dependent kinetic experiments revealed that inhibition or supplementation of BH_4 synthesis had no effects on iNOS induction or transcription rate. However, iNOS mRNA was present over a prolonged time in sepiapterin-supplemented SMC. Analysis of iNOS mRNA levels showed stable iNOS mRNA in sepiap-terin-treated cells 8 h after transcription inhibition, while in dicumarol-treated cells iNOS mRNA disappeared. The decrease of iNOS mRNA by dicumarol was abolished by sepiapterin. These data indicate that BH_4 post-transcriptionally stabilizes iNOS mRNA in smooth muscle cells. However, the actual mechanism by which iNOS mRNA is stabilized by BH_4 remains yet to be defined. That a cofactor of an enzyme controls the enzyme's mRNA and thereby its expression, is a little known feature. But such a mechanism would also explain the recent observation that, in addition to their reduced activities, the absolute amounts of the BH_4-dependent aromatic amino acid hydroxylases, phenylalanine, tyrosine, and tryptophan hydroxylase, are decreased in BH_4 deficient mice (*hph-1* mice) [52].

In summary of the possible hypotheses of BH_4-responsiveness, what we most likely will find upon investigation is that the response to BH_4 supplementation is multi-factorial and the relationship between the BH_4-responsive genotype/mutation will have to be studied in detail, with in vitro characterization of mutant PAH enzyme and in vivo characterization in PAH and BH_4 knockout mice and/or cell-lines, for a complete understanding of BH_4-responsiveness in PKU patients.

Toxicology of BH_4

BH_4 has been used successfully for treatment of patients with BH_4 deficiency for over 20 years [2]. Based on the information from the BIODEF database (www.bh4.org/biodef.html) there are no side effects reported. Also, in 1998 a questionnaire was sent to all clinics in Germany who used Schircks BH_4 and there was only one report of a transient rush in a child on 2.5–10.0 mg/kg BH_4 (Blau N, personal communication). However, the product information of the Suntory Biopterin BH_4 product lists a number of adverse reactions observed in 318 patients enrolled in clinical trial. Major symptoms were psychoneurotic (e.g., sleep disorders) in 13.8% (44/318), urological (e.g., pollakisuria) in 9.1% (29/318), and gastrointestinal (e.g., loose bowels) in 2.8% (9/318).

The BH_4 toxicology in mice was analyzed by acute and subchronic intraperitoneal and acute oral survival studies [53]. Using the 6R,S-BH_4, an LD_{50} of approximately 260 mg/kg was obtained from acute (14-day) intraperitoneal studies. Acute oral administration of up to 1318 mg/kg BH_4 did not cause any significant morbidity or mortality, nor did subchronic (92-day) i.p. administration of 10–50 mg/kg BH_4 [53].

Subcutaneous administration of BH_4 (30 mg/kg) to the *hph-1* mouse (BH_4-deficient mouse) resulted in a 2-fold increase in brain cofactor concentrations without any effect on the monoamine turnover [54]. Thus, with the doses currently used therapeutically, acute BH_4 administration does not directly influence tyrosine and

tryptophan hydroxylases in the *hph-1* mouse. However, increasing the amount of BH_4 to 300 mg/kg resulted in a brief activation of the monoamine turnover and 2 of 12 mice died.

Recently, Kim et al. [55] showed that direct injection of BH_4 into the substantia nigra of rats caused a selective and dose-dependent loss of dopaminergic terminals and decrease of dopamine content in the striatum. Thus, this animal model exhibited morphological, biochemical, and behavioral characteristic associated with Parkinson disease. However, the lowest concentration of BH_4 tested by Kim et al. [56] was 6 mM which is about 20,000 times higher than the concentration found in CSF of patients after oral administration of 20 mg/kg. Other investigations suggest that BH_4 may be cytoprotective at least in some cell systems [57,58].

Treatment

There are only a few reports on the long-term follow-up of HPA/PKU patients on treatment with BH_4 [5,28,59,60]. However, according to unpublished communications, a number of patients with BH_4-responsive PAH deficiency are presently on different BH_4 treatment protocols, either under monotherapy (7–20 mg/kg) or in a combination with the low-phenylalanine or low-protein diet. Potentially, two-thirds of mild PKU patients can be treated with BH_4. In addition, pregnant women with mild PKU may also benefit from BH_4 administration [24]. Unfortunately, BH_4 is expensive and not available for all patients at low or no costs. Lack of well-designed long-term studies with patients with BH_4-responsive HPA/PKU makes the registration of BH_4 as an orphan drug even more difficult. However, several long-term crossover or double-blind studies are currently running in different countries and there is hope that BH_4 will be available for pharmacological therapy of the mild variant of PAH deficiency in the next 3–5 years.

Acknowledgments

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