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A hypothesis on the biochemical mechanism of BH_4-responsiveness in phenylalanine hydroxylase deficiency

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Summary. We describe six children with tetrahydrobiopterin (BH_4) responsive phenylalanine hydroxylase (PAH) deficiency. All patients carry two mutant alleles in the PAH gene. Cofactor deficiency was excluded. The effect of BH_4 administration was studied by correlating different oral BH_4 doses with plasma phenylalanine levels under defined protein intake. Our results indicate that oral BH_4 supplementation may be used as long-term treatment for individuals with BH_4-responsive PAH deficiency, either without or in combination with a less restrictive diet. Previous *in vitro* studies have demonstrated that BH_4 inhibits PAH tetramers but activates PAH dimers. This may indicate, that BH_4-responsiveness results from BH_4 induced stabilization of mutant PAH dimers. In addition, interindividual differences in the cellular folding apparatus may determine the tertiary structure and the amount of mutant PAH dimers and hence may account for divergent BH_4-responsiveness reported for the same *PAH* genotype.

Keywords: BH_4-responsive PKU – Tetrahydrobiopterin – Mechanism

List of abbreviations: BH_4, Tetrahydrobiopterin; DGGE, Denaturing gradient gel electrophoresis; DHPR, Dihydropteridine reductase; HPA, Hyperphenylalaninemia; MHP, Mild hyperphenylalaninemia; PAH, Phenylalanine hydroxylase; Phe, Phenylalanine; PKU, Phenylketonuria; Tyr, Tyrosine

Introduction

Phenylalanine hydroxylase (EC 1.14.16.1), a non-heme iron(II)-containing enzyme, hydroxylates phenylalanine to tyrosine in the presence of the cofactor (*6R*)-L-*erythro*-5,6,7,8-tetrahydrobiopterin (BH_4). The 50 kDa enzyme monomer consists of three domains, a regulatory (amino acid 1–142), a catalytic (amino acid 143–410) and a tetramerization domain (amino acid 411–452). The latter is crucial for the formation of a homotetramer displaying higher turnover of phenylalanine than the dimer which however, still possesses considerable enzymatic activity. More than 400 mutations causing hyperphenylalaninemia are known in the phenylalanine hydroxylase gene (Erlandsen, 1999; Scriver, 2000). They are distributed throughout all three domains of each subunit with most of the pathologically significant mutations being located in the catalytic domain (Zschocke, 1999).

Primary hyperphenylalaninemias (HPA) are either caused by loss of activity of phenylalanine hydroxylase or by lack of its cofactor BH_4. PAH-deficiency and disorders of the BH_4 metabolism can often be differentiated by a BH_4 loading test, since plasma phenylalanine levels fall after BH_4 application in BH_4 deficiency, but remain unaffected in case of PAH deficiency. However, recently several patients with BH_4 responsive HPA have been described (Kure, 1999; Spaapen, 2001; Trefz, 2001; Lindner 2001). It has been hypothesised that the mutant PAH enzyme shows a decreased affinity for its cofactor BH_4 which can be compensated for by oral BH_4 supplementation (Erlandsen, 2001).

We have identified six children with mutations in the *PAH* gene and responsiveness to oral BH_4 administration. On the basis of data assembled from our patients and recent publications, we present a hypothesis on the mechanism of BH_4 responsiveness involving changes in the tertiary and quarternary structure.

Materials and methods

Patients

Hyperphenylalaninemia was detected in newborns by routine screening from dried blood spots. To further differentiate the type of hyperphenylalanemia, infants were admitted and an oral BH_4-loading test (described below) was performed. Six unrelated individuals were identified as being responsive to BH_4-loading while urinary pterins and blood DHPR activity did not indicate a defect in BH_4 metabolism. The presence of *PAH* mutations was subsequently confirmed (as described below).

Patient one, BS, is the second child of non-consangious parents born spontaneously at the 42nd week of gestation. Postnatal and newborn period passed without any abnormalities. Since the initial blood phenylalanine levels remained just below 600 μmol/L, BS was fed with normal infant formulas. However, during the second half of his first year of life plasma phenylalanine levels were found to be consistently above 600 μmol/L.

Patient two, LW, is the first child of unrelated parents. LW was born spontaneously at the 38th week of gestation, forceps was used for the delivery. LW developed supraventricular tachycardias and an *E. coli* sepsis during his second month of life. Since then no further complications have occurred. Plasma phenylalanine levels were above 600 μmol/L from the very beginning.

Patient three, TJ, the first child of unrelated parents, was born at the 41st week of gestation and showed no abnormalities so far. On regular controls the plasma phenylalanine levels have not exceeded 600 μmol/L during the first two years of his life.

Patient four, KT, born at the 42nd week of gestation postnatally suffered from slight respiratory distress but gradually adapted during the first hours of life. Plasma phenylalanine levels have never reached 600 μmol/L during the first year of life.

Patent five, EM, the fourth child of unrelated parents was born at term without complications. Plasma phenylalanine levels remained below 600 μmol/L during the first 6 months of life without any protein restrictive diet.

Patient six, AJ, the second child of unrelated parents has been treated with phenylalanine-free formulas and protein restriction since her newborn period. Plasma phenylalanine concentrations would reach up to 1816 μmol/L without diet.

Amino acid measurements

Neonatal screening was performed on filter paper blood spots using the phenylalanine kit from PerkinElmer Life Sciences (Turku, Finland). The PerkinElmer assay was adapted for whole blood samples. Briefly, ninhydrine reacts with phenylalanine to yield the fluorescent hydrindantine. The conditions used in this assay ensure high selectivity for phenylalanine. Blood samples from the BH_4-loading test and from the BH_4-optimization assay (s.below) were analyzed for phenylalanine. In addition, samples were assayed by ion-exchange chromatography on a Biochrom 20 (Pharmacia, Freiburg, Germany) to measure tyrosine concentrations.

BH_4-loading test

Urine samples and blood specimens were taken immediately before oral application of 20 mg/kg body weight BH_4 (administered 30 min before a regular meal). Blood specimens were acquired 4 hours and 8 hours after BH_4-loading. Urine was collected between 4 and 8 hours as well as between 8 and 12 hours after BH_4 administration. The phenylalanine and tyrosine concentrations of the collected blood samples were determined as indicated above. Further, the pattern of urinary pterines was analysed and DHPR (dihydropteridine reductase) activity in erythrocytes was measured.

BH_4-optimization assay

BH_4-responsive patients were supplied with a defined intake of phenylalanine per day (100–150 mg/kg/d) and were given a certain amount of oral BH_4 (5 or 10 mg/kg/d) in six single doses. The blood phenylalanine levels were monitored every 4 h. BH_4 doses were changed after about 48 h when stabilisation of blood phenylalanine levels had occurred (s. Fig. 2).

Mutational analysis

Mutations of the *PAH*-gene were determined by DGGE and subsequent sequencing as previously described (Zschocke, 1999).

Results

BH_4-responsiveness was initially demonstrated for all six children (BS, LW, TJ, KT, EM, and AJ) by oral BH_4 loading tests (s. Fig. 1). Cofactor deficiency was excluded by normal urinary pterin concentrations, normal DHPR activity in erythrocytes and (for patient LW) normal neurotransmitter concentrations in CSF (data not shown, samples analyzed by N. Blau, University of Zürich). Patients TJ, EM and KT showed a MHP phenotype while BS and LW showed Phe values above the therapeutic threshold indicative of mild PKU. Patient AJ had plasma Phe compatible with a moderate classical PKU. Phenotypes are in line with the *PAH* genotypes identified (cf. Table 1): both A104D and Y414C are mild PKU mutations, while

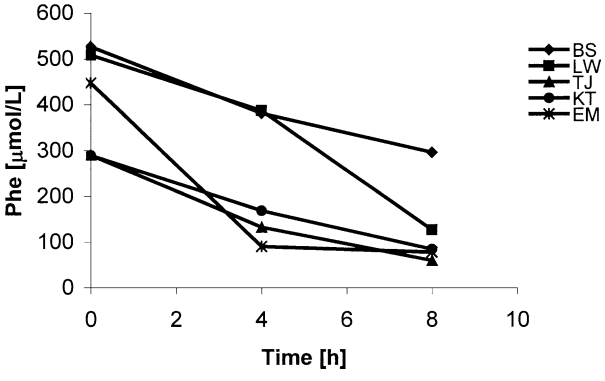

Fig. 1. Results of the BH_4-loading test for patients BS, LW, TJ, KT and EM. Phenylalanine concentrations in blood samples have been analysed before (0 h) as well as 4 h and 8 h after BH_4 treatment. The tests were repeated once for patients BS and LW and reproducible results were obtained. No abnormalities in urine pteridines and neurotransmitters have been detectable. Phenylalanine levels for patient AJ fell within 8 h from 1942 μmol/L to 1258 μmol/L after BH_4 treatment. Data has been omitted from the chart for reasons of clarity

A403V and D415N are common MHP mutation associated with Phe values below treatment levels irrespective of the mutation on the other allele (Guldberg, 1998).

Patient BS and LW, who showed persistently high Phe concentrations in blood, were selected for the investigation of their BH_4-response in detail, to elucidate the optimal BH_4 dose and, if successfully implemented, to initiate a long-term BH_4 treatment. To monitor the BH_4 effect, plasma phenylalanine was correlated with different oral BH_4 doses under a defined protein intake corresponding to 100–150 mg phenylalanine per kg body weight and day. Figure 2a depicts the response of patient BS to different amounts of BH_4. After a brief delay of about 4 h, there was a marked reduction of plasma phenylalanine levels after application of 10 mg/kg BH_4. Daily doses of no more than 5 mg/kg BH_4 were adequate to keep the plasma phenylalanine values below 600 μmol/L. In case of patient LW the BH_4-response showed slightly different characteristics. In particular, a daily BH_4 dose of 5 mg/kg body weight was not sufficient to keep phenylalanine values below the threshold level of 600 μmol/L. Also, reduction in plasma phenylalanine after the initiation of oral BH_4 applications lagged behind that of patient BS (s. Fig. 2b). No increase in plasma tyrosine concentrations were detected in blood samples from both infants during the BH_4 optimization protocol (data not shown). Interestingly, a 18 h–24 h delayed rise in blood Phe levels has been observed after discontinuing BH_4 supplementation.

Both children with mild PKU (BS, LW) were continued on oral BH_4 supplementation and were since fed without protein restriction or special phenylalanine free formulas. During the past year, plasma phenylalanine concentrations of patient BS kept within the desirable range at daily BH_4 doses between 5–10 mg/kg b.w. (s. Fig. 3). For patient LW, daily BH_4 supplementation of 15–20 mg/kg b.w. was required to keep phenylalanine values below 600 μmol/L. At lower BH_4 doses plasma Phe levels rose above 600 μmol/L, in particular during febrile infections (data not shown). Both children showed no neurological symptoms and have developed normally so far.

Discussion

We have identified six different PAH genotypes which were BH_4-responsive in a BH_4 loading test. Our patients carry mutations within either of the three PAH domains. Taking into consideration other reported PAH alleles associated with BH_4 responsiveness, no defined structural motif responsible for BH_4 affinity can be located. All six genotypes contained at least one mutation associated with some residual enzyme activity (s. Table 1). Together with the fact that classical PKU does not

Table 1. PAH gene mutations associated with BH_4 responsiveness, ordered by increasing amino acid residues

PAH gene mutations allele 1/allele 2	Domain position of mutation allele 1/allele 2	Clinical phenotype	Reference
A104D/K320N	regulatory/catalytic	variant PKU	TP, patient BS
R176X/A403V*	catalytic/catalytic	mild HPA	TP, patient EM
V190A/R243X	catalytic/catalytic	variant PKU	[Spaapen, 2001]
IVS4nt-1 g>a/A373T	catalytic/catalytic	mild HPA	[Kure, 1999]
R241C/A403V*	catalytic/catalytic	variant PKU	[Spaapen, 2001]
R241C/R413P	catalytic/tetramerization	mild HPA	[Kure, 1999]
R243Q/D415N	catalytic/tetramerization	mild HPA	TP, patient KT
R252W/P407S	catalytic/catalytic	mild HPA	[Kure, 1999]
A300S*/A403V*	catalytic/catalytic	variant PKU	[Spaapen, 2001]
A313T/L367fsinsC	catalytic/catalytic	variant PKU	[Spaapen, 2001]
IVS10nt-11 g>a/R408W	catalytic/catalytic	classical PKU	TP, patient AJ
IVS10nt-11 g>a/E390G*	catalytic/catalytic	mild HPA	[Trefz, 2001]
A395P/A403V*	catalytic/catalytic	mild HPA	TP, patient TJ
R408W/Y414C*	catalytic/tetramerization	variant PKU, mild HPA	[Lindner, 2001]
Y414C*/Y414C*	tetramerization	variant PKU	TP, patient LW

PKU phenylketonuria, *HPA* hyperphenylalaninemia, *TP* this publication
* PAH alleles with reported ambiguous phenotype

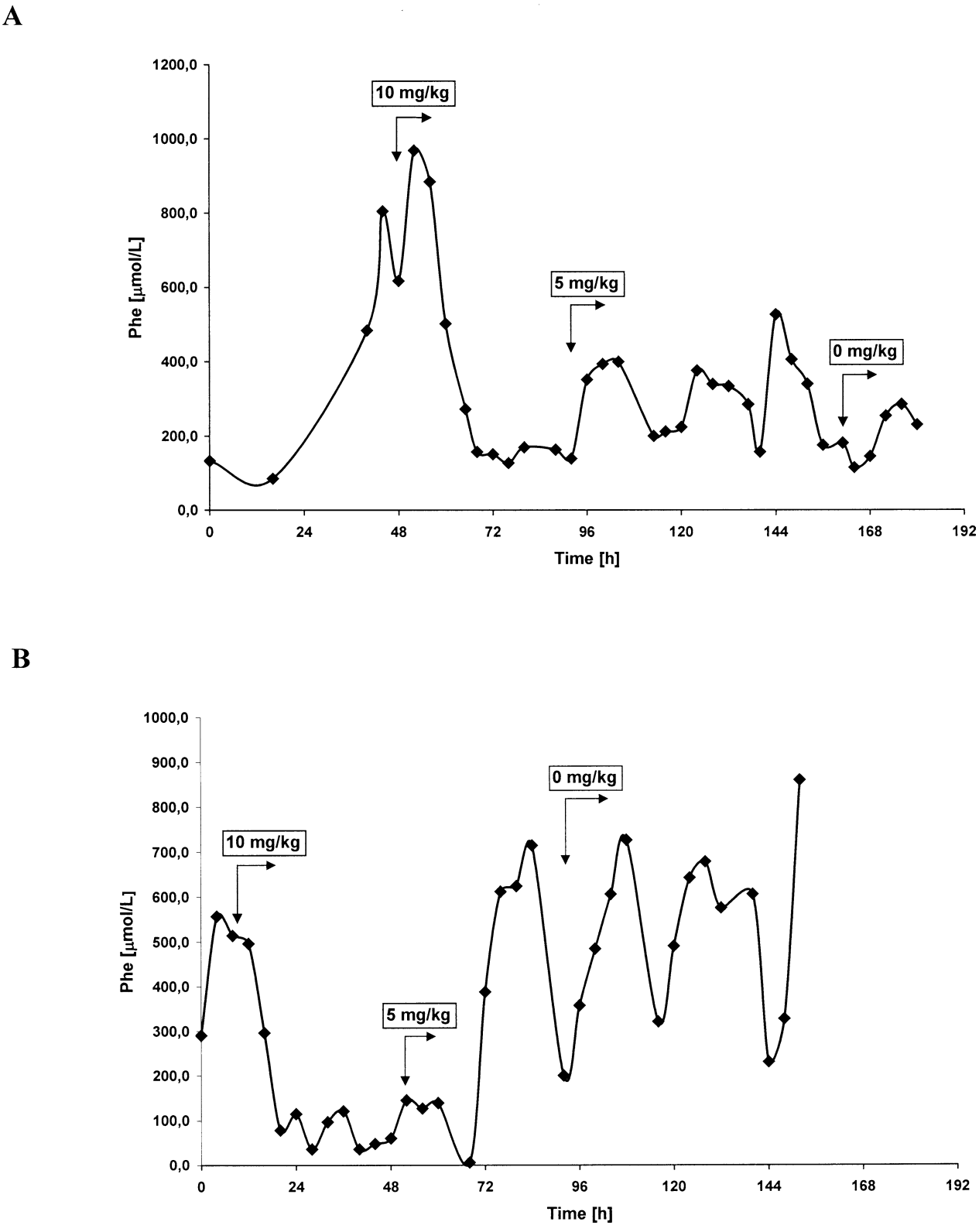

Fig. 2. BH_4-titration curves for patients BS (**A**) and LW (**B**). The daily protein intake has been 100–150 mg per kg b.w. for both patients. Samples have been taken and analysed for phenylalanine content approx. every four hours before another dose of BH_4 has been administered. BH_4 dosage is shown in boxes above the curve and proceeds unaltered until a change has been indicated. Patient BS responded well to doses as low as 5 mg/kg*d of BH_4 (**A**). In contrast, patient LW showed no response to a 5 mg/kg*d dose of BH_4 despite having low and stable blood phenylalanine levels at a dose of 10 mg/kg*d (**B**). This difference in responsiveness to BH_4 is also reflected in the longer delay for patient BS until blood phenylalanine levels rise after the end of treatment

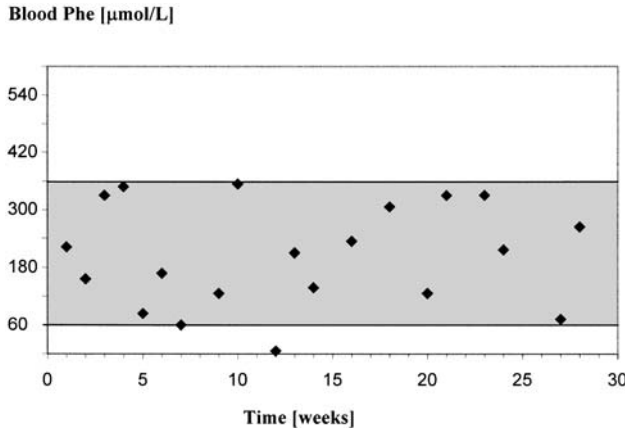

Fig. 3. Patient BS was supplemented with 10 mg/kg BH_4 per day given in three single doses. No dietary restrictions were applied. Blood phenylalanine levels were determined once a week. Phenylalanine values have been kept in the desired range of 60 to 360 μmol/L (shaded area) over a period of 7 months by this regimen alone

show a compensatory effect of high doses of BH_4 it is unlikely that tyrosine hydroxylase or other hydroxylases are stimulated to replace PAH in case of its deficiency.

We have found one patient, LW, with BH_4 sensitivity who is homozygous for the Y414C mutation. Functionally, residue Y414C is important for keeping the tetramerization domain of the enzyme close to the catalytic domain. Though, the Y414C mutation has been reported to be the second most common in PKU patients of northern Europe, its association with BH_4-responsiveness has been reported only very recently. Further, various degrees of BH_4-responsiveness were described for patients hemizygous for Y414C (Lindner, 2001). This indicates that BH_4-responsiveness is not strictly correlated to the PAH genotype.

Patient BS is compound heterozygous for the mutations A104D and K320N. The former affects structural features of the regulatory domain, whereas the latter is located on the surface of the protein towards the end of the catalytic domain with no predictable effect of the substitution. In addition, mutations of the other three mildly affected patients as well as mutations reported by Kure et al. for patients with BH_4-responsive PAH deficiency show no conclusive pattern (Kure, 1999). Three of the latter, namely Y204C, R241C and R413P are situated on the surface of the protein with no interactions to other amino acids and merely the mutation R243Q is located in the catalytic domain. Since no defined structural motif responsible for BH_4 sensitivity can be recognized and since the same PAH genotype is not consistently responsive to BH_4, additional factors independent of the PAH genotype and thus independent of primary and secondary PAH protein structures have to determine BH_4-responsiveness. In fact, two large studies on genotype-phenotype correlation revealed several PAH alleles with inconsistent phenotypes (Guldberg, 1998; Kayaalp, 1997).

The importance of chaperone-assisted folding in the determination of the tertiary and quarternary structures of mutant proteins and hence in the phenotypic expression of many inborn errors of metabolism has been well documented (Gregersen, 2001). PAH deficiency belongs to this group of protein folding disorders for which largely the interaction between structural alterations in the mutant PAH protein and cellular quality control system determines the residual enzyme activity. In the following, we present a model how BH_4 might influence the tertiary and quarternary structure of mutant PAH proteins and hence may confer enhanced residual enzymatic activity.

Regulatory binding sites for Phe and BH_4 have been deduced from kinetic studies of PAH (Philips and Kaufman, 1984; Xia, 1994). Binding of BH_4 to wild type PAH in the absence or prior to Phe binding inhibits PAH tetramerization and favours a low-activity dimeric conformation. It has been described that physical destruction of PAH tetramers by low doses of irradiation results in BH_4-dependent activation of PAH (Davis, 1996 and 1997). Therefore, it can be concluded that BH_4 may activate mutant PAH by stabilizing dimeric PAH conformations if BH_4 concentrations are high enough to assure binding to its regulatory site prior to Phe binding. BH_4 induced conformational changes of the mutant PAH dimer may either delay its degradation by the cellular quality control system or directly increase residual enzymatic activity.

Our suggested mechanism is compatible with several observations concerning BH_4-responsiveness. The delayed response to BH_4 intake can be explained by the need for newly synthesized PAH that is not in its tetrameric structure and can be activated by BH_4. Milder cases of PKU are more likely to be responsive to BH_4 treatment, since lower Phe levels and higher amounts of residual PAH can be expected. The required BH_4 dose is dependent on the concentration

of Phe and of available PAH, hence BH_4 must overcome a certain threshold to be activating. Finally, the persisting effect of BH_4 for up to 24 h after ceasing its supplementation might indicate a reduced degradation rate of mutant PAH or of the cofactor when it is associated to the protein.

In summary, we have observed BH_4-responsiveness with various degrees of PAH deficiency, including mild HPA, variant PKU and classical PKU. BH_4-responsiveness occurs more frequently in milder phenotypes of PAH deficiency (Blau, personal communication). Variant PKU (as observed for patient LW and BS) can be treated with BH_4 monotherapy; patients with classical PKU should require a combination of BH_4 treatment and protein restriction but may benefit from BH_4 application. Daily BH_4 supplementation ranging from 5 to 20 mg per kg b.w. have to be applied and optimal doses have to be elucidated for each patient individually whereby, the more severe PKU phenotypes will require higher doses of BH_4 than milder cases. Our findings can be explained by a comprehensive model of PAH activation by BH_4. Hereby, binding of BH_4 to a regulatory site induces changes in the tertiary and quarternary structure yielding increased enzymatic turnover when preceded by a certain folding of PAH monomers. The resulting overall conformational change may either stabilize PAH dimers by preventing their degradation or may increase the intrinsic enzymatic activity of these dimers. Further research is required to validate our suggested mechanism of BH_4-responsiveness in PAH defects and might facilitate new strategies in treatment of inherited metabolic diseases.

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