

## TETRAHYDROBIOPTERIN AS AN ALTERNATIVE TREATMENT FOR MILD PHENYLKETONURIA

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### ABSTRACT

**Background** Hyperphenylalaninemia is a common inherited metabolic disease that is due to phenylalanine hydroxylase deficiency, and at least half the affected patients have mild clinical phenotypes. Treatment with a low-phenylalanine diet represents a substantial psychosocial burden, but alternative treatments have not been effective.

**Methods** To explore the therapeutic efficacy of tetrahydrobiopterin, we performed a combined phenylalanine-tetrahydrobiopterin loading test and analyzed the in vivo rates of [<sup>13</sup>C]phenylalanine oxidation in 38 children with phenylalanine hydroxylase deficiency (age range, 1 day to 17 years). We assessed whether responsiveness to tetrahydrobiopterin was associated with specific genotypes, and we mapped mutations using a structural model of the phenylalanine hydroxylase monomer.

**Results** In 27 (87 percent) of 31 patients with mild hyperphenylalaninemia (10 patients) or mild phenylketonuria (21 patients), tetrahydrobiopterin significantly lowered blood phenylalanine levels. Phenylalanine oxidation was significantly enhanced in 23 of these 31 patients (74 percent). Conversely, none of the seven patients with classic phenylketonuria had a response to tetrahydrobiopterin as defined in this study. Long-term treatment with tetrahydrobiopterin in five children increased daily phenylalanine tolerance, allowing them to discontinue their restricted diets. Seven mutations (P314S, Y417H, V177M, V245A, A300S, E390G, and IVS4-5C→G) were classified as probably associated with responsiveness to tetrahydrobiopterin, and six mutations (A403V, F39L, D415N, S310Y, R158Q, and I65T) were classified as potentially associated. Four mutations (Y414C, L48S, R261Q, and I65V) were inconsistently associated with this phenotype. Mutations connected to tetrahydrobiopterin responsiveness were predominantly in the catalytic domain of the protein and were not directly involved in cofactor binding.

**Conclusions** Tetrahydrobiopterin responsiveness is common in patients with mild hyperphenylalaninemia phenotypes. Responsiveness cannot consistently be predicted on the basis of genotype, particularly in compound heterozygotes. (N Engl J Med 2002;347:2122-32.)

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**H**YPERPHENYLALANINEMIA, a common inherited metabolic disease, was one of the first genetic disorders that could be treated.<sup>1</sup> In most cases, hyperphenylalaninemia results from phenylalanine hydroxylase deficiency due to mutations in the phenylalanine hydroxylase gene.<sup>2</sup> The associated phenotypes range in severity from classic phenylketonuria (Online Mendelian Inheritance in Man number 261600) to mild phenylketonuria and mild hyperphenylalaninemia. At least half of affected patients have one of the milder clinical phenotypes. Patients with both classic and mild phenylketonuria require lifelong dietary protein restriction to prevent neurologic sequelae and to ensure normal cognitive development, whereas patients with mild hyperphenylalaninemia may not require treatment.<sup>3</sup> The highly restrictive diet is associated with a risk of nutritional deficiencies and represents a burden for the patients and their families. Therefore, a search for non-dietary treatment alternatives has been encouraged.<sup>4</sup>

In approximately 50 genetic diseases of humans involving enzyme deficiencies, treatment with high doses of a cofactor can increase enzyme activity.<sup>5</sup> Tetrahydrobiopterin is a natural cofactor of aromatic amino acid hydroxylases and nitric oxide synthase. Supplementation with this compound is an established treatment for the rare patients with hyperphenylalaninemia that is due to defects in the biosynthesis of tetrahydrobiopterin.<sup>6,7</sup> However, more than 98 percent of patients with hyperphenylalaninemia have mutations in the phenylalanine hydroxylase gene, and they have elevated rather than decreased plasma concentrations of biopterin owing to the action of guanosine triphosphate cyclohydrolase I feedback regulatory protein.<sup>8</sup> The therapeutic use of tetrahydrobiopterin in patients with phenylalanine hydroxylase deficiency had therefore not been considered.

Recently, however, individual patients with mutations in the phenylalanine hydroxylase gene have been shown to have a decrease in blood phenylalanine con-

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centrations after tetrahydrobiopterin loading.<sup>9-13</sup> Phenylalanine concentrations in the peripheral circulation, however, are governed by various genetic loci and modifying factors,<sup>1,14</sup> and there is no evidence that the beneficial effect of tetrahydrobiopterin occurs at the level of phenylalanine hydroxylation. Therefore, we prospectively studied 38 children with phenylalanine hydroxylase deficiency in an effort to determine the frequency of sensitivity to tetrahydrobiopterin in these patients, whether tetrahydrobiopterin restores their oxidative capacity for phenylalanine, whether responsiveness to tetrahydrobiopterin is related to specific genotypes, and whether associated mutations map to distinct regions of the protein.

## METHODS

### Patients

The study was conducted from December 2000 through December 2001 and was approved by the medical board of the Children's Hospital Research Center. We obtained written informed consent from the families of 38 children with various classes of hyperphenylalaninemia stratified according to the plasma phenylalanine concentration before treatment (normal, 30 to 120  $\mu\text{mol}$  per liter): 10 patients had mild hyperphenylalaninemia (phenylalanine, less than 600  $\mu\text{mol}$  per liter; age, 15 days to 10 years), 21 had mild phenylketonuria (phenylalanine, 600 to 1200  $\mu\text{mol}$  per liter; age, 8 days to 17 years), and 7 had classic phenylketonuria (phenylalanine, more than 1200  $\mu\text{mol}$  per liter; age, 1 day to 9 years). A defect in the synthesis or recycling of tetrahydrobiopterin was excluded by analysis of urinary pterins and dihydropteridine reductase activity in erythrocytes. We analyzed 7 patients during the newborn period and 31 at older ages. Five affected siblings from four families were included, because nongenetic factors are known to influence phenylalanine homeostasis. Mean daily phenylalanine tolerance was determined by calculating the dietary phenylalanine intake according to nutritional protocols.

### Combined Phenylalanine and Tetrahydrobiopterin Loading Test

Phenylalanine loading was accomplished by having patients consume a meal containing 100 mg of phenylalanine per kilogram of body weight. One hour after the end of the meal the patients ingested 20 mg of tetrahydrobiopterin per kilogram (Schircks Laboratories). Blood phenylalanine concentrations were determined by electrospray ionization–tandem mass spectrometry before phenylalanine loading and before and 4, 8, and 15 hours after the tetrahydrobiopterin challenge. During the test period newborns were breast-fed, while older children received a standardized protein intake (10 mg of phenylalanine per kilogram) between six and eight hours after the challenge with tetrahydrobiopterin.

### In Vivo Analysis of Phenylalanine Oxidation

The rate of phenylalanine oxidation was determined twice (on two different days) in each child — once without treatment and once during treatment with tetrahydrobiopterin (10 mg per kilogram over a 24-hour period). The tests were performed after a four-hour fast in infants and an overnight fast in older children. A total of 6 mg per kilogram of L-[1-<sup>13</sup>C]phenylalanine (Euriostop), dissolved in a 25 percent dextrose solution (2 mg per milliliter), was given orally. Breath samples were subsequently collected over a period of 180 minutes and stored in evacuated glass tubes until analysis by isotope-ratio mass spectrometry (deltaS, Thermoquest). The recovery of carbon-13 in breath samples was calculated as described

by Treacy et al.,<sup>15</sup> assuming a total carbon dioxide production of 300 mmol per hour per square meter of body-surface area.<sup>16,17</sup> The amount of labeled carbon dioxide formed was expressed as the cumulative percentage of the dose administered as a function of time. The validity of results in newborns might be influenced by the diet or by the fact that breath sampling is more challenging than in older subjects. The base-line percentage of carbon-13 measured at time 0, however, did not differ significantly between newborns and older children. Values were considered to be below the limit of detection when the signal intensity of the atom excess (expressed as a percentage at time t, obtained by subtraction of the mean base-line value) did not allow sufficient distinction from atmospheric carbon-13 dioxide. On average, fewer than 1 of 27 consecutive measurements of carbon dioxide obtained during the 180 minutes of individual testing was uninterpretable in older children and fewer than 2 of 27 were uninterpretable in newborns, and these variations had a negligible influence on the final calculation. For the comparison among patients, we normalized the data by expressing individual results as a percentage of the mean value for the control group, which consisted of 12 healthy children (age, 2 days to 13 years).

### Mutational Analysis

DNA was extracted from leukocytes according to standard protocols. Thirteen genomic fragments covering the entire coding sequence and the exon-flanking intronic sequences of the phenylalanine hydroxylase gene were amplified by the polymerase chain reaction followed by direct sequencing.<sup>18</sup>

### Mapping of Phenylalanine Hydroxylase Gene Mutations

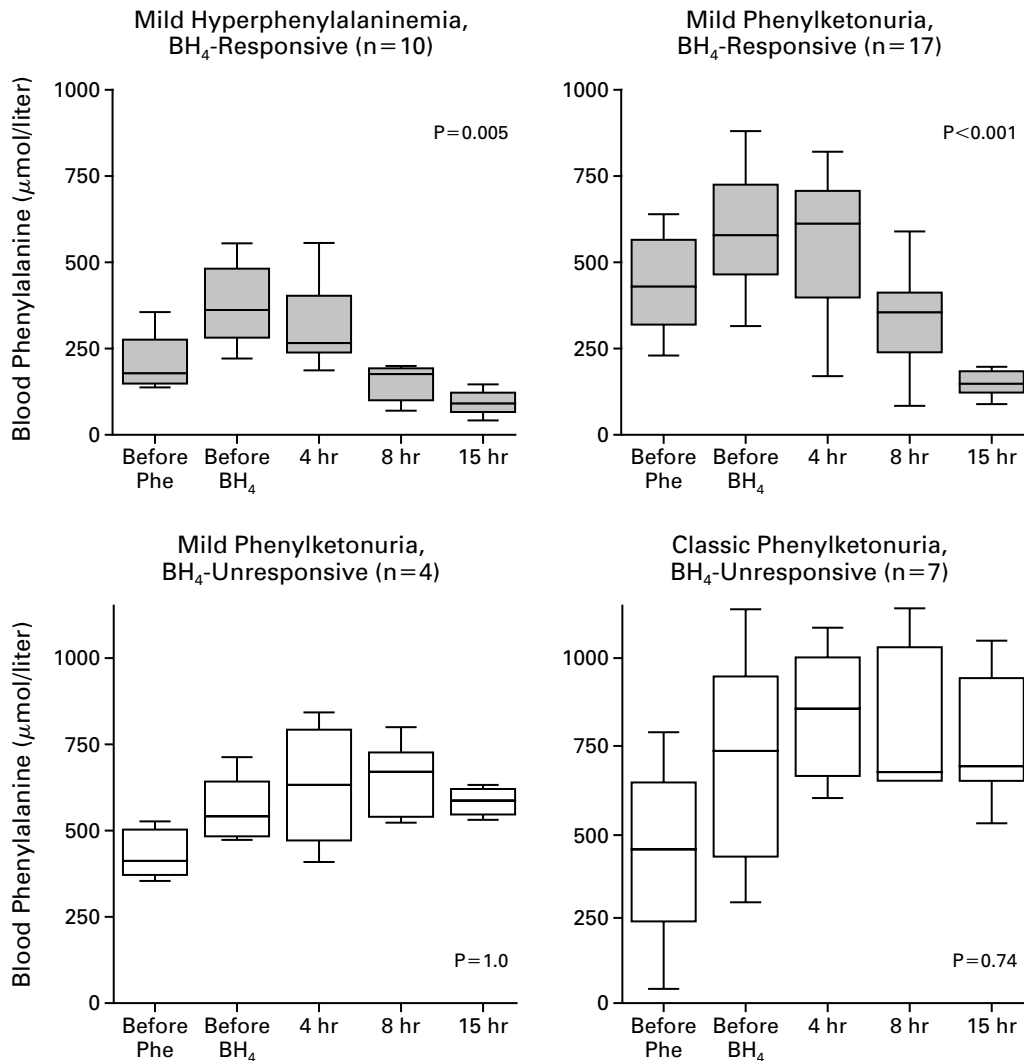
We constructed a model of the full-length, tetrahydrobiopterin-bound phenylalanine hydroxylase monomer from the crystal structures of several truncated forms<sup>19-22</sup> by superimposing the catalytic domains using the tools provided by SWISS-MODEL/Swiss-Pdb Viewer.<sup>23</sup>

## RESULTS

### Effects of Tetrahydrobiopterin on Blood Phenylalanine Levels and Rates of Phenylalanine Oxidation

Patients were classified as responsive to tetrahydrobiopterin when blood phenylalanine levels 15 hours after tetrahydrobiopterin challenge had decreased by more than 30 percent from the value obtained before the administration of tetrahydrobiopterin. An improvement in the rate of phenylalanine oxidation was considered to be significant when supplementation with tetrahydrobiopterin increased the individual normalized value by at least 15 percent. Tetrahydrobiopterin sensitivity was observed during the loading test in all 10 patients with mild hyperphenylalaninemia and in 17 of 21 patients with mild phenylketonuria (27 of 31, or 87 percent). Only four patients with mild phenylketonuria and all seven patients with classic phenylketonuria did not fulfill the criterion of responsiveness to tetrahydrobiopterin (Fig. 1). Some patients had a rapid decrease in phenylalanine resembling that seen in patients with defects in the synthesis of tetrahydrobiopterin, whereas others had a slow response, which reached a maximum 15 hours after the administration of the cofactor (data not shown).

The basal cumulative recovery of labeled carbon dioxide reflected the various levels of residual phenyl-

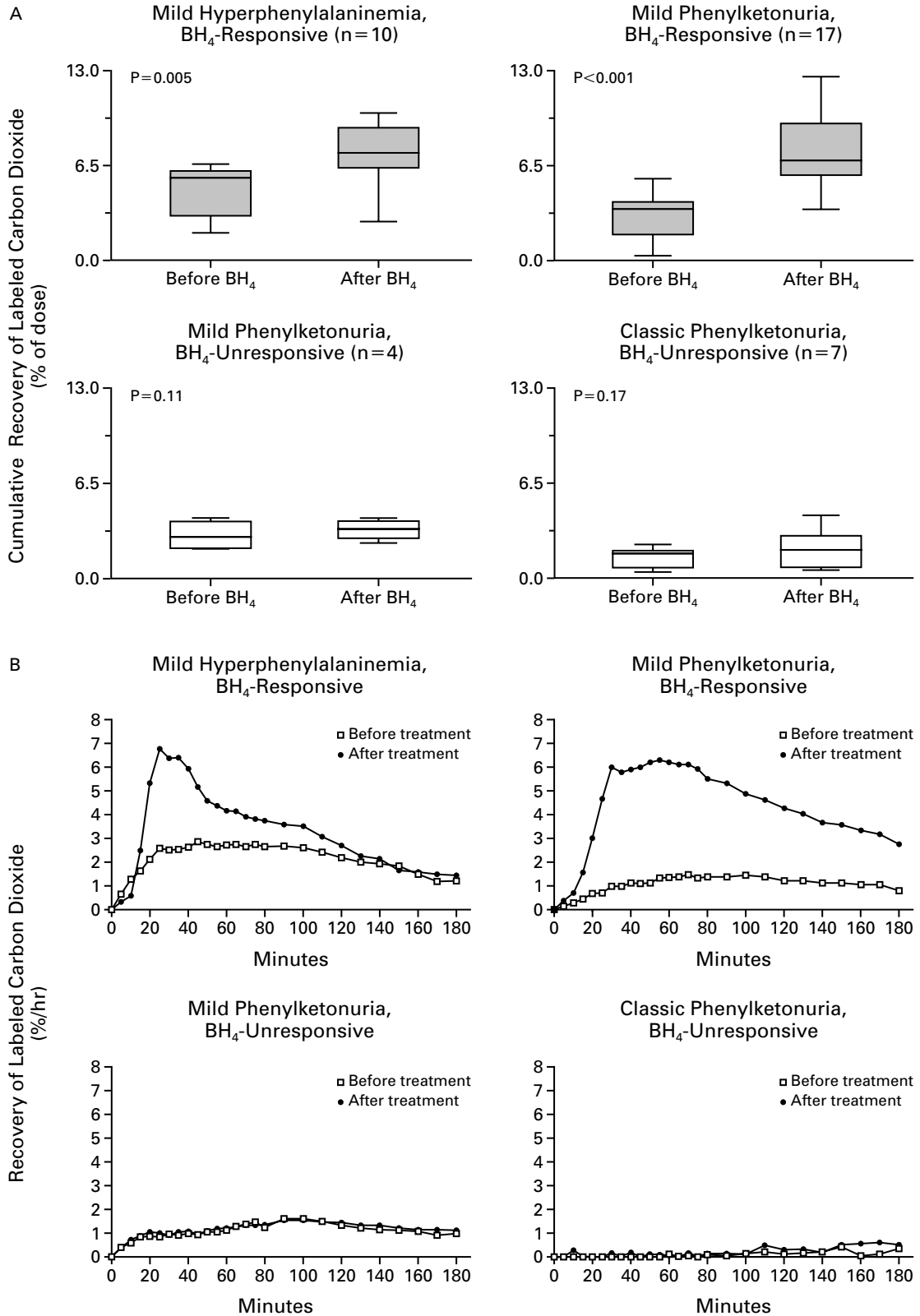


**Figure 1.** Blood Phenylalanine Concentrations before Phenylalanine Loading (Phe) and before and after Challenge with Tetrahydrobiopterin (BH<sub>4</sub>).

The boxes show the interquartile ranges (25th to 75th percentiles), the horizontal black bars represent the medians, and the I bars indicate the range. The P value refers to the difference between the blood phenylalanine concentration before and 15 hours after the administration of tetrahydrobiopterin.

**Figure 2 (facing page).** Effect of Short-Term Treatment with Tetrahydrobiopterin (BH<sub>4</sub>) on Phenylalanine Oxidation.

Panel A shows the cumulative recovery of labeled carbon dioxide during the 180 minutes after the ingestion of labeled phenylalanine before and after tetrahydrobiopterin treatment. The boxes show the interquartile ranges (25th to 75th percentile), the horizontal black bars represent the medians, and the I bars indicate the range. The P value refers to the difference between the phenylalanine oxidation values before and after tetrahydrobiopterin treatment. Panel B shows the fractional formation rate of labeled carbon dioxide during the 180 minutes after the ingestion of labeled phenylalanine in four representative patients with impaired phenylalanine hydroxylase activity before and after treatment with tetrahydrobiopterin.

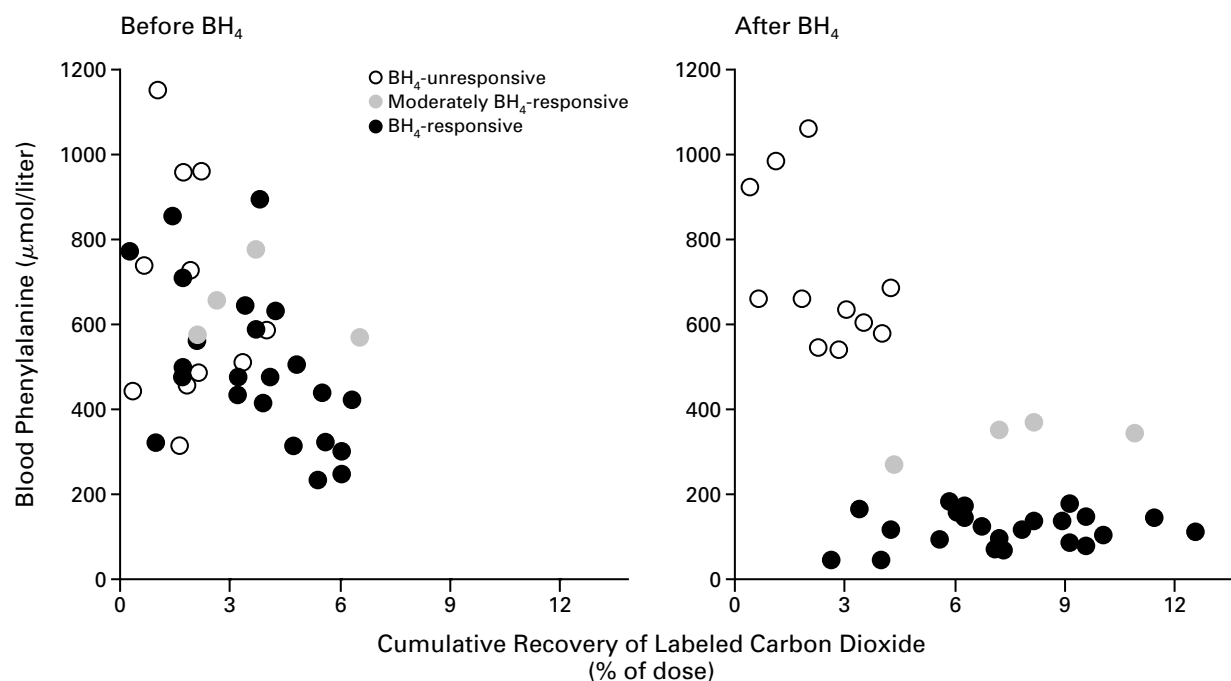


alanine oxidation and ranged from a mean ( $\pm$ SD) of  $1.4\pm 0.7$  percent in the 7 patients with classic phenylketonuria to  $3.0\pm 1.4$  percent in the 21 patients with mild phenylketonuria and to  $4.8\pm 1.8$  percent in the 10 patients with mild hyperphenylalaninemia (mean value in 12 healthy controls,  $8.3\pm 2.8$  percent). During treatment with tetrahydrobiopterin (10 mg per kilogram over 24 hours), the cumulative recovery of labeled carbon dioxide significantly increased in the same groups that had had a response to the loading test. The increase was more pronounced in those with mild phenylketonuria than in those with mild hyperphenylalaninemia (Fig. 2A). The curves of the fractional formation of labeled carbon dioxide deviated markedly from that of the normal-oxidation phenotype (Fig. 2B). With cofactor treatment, the curves reverted toward normal in patients who had a response to tetrahydrobiopterin but remained unchanged in patients who did not have a response (Fig. 2B).

Before tetrahydrobiopterin treatment, all patients had blood phenylalanine concentrations above  $200\ \mu\text{mol}$  per liter and cumulative rates of recovery of labeled carbon dioxide below 7 percent, with considerable overlap between patients with a response and those without a response. After tetrahydrobiopterin treatment, the values in these two groups no longer overlapped (Fig. 3).

The degree of intersubject variability was large: tetrahydrobiopterin challenge reduced phenylalanine levels by 37 to 92 percent when blood values were compared before and 15 hours after the administration of tetrahydrobiopterin. In 23 of 27 patients with a response to tetrahydrobiopterin, blood phenylalanine concentrations decreased below  $200\ \mu\text{mol}$  per liter, whereas in 4 patients the response was moderate, and values were between  $200$  and  $400\ \mu\text{mol}$  per liter. In patients with no response, blood phenylalanine concentrations always exceeded  $400\ \mu\text{mol}$  per liter after tetrahydrobiopterin challenge. Tetrahydrobiopterin enhanced the oxidation rates of labeled phenylalanine by 10 to 91 percent and resulted in rates within the normal range in 22 of the 27 patients with a response to tetrahydrobiopterin. The remaining five patients had an improvement, but the rates did not reach the normal range. In 33 of 38 patients we observed full concordance between the two end points analyzed.

Values for individual patients and examples of notable imbalances in the effect of tetrahydrobiopterin are shown in Figure 4. In four patients (Patients 2, 4, 7, and 26) who were responsive to tetrahydrobiopterin, the normalized increase in phenylalanine oxidation was in the range of 8 to 14 percent (data not shown) and therefore below our predefined level of significance. One patient with classic phenylketonuria (Patient 35)



**Figure 3.** Relation between the Cumulative Recovery of Labeled Carbon Dioxide during the 180 Minutes after the Ingestion of Labeled Phenylalanine and the Blood Phenylalanine Concentration before and after the Administration of Tetrahydrobiopterin ( $\text{BH}_4$ ), According to the Response to Tetrahydrobiopterin.

had a slight decrease in the blood phenylalanine concentration that did not fulfill the criterion of responsiveness, whereas the increase in the oxidation rate (24 percent) was significant. Notably, 7 of the 11 patients who did not meet the criterion of responsiveness in the loading test had a slight increase in the rate of phenylalanine oxidation (range, 2 to 14 percent) with short-term treatment with tetrahydrobiopterin.

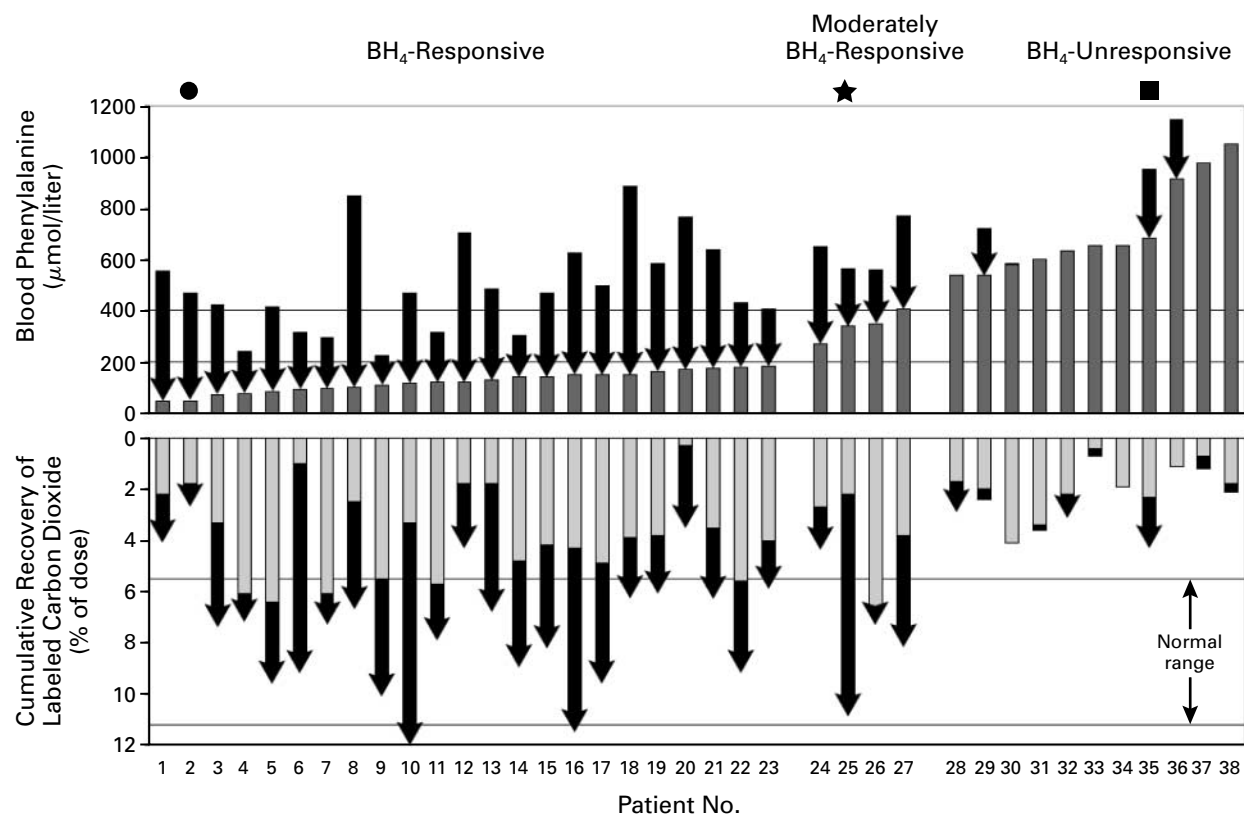
**Long-Term Treatment with Tetrahydrobiopterin**

The parents of five children with mild phenylketonuria (age, 4 to 14 years) provided written informed consent for their children to participate in a therapeutic trial replacing dietary phenylalanine restriction with the oral administration of tetrahydrobiopterin. Cofactor treatment at daily doses of 7.1 to 10.7 mg per kil-

ogram for a mean of  $207.0 \pm 51.3$  days (range, 166 to 263) led to an increase in the mean daily phenylalanine tolerance, from  $18.7 \pm 8.6$  mg per kilogram (range, 8.5 to 30.0) before treatment to  $61.4 \pm 27.9$  mg per kilogram (range, 17.9 to 90.0) during treatment ( $P = 0.04$ ), with little effect on the mean blood concentrations of phenylalanine ( $366 \pm 120 \mu\text{mol}$  per liter during dietary treatment and  $378 \pm 173 \mu\text{mol}$  per liter during cofactor treatment).

**Identification and Mapping of Phenylalanine Hydroxylase Gene Mutations**

In 37 of 38 patients, two mutant alleles were identified (Table 1). We classified seven mutations (P314S, Y417H, V177M, V245A, A300S, E390G, and IVS4-5C→G) as probably responsible for responsiveness to



**Figure 4.** Effect of Tetrahydrobiopterin (BH<sub>4</sub>) on Peripheral Phenylalanine Clearance and Oxidation Rates in the 38 Patients with Hyperphenylalaninemia.

The upper graph shows blood phenylalanine concentrations before (bars and arrows) and 15 hours after the administration of tetrahydrobiopterin (bars alone). The lower graph shows the cumulative recovery of labeled carbon dioxide after the ingestion of labeled phenylalanine before (bars alone) and after (bars and arrows) tetrahydrobiopterin treatment. The normal range of phenylalanine oxidation in 12 healthy controls (age range, 2 days to 13 years) is shown (mean [ $\pm$ SD],  $8.3 \pm 2.8$  percent). The benefit resulting from tetrahydrobiopterin treatment in individual patients is depicted by a black arrow in both panels. Two examples of an imbalance in the effect of tetrahydrobiopterin are indicated by the circle (a distinct decrease in blood phenylalanine concentrations but little enhancement of phenylalanine oxidation) and the star (little effect on the blood phenylalanine concentration and a large increase in phenylalanine oxidation). The square indicates one patient with classic phenylketonuria who had a slight response to tetrahydrobiopterin that missed the criterion of tetrahydrobiopterin responsiveness.

**TABLE 1.** GENOTYPES IN 38 PATIENTS WITH HYPERPHENYLALANINEMIA, ACCORDING TO WHETHER THEY WERE RESPONSIVE TO TETRAHYDROBIOPTERIN.

PATIENT No.*	ALLELE 1	ALLELE 2	PHENOTYPE	RESPONSIVE TO TETRAHYDRO-BIOPTERIN
1	A403V†	IVS4+5G→T	Mild hyperphenylalaninemia	Yes
2	A403V†	Not identified	Mild hyperphenylalaninemia	Yes
3	P314S‡§	R408W¶	Mild hyperphenylalaninemia	Yes
4	F39L†	D415N†	Mild hyperphenylalaninemia	Yes
5	Y414C	D415N†	Mild hyperphenylalaninemia	Yes
6	Y417H‡§	Y417H‡§	Mild phenylketonuria	Yes
7	F55L	S310Y†§	Mild hyperphenylalaninemia	Yes
8	R261Q	Y414C	Mild phenylketonuria	Yes
9	V177M‡	R408W¶	Mild hyperphenylalaninemia	Yes
10	P275L§	Y414C	Mild phenylketonuria	Yes
11	V245A‡	R408W¶	Mild hyperphenylalaninemia	Yes
12	L48S	R158Q†	Mild phenylketonuria	Yes
13	Y417H‡§	Y417H‡§	Mild phenylketonuria	Yes
14	V245A‡	R408W¶	Mild hyperphenylalaninemia	Yes
15	R261X¶	A300S‡	Mild phenylketonuria	Yes
16	R158Q†	E390G‡	Mild phenylketonuria	Yes
17	R261X¶	A300S‡	Mild phenylketonuria	Yes
18	Y414C	IVS12+1G→A¶	Mild phenylketonuria	Yes
19	I65S§, H170Q§	A300S‡	Mild phenylketonuria	Yes
20	R261Q	Y414C	Mild phenylketonuria	Yes
21	K274fsdel11bp¶	E390G‡	Mild phenylketonuria	Yes
22	IVS4-5C→G‡	R408W¶	Mild phenylketonuria	Yes
23	R261X¶	A300S‡	Mild phenylketonuria	Yes
24	I65T†	Y414C	Mild phenylketonuria	Moderately
25	E390G‡	IVS12+1G→A¶	Mild phenylketonuria	Moderately
26	I65V	R261Q	Mild hyperphenylalaninemia	Moderately
27	R158Q†	Y414C	Mild phenylketonuria	Moderately
28	Y414C	IVS12+1G→A¶	Classic phenylketonuria	No
29	P281L¶	Y414C	Mild phenylketonuria	No
30	I65V	IVS12+1G→A¶	Mild phenylketonuria	No
31	I65V	IVS12+1G→A¶	Mild phenylketonuria	No
32	N61D§	R261Q	Mild phenylketonuria	No
33	R408W¶, R413P	Y414C	Classic phenylketonuria	No
34	P281L¶	P281L¶	Classic phenylketonuria	No
35	R243X¶	Y414C	Classic phenylketonuria	No
36	L48S	P281L¶	Classic phenylketonuria	No
37	R261Q	R408W¶	Classic phenylketonuria	No
38	R243X¶	IVS7+1G→A	Classic phenylketonuria	No

\*Patient numbers correspond to those in Figure 4.

†This mutation was potentially associated with responsiveness to tetrahydrobiopterin.

‡This mutation was probably associated with responsiveness to tetrahydrobiopterin.

§This mutation has not been described previously.

¶This is a putative null mutation.

||This mutation was inconsistently associated with responsiveness to tetrahydrobiopterin.

tetrahydrobiopterin, because they were present in either the homozygous or a functional hemizygous state. Six additional mutations were potentially connected to tetrahydrobiopterin responsiveness because of considerable residual in vitro enzyme activity (A403V, F39L, D415N, R158Q, and I65T), as previously proposed,<sup>24</sup> or (in the case of S310Y) because of a known

severe mutation on the second allele. Four mutations (Y414C, L48S, R261Q, and I65V) were inconsistently associated with tetrahydrobiopterin responsiveness. Eight of 12 missense mutations connected to tetrahydrobiopterin responsiveness mapped to the catalytic domain, whereas 2 mapped to the regulatory domain and 2 to the tetramerization domain. None of them

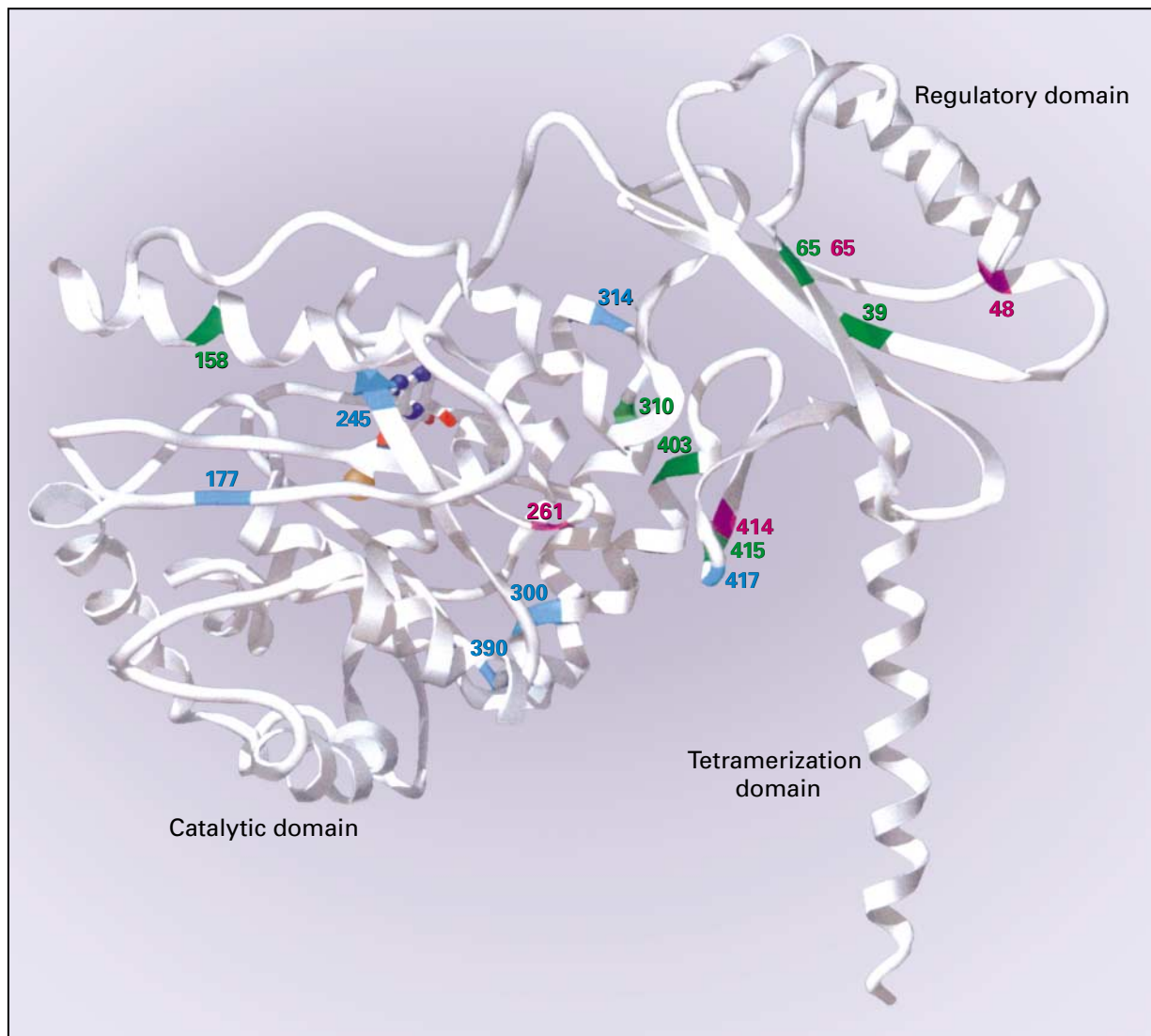
affected residues at the active site or amino acids that interacted directly with the cofactor (Fig. 5).

### DISCUSSION

We present two lines of evidence that the metabolic phenotype of phenylalanine hydroxylase deficiency can be modified by pharmacologic doses of tetrahydrobiopterin. First, tetrahydrobiopterin loading led to

normal or nearly normal blood phenylalanine concentrations in most patients with residual phenylalanine hydroxylase activity, suggesting that responsiveness to tetrahydrobiopterin is a common feature of mild hyperphenylalaninemia phenotypes. Second, tetrahydrobiopterin enhanced residual phenylalanine oxidative capacity in these patient groups.

Our findings suggest that the in vivo phenylalanine



**Figure 5.** Structural Localization of Phenylalanine Hydroxylase Missense Mutations.

The phenylalanine hydroxylase monomer, shown as a ribbon, is composed of three functional domains: the regulatory domain (residues 1 to 142), the catalytic domain (residues 143 to 410), and the tetramerization domain (residues 411 to 452). The active-site iron (partially obscured brown sphere) and the cofactor analogue 7,8-dihydro-tetrahydrobiopterin (stick model) are in the catalytic domain. Mutations that are probably associated with responsiveness to tetrahydrobiopterin are blue. Mutations that are potentially associated with responsiveness to tetrahydrobiopterin are green. Mutations that are inconsistently associated with responsiveness to tetrahydrobiopterin are magenta. The mutations at residue 65 may be either potentially or inconsistently associated with responsiveness to tetrahydrobiopterin.

oxidation test can discriminate among classes of hyperphenylalaninemia of different severity. This observation is in accordance with data on the ability of the method to measure the dose effects of the phenylalanine hydroxylase gene.<sup>15</sup> However, because of the multifactorial nature of hyperphenylalaninemia,<sup>25,26</sup> the whole-body rate of phenylalanine oxidation is not a simple equivalent of phenylalanine hydroxylase activity. The decrease in blood phenylalanine concentrations was accompanied by a significant increase in phenylalanine oxidative capacity in the majority of patients who were identified as responsive to tetrahydrobiopterin. Taken together, these observations are consistent with the hypothesis that impaired phenylalanine hydroxylation is corrected by tetrahydrobiopterin therapy.

The extent of the fractional change in the disposal of phenylalanine did not always correspond to the change in phenylalanine oxidation — a finding not unexpected with respect to genetically determined enzyme deficiencies in general<sup>27</sup> and phenylalanine hydroxylase deficiency in particular.<sup>26</sup> We observed slow and rapid responses as well as differences in the time course and relative extent of formation of labeled carbon dioxide, suggesting that tetrahydrobiopterin may exert its effects through various mechanisms and with different degrees of efficacy. In addition to the proposal that high-dose tetrahydrobiopterin treatment may compensate for the decreased affinity of the mutant phenylalanine hydroxylase for tetrahydrobiopterin,<sup>28</sup> other mechanisms need to be considered. Tetrahydrobiopterin treatment may up-regulate the expression of the phenylalanine hydroxylase gene,<sup>24</sup> stabilize phenylalanine hydroxylase messenger RNA,<sup>29</sup> facilitate the formation of functional phenylalanine hydroxylase tetramers, or protect a misfolded enzyme protein from proteolytic cleavage.<sup>30,31</sup>

The use of genotyping to predict the phenotype may present difficulties in the case of complex traits such as hyperphenylalaninemia,<sup>32</sup> particularly in compound heterozygotes. We identified predominantly “mild” genotypes in the group of patients with a response to tetrahydrobiopterin, whereas most of the patients without a response had “severe” genotypes.<sup>2</sup> The weight of the evidence of the association of distinct mutations with responsiveness to tetrahydrobiopterin varied. The Y414C mutation occurs in more than one clinical phenotype.<sup>33,34</sup> We and others<sup>12</sup> have identified this mutation in a functional hemizygous state in two patients with identical genotypes but discordant responses to tetrahydrobiopterin. This observation may be explained by the influences of modifying loci in hyperphenylalaninemia, since this trait is polygenic.<sup>1</sup> In a homozygous state, and thus one in which homopolymeric tetramers are formed, the Y414C and the L48S mutations were reported to confer respon-

siveness to tetrahydrobiopterin.<sup>24,35</sup> However, we detected these mutations in a functional hemizygous state in patients with classic phenylketonuria who had no response to tetrahydrobiopterin. Under these conditions, heteropolymerization may impede the formation of functional tetramers.

Our data confirm that most missense mutations associated with sensitivity to tetrahydrobiopterin are in the catalytic domain of the protein, but they do not map to residues at the active site and are not directly involved in cofactor binding.<sup>28</sup> These mutations may affect interactions between domains in a monomer or influence residues in the dimer or tetramer interfaces,<sup>36</sup> resulting in the misfolding of the protein and reduced enzyme activity. Tetrahydrobiopterin may act as a chemical chaperone and thus prevent misfolding.

In vitro expression analysis has been used to predict the functional effect in vivo of mutations in the phenylalanine hydroxylase gene.<sup>37-39</sup> This type of analysis may result in the overestimation of phenylalanine hydroxylase activity in vitro,<sup>38</sup> perhaps because such analyses have been carried out almost exclusively in the presence of high concentrations of natural or synthetic cofactors,<sup>2</sup> thereby contributing to genotype-phenotype inconsistencies.<sup>33</sup> Revised experimental protocols to assess the intrinsic severity of mutations should include a range of tetrahydrobiopterin concentrations.<sup>40</sup>

Since responsiveness to tetrahydrobiopterin cannot be predicted on the basis of pretreatment phenylalanine concentrations, we would suggest a new clinical classification: tetrahydrobiopterin-unresponsive hyperphenylalaninemia and tetrahydrobiopterin-responsive hyperphenylalaninemia, which includes tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency and defects in the synthesis of tetrahydrobiopterin. A phenylalanine-tetrahydrobiopterin loading test with an extended observation period (at least 15 hours) can reliably and safely discriminate between patients with a response and patients without a response and ought to be performed in all patients with hyperphenylalaninemia to identify those who may benefit from tetrahydrobiopterin treatment. Our short-term study design does not exclude the possibility of subtle effects, which may become evident only after prolonged treatment even in some patients with classic phenylketonuria.

Our data suggest that long-term therapy with tetrahydrobiopterin could lead to an increase in phenylalanine tolerance. Cofactor treatment instead of a phenylalanine-restricted diet might be possible in many patients and would be expected to improve their quality of life substantially. Tetrahydrobiopterin treatment may also be helpful in cases of maternal phenylketonuria, since metabolic control, which is key to the prevention of serious adverse effects in the offspring,<sup>41</sup> is dif-

difficult to maintain during pregnancy. However, the safety of tetrahydrobiopterin therapy during pregnancy has not been established. Worldwide, more than 350 patients with tetrahydrobiopterin deficiency have been treated with the cofactor.<sup>42</sup> Some dose-dependent adverse reactions, including sleep disorders, polyuria, and loose stools, were reported in a safety evaluation.<sup>43</sup>

Several obstacles must be overcome before tetrahydrobiopterin treatment can be used routinely. First, tetrahydrobiopterin has not yet been approved for therapeutic use in most countries. Second, this compound is expensive. Third, dose-finding studies and clinical trials are needed to determine the bioavailability and long-term effects of tetrahydrobiopterin therapy in patients with phenylalanine hydroxylase deficiency.

In conclusion, we found that pharmacologic doses of tetrahydrobiopterin corrected impaired phenylalanine oxidation in the majority of patients with mild hyperphenylalaninemia phenotypes. Our findings have implications for the diagnostic workup and clinical classification of this defect as well as for therapeutic interventions. In the near future, in a large number of patients with hyperphenylalaninemia, cofactor treatment may obviate the need for the most burdensome dietary restrictions.

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