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Missense Mutations in the N-Terminal Domain of Human Phenylalanine Hydroxylase Interfere with Binding of Regulatory Phenylalanine

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Hyperphenylalaninemia due to a deficiency of phenylalanine hydroxylase (PAH) is an autosomal recessive disorder caused by >400 mutations in the *PAH* gene. Recent work has suggested that the majority of *PAH* missense mutations impair enzyme activity by causing increased protein instability and aggregation. In this study, we describe an alternative mechanism by which some *PAH* mutations may render PAH defective. Database searches were used to identify regions in the N-terminal domain of PAH with homology to the regulatory domain of prephenate dehydratase (PDH), the rate-limiting enzyme in the bacterial phenylalanine biosynthesis pathway. Naturally occurring N-terminal *PAH* mutations are distributed in a nonrandom pattern and cluster within residues 46–48 (GAL) and 65–69 (IESRP), two motifs highly conserved in PDH. To examine whether N-terminal *PAH* mutations affect the ability of PAH to bind phenylalanine at the regulatory domain, wild-type and five mutant (G46S, A47V, T63P/H64N, I65T, and R68S) forms of the N-terminal domain (residues 2–120) of human PAH were expressed as fusion proteins in *Escherichia coli*. Binding studies showed that the wild-type form of this domain specifically binds phenylalanine, whereas all mutations abolished or significantly reduced this phenylalanine-binding capacity. Our data suggest that impairment of phenylalanine-mediated activation of PAH may be an important disease-causing mechanism of some N-terminal *PAH* mutations, which may explain some well-documented genotype-phenotype discrepancies in PAH deficiency.

Introduction

Phenylalanine hydroxylase (PAH; phenylalanine 4-mono-oxygenase, EC 1.14.16.1) is responsible for the enzymatic conversion of L-phenylalanine to L-tyrosine in humans. Defects in PAH enzymatic activity caused by mutations in the *PAH* gene are associated with hyperphenylalaninemia and phenylketonuria (PKU [MIM 261600]). The disorder is transmitted in an autosomal recessive pattern and is the most common inborn error of amino acid metabolism among whites, with an average incidence of 1 in 10,000 (Scriver and Kaufman 2001). More than 400 *PAH* mutations and a much larger number of *PAH*-mutation genotypes have been identified in patients with hyperphenylalaninemia (Scriver et al. 2000), which may explain the broad continuum of metabolic and biochemical phenotypes associated with PAH deficiency (Okano et al. 1991; Kayaalp et al. 1997; Guldberg et al. 1998). Of the known *PAH* mutations, ~60% are missense variants, of which the molecular consequences are not directly evident.

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Monomeric PAH is organized into three functional domains, including an N-terminal regulatory domain (residues 2–120), a catalytic domain (residues 121–427), and an oligomerization domain at the extreme C-terminal end (residues 428–452) (Hufton et al. 1995; Erlandsen et al. 1997; Fusetti et al. 1998; Kobe et al. 1999). At physiological conditions, PAH is found in an equilibrium between dimeric, tetrameric, and—to some extent—higher oligomeric forms (Martinez et al. 1995). Stability studies have demonstrated that even low concentrations of chemical denaturants cause dramatic shifts in the oligomeric composition (Kleppe et al. 1999). This relatively low stability of native PAH may explain recent findings that many naturally occurring mutant variants show a high degree of instability and susceptibility towards aggregation and degradation (Eiken et al. 1996; Bjørgo et al. 1998; Waters et al. 1998*a*, 1998*b*, 1999, 2000; Gjetting et al. 2001). The notion that misfolding and structural instability are important mechanisms of enzyme dysfunction is also supported by the mapping of disease-causing missense mutations onto the proposed model of tetrameric PAH. With very few exceptions, mutations affect residues that are located outside the active site and appear important for the overall integrity of the enzyme (Fusetti et al. 1998; Erlandsen and Stevens 1999; Jennings et al. 2000).

Recent crystallographic analysis of rat PAH has provided important insights into the functional basis for

regulation of this enzyme (Kobe et al. 1999). Notably, the N-terminal regulatory domain exhibits a double $\beta\alpha\beta$ motif that is common to a number of proteins, including several involved in amino acid metabolism. This observation is compatible with previous biochemical evidence (Shiman 1980; Kaufman 1993) that PAH binds phenylalanine at two physically distinct sites: an allosteric regulatory or activating site and a catalytic site. According to the proposed model (Kobe et al. 1999), the regulatory domain and the catalytic domain of PAH are connected by a flexible hinge joint. The extreme N-terminal end (residues 1–33) constitutes an autoregulatory sequence that extends from the core of the regulatory domain into the active site and serves to restrict access of phenylalanine to the active site. Removal of this sequence gives rise to an enzyme that does not require activation by phenylalanine and is not inhibited by (6R)-5,6,7,8-tetrahydrobiopterin (BH_4), the natural cofactor of PAH (Jennings et al. 2001; Wang et al. 2001). These findings suggest that binding of phenylalanine to the regulatory domain of PAH induces conformational changes that displace the autoregulatory sequence from its position at the active site. Activation of PAH is a cooperative, reversible process involving all three functional domains and all four subunits in the holoenzyme (Shiman et al. 1990; Kaufman 1993; Davis et al. 1997).

Although there is now substantial evidence that many missense mutations in the catalytic domain of PAH cause enhanced aggregation and rapid degradation in vitro, presumably due to protein misfolding (Knappskog et al. 1996; Bjørgo et al. 1998; Waters et al. 1998*a*), the effects of missense mutations in the N-terminal regulatory domain are less clear. Size-exclusion chromatography and pulse-chase experiments have clearly demonstrated that some mutations in the N-terminal domain reduce PAH protein levels in cultured cells and that the increased protein turnover can be inhibited by the addition of protease inhibitors (Eiken et al. 1996; Waters et al. 1998*a,* 1999, 2000). Nevertheless, the rate of protein turnover appeared to be significantly lower for N-terminal mutations than for C-terminal mutations (Waters et al. 1998*a*). Furthermore, recent stability studies of mutant proteins expressed in *Escherichia coli* showed that, in general, mutations in the regulatory domain were associated with relatively stable proteins compared to most mutations in the catalytic domain (Gjetting et al. 2001). Collectively, these data suggest that a distinct mechanism may operate, alone or in concert, to cause loss of PAH function, leading to hyperphenylalaninemia. Here, we show that many naturally occurring N-terminal *PAH* mutations map to residues highly conserved in prephenate dehydratase (PDH), a key enzyme in bacterial phenylalanine biosynthesis, and that these mutations reduce or abolish the ability of the N-terminal domain of PAH to bind phenylalanine.

Materials and Methods

Recombinant DNA Techniques

Construction of mutant human PAH cDNAs inserted into pET-11a has been described in detail elsewhere (Gjetting et al. 2001). To generate wild-type and mutant forms of the N-terminal region of PAH (residues 2–120), the mutant PAH cDNAs in pET-11a were used as templates for PCR amplification, using *pfu* DNA polymerase (Stratagene) and the primers PAH-N-*Xmn*I-for (5′-GAG GGA AGG ATT TCC ACT GCG GTC CTG GAA AAC CC-3′) and PAH-N-*Bam*HI-rev (5′-CGG GAT CCT TAG AAC CAG GGC ACT GTG TCT TT-3′). The PCR products were digested with *Xmn*I and *Bam*HI and were ligated into the vector, pMAL-c2 (New England Biolabs), also prepared with these enzymes. Control sequencing of all DNA extended in vitro was performed with a ^{32}P-labeled primer, using the Thermo Sequenase cycle sequencing kit (Amersham Pharmacia Biotech).

Expression and Purification

The N-terminal domain of PAH fused to maltose-binding protein (MBP) was expressed in freshly transformed *E. coli* (Strain TB-1). Overnight cultures were diluted 20 times in fresh medium and grown for 2 h at 30°C before expression was induced by addition of IPTG (0.5 mM). The cells were harvested by centrifugation after 2 h of incubation at 30°C. Subsequent protein extraction and affinity chromatography were carried out essentially as recommended by the supplier (New England Biolabs). Eluted protein was concentrated by ultrafiltration with a cut-off of 10 kDa (Millipore), and the protein concentration was measured spectrophotometrically using a theoretical extinction coefficient of 73,000 $ABS_{280\,nm}\ cm^{-1}M^{-1}$, calculated with the program PEPTIDESTRUCTURE (Genetics Computer Group).

Protein Gel Analysis

The protocol used for SDS-PAGE was adopted from previously described procedures (Harlow and Lane 1988). For nondenaturing PAGE, purified protein was loaded onto precast gels containing a 4%–15% polyacrylamide gradient (Bio-Rad) and was subjected to electrophoresis at 100 V for 2 h in a Tris-glycine running buffer without SDS, as described elsewhere (Gjetting et al. 2001). The separated proteins were stained with GelCode (Pierce Chemical). For quantitative analyses, the gels were examined by use of the 1D Gel Analysis PHORETIX Software (Phoretix).

Phenylalanine Binding Assays

Purified fusion protein (50 μM) and substrate (25–400 μM unlabeled phenylalanine, 0.1 μCi ^{14}C-labeled phenylalanine [NEN Life Science Products] were mixed in a total volume of 50 μl, were incubated at 20°C for 1 h, and then were transferred to an ultrafiltration filter with a 10 kDa cutoff (Microcon). After an additional incubation at 20°C for 1 h, the samples were centrifuged for 5 min at 10,000 × *g*, resulting in ~25 μl centrifugate containing unbound phenylalanine and 25 μl retentate containing unbound and bound phenylalanine. Ten-microliter aliquots of these samples were counted by scintillation, using Ultima Gold scintillation liquid (Packard BioScience). The concentration of bound phenylalanine was calculated according to the formula $(cpm_r - cpm_c)/(cpm_r + cpm_c) \times C_{phe}$, where cpm_r is radioactivity in the retentate, cpm_c is radioactivity in the centrifugate, and C_{phe} is the initial phenylalanine concentration. Under the assumption of one phenylalanine-binding site per monomer, the percentage of occupied binding sites was calculated as (concentration of bound phenylalanine/protein concentration) × 100%. Thus, the occupancy of binding sites was used as a measure for binding.

Results

Homology between the Regulatory Domains of PAH and PDH

Protein database searches were used to identify proteins with sequence homology to human PAH. Overall, weak homology was found between the N-terminal regulatory domain of PAH and the regulatory domain of PDH in a wide range of microorganisms. Nevertheless, very strong homology was found for two motifs, GAL (residues 46–48 in human PAH) and (I/L)ESRP (residues 65–69 in human PAH) (fig. 1). The residues in these regions were conserved in PAH from all examined eukaryotic species and in PDH from all prokaryotic species, with the exception of *Corynebacterium glutamicum* (G*S*L instead of G*A*L) and *Amycolatopsis methanolica* (G*T*L instead of G*A*L and L*AD*RP instead of L*ES*RP). In addition to these nearly invariant motifs, several additional residues showed very high degrees of evolutionary conservation, including those corresponding to I38, L52, F55, V60, N61, L62, T63, Y77, F79, I95, and I102 in human PAH. The overall structure of the region comprising the GAL and (I/L)ESRP motifs were conserved in human tyrosine hydroxylase (TH) and tryptophan hydroxylase (TRH), but both motifs were changed at single residues in both cases (fig. 1). Examination of the crystal structure of the N-terminal domain of dimeric rat PAH (Kobe et al. 1999) revealed that the GAL and IESRP motifs are in close proximity to each other on one side of the domain near the surface involved in dimerization (fig. 2).

Many N-Terminal PAH Mutations Affect Residues Conserved in PDH

As of October 2000, the PAH Mutation Analysis Consortium had identified 256 different *PAH* missense mutations in patients with hyperphenylalaninemia, including 40 (16%) single–amino acid substitutions affecting a total of 33 residues in the regulatory domain of PAH (residues 2–120) (fig. 1). Of these N-terminal mutations, 10 (25%) map to the eight residues forming the GAL and IESRP motifs, suggesting a nonrandom distribution of mutations ($P = .0002$). Two or three different disease-related amino acid substitutions have been recorded at three of these residues (A47, I65, and R68).

Expression of the PAH Regulatory Domain as Fusion Protein in E. coli

To examine the molecular consequences of N-terminal *PAH* missense mutations, wild-type and mutant (G46S, A47V, T63P/H64N, I65T, and R68S) forms of the regulatory domain of PAH (residues 2–120; designated "PAHRD") were expressed as fusion proteins with MBP in *E. coli* and purified by affinity chromatography. The preparations were subjected to SDS-PAGE and nondenaturing PAGE to address the purity, homogeneity, and oligomeric composition of the fusion proteins. All purified fusion proteins boiled in SDS migrated as one band of ~56 kDa, the expected size of MBP fused to the regulatory domain of PAH (fig. 3*A*). When the proteins were analyzed by native PAGE, some bands and smears were observed, in addition to the ~120 kDa dimer, that probably represent aggregated forms (fig. 3*B*). Nevertheless, quantitative examination of the gels showed that the relative amount of aggregates was increased by less than one-third for all the mutant proteins when compared with the wild-type protein.

N-Terminal PAH Mutations Affect Binding of Regulatory Phenylalanine

To test whether phenylalanine binds to the wild-type and mutant forms of the N-terminal PAH domain, purified MBP-PAHRD fusion proteins were incubated with different concentrations of radiolabeled phenylalanine, followed by ultracentrifugation. Figure 4 shows the binding of phenylalanine to wild-type MBP-PAHRD. Half maximal binding was obtained at phenylalanine concentrations of ~125 μM. The normal plasma phenylalanine level under physiological conditions is 60–120 μM, and the therapeutic range in children with PAH deficiency is 120–400 μM. In subsequent experiments, phenylalanine binding of the five mutant forms of MBP-PAHRD was examined at substrate concentrations of

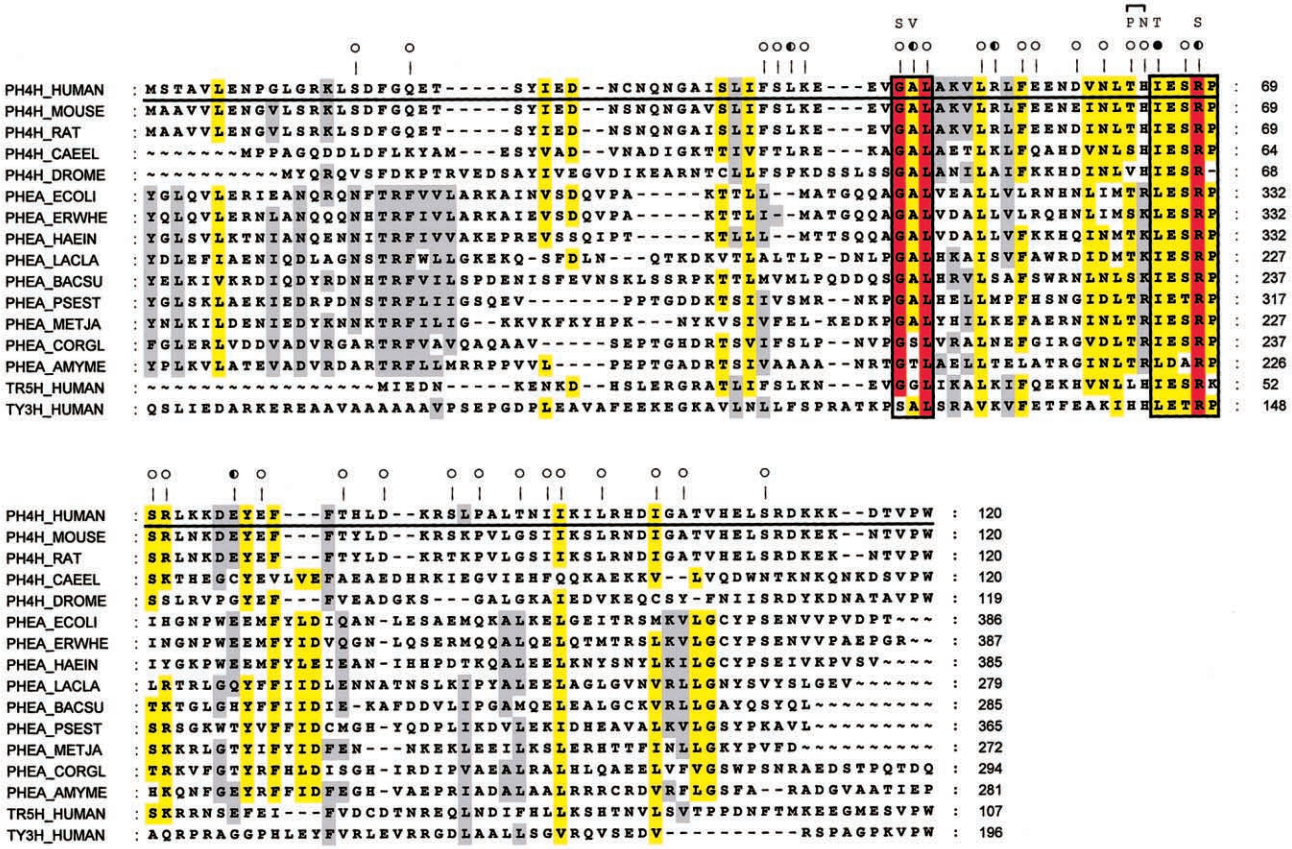

Figure 1 Amino acid–sequence alignment of the N-terminal region of aromatic amino acid hydroxylases and the C-terminal region of bacterial prephenate dehydratases, using the program PILEUP (Genetics Computer Group). Labeled with their accession names in the SWISS-PROT database are phenylalanine hydroxylase of *Homo sapiens* (underlined, PH4H_HUMAN), *Mus musculus* (PH4H_MOUSE), *Rattus norvegicus* (PH4H_RAT), *Caenorhabditis elegans* (PH4H_CAEEL), and *Drosophila melanogaster* (PH4H_DROME); prephenate dehydratase of *Corynebacterium glutamicum* (PHEA_CORGL), *Amycolatopsis methanolica* (PHEA_AMYME), *Escherichia coli* (PHEA_ECOLI), *Erwinia herbicola* (PHEA_ERWHE), *Haemophilus influenzae* (PHEA_HAEIN), *Lactococcus lactis* (PHEA_LACLA), *Bacillus subtilis* (PHEA_BACSU), *Pseudomonas stutzeri* (PHEA_PSEST), and *Methanococcus jannasch* (PHEA_METJA); tyrosine hydroxylase of *Homo sapiens* (TY3H_HUMAN) and tryptophan hydroxylase of *Homo sapiens* (TR5H_HUMAN). Naturally occurring *PAH* missense mutations recorded in PAHdb are indicated by circles (*open,* one known mutation; *partially filled,* two known mutations; *filled,* three known mutations). Mutations analyzed in the present study (G46S, A47V, T63P/H64N, I65T, and R68S) are indicated by the substituting amino acid. Residues conserved in 100%, 70%–99%, or 40%–69% of the aligned PAH and PDH sequences are shaded red, yellow, or grey, respectively.

100 μM and 400 μM. As shown in table 1, reduced phenylalanine binding was observed for all mutant proteins. For three of the mutations, G46S, A47V, and R68S, phenylalanine binding was not completely abolished, and binding saturation increased significantly with increasing phenylalanine concentrations. We noted with interest that these mutations are all associated with mild forms of PAH deficiency (Kayaalp et al. 1997; Guldberg et al. 1998). Although the binding studies presented here should be considered mainly qualitative, they suggest that some N-terminal *PAH* mutations interfere with binding of phenylalanine to this domain.

Discussion

We provide direct and indirect evidence for a novel mechanism by which a subset of *PAH* mutations may cause enzyme dysfunction and lead to disease. Using protein database searches, we delineated a region in the N-terminal regulatory domain of PAH with overall weak homology to the regulatory domain of PDH, the rate-limiting enzyme in bacterial phenylalanine biosynthesis. Two motifs, GAL (residues 46–48 in human PAH) and (I/L)ESRP (residues 65–69 in human PAH) were conserved in PAH from all examined eukaryotic organisms,

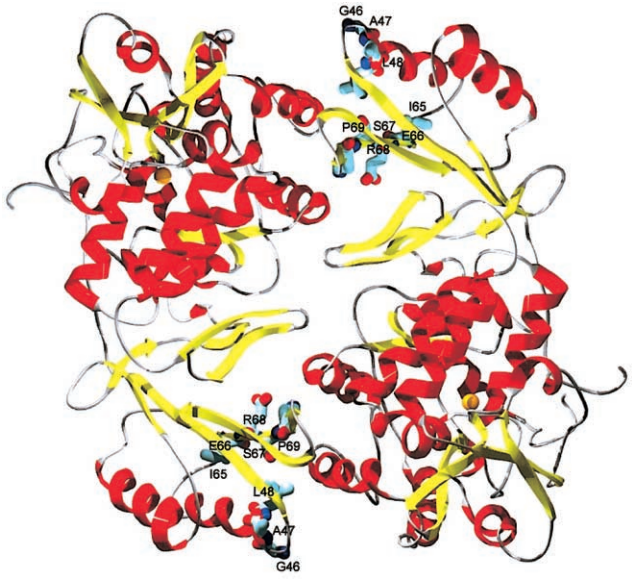

Figure 2 Crystal structure modelling of dimeric rat PAH (PDB file: Kobe et al. 1999). The conserved motifs, GAL (residues 46–48) and IESRP (residues 65–69) are located in close proximity to each other in the regulatory domain near the dimerization interface. The figure was generated with the SWISS-MODEL and the SWISS-PdbViewer (Guex and Peitsch 1997).

including the distantly related *Caenorhabditis elegans*, and, with very few exceptions, in PDH from a wide range of microorganisms (Pohnert et al. 1999). In addition, four residues (60–63) in the region preceding the IESRP motif showed a high degree of evolutionary conservation. A large proportion of *PAH* mutations identified in patients with hyperphenylalaninemia cluster within or around the GAL and IESRP motifs, further suggesting that these residues are important for the function of PAH.

The binding of phenylalanine to the regulatory domain of PDH has been characterized in detail in *E. coli* (Nelms et al. 1992; Zhang et al. 1998; Pohnert et al. 1999). In this organism, the first two enzymes involved in the conversion of phenylalanine from chorismate, chorismate mutase, and PDH, are combined in the bifunctional P-protein encoded by the *pheA* gene. Phenylalanine biosynthesis is controlled by phenylalanine-mediated feedback inhibition of the chorismate mutase and PDH activities. The PDH regulatory domain of the P-protein was purified as a dimer and was shown to possess a phenylalanine-binding capacity comparable with those of other amino acid–binding proteins (Pohnert et al. 1999). The GAL and LESRP regions represent, respectively, the hydrophobic and hydrophilic maxima in this domain, and R331 (homologous to R68 in human PAH) has been suggested to directly interact with the carboxylic acid group in phenylalanine (Pohnert et al. 1999). Site-directed mutagenesis experiments showed that changes in the GAL and LESRP regions affected phenylalanine binding, subsequent conformation changes, and feedback inhibition, to different extents (Pohnert et al. 1999). Furthermore, nitrous-acid–induced *pheA* mutations causing almost total resistance of the P-protein to feedback inhibition mapped to residues 304–310, corresponding to residues 41–47 in human PAH (Nelms et al. 1992).

Taken together, the data from these studies of the *E. coli* P-protein strongly corroborate the data from our studies of human PAH. First, the regulatory domain of PAH (residues 2–120), like the regulatory domain of PDH, formed a dimer when expressed as a fusion protein in *E. coli*. Second, this fusion protein was able to specifically bind phenylalanine at physiological concentrations. Third, naturally occurring *PAH* mutations completely abolished or significantly reduced the capacity of this domain to bind phenylalanine. We noted

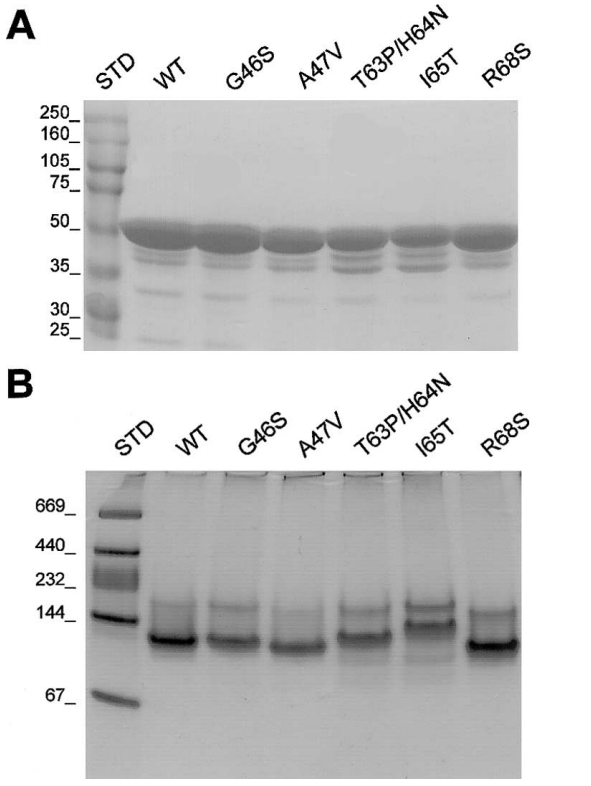

Figure 3 Analysis of purified MBP-PAHRD fusion proteins (wild-type and mutant forms) by SDS-PAGE (*A*) and nondenaturing PAGE (*B*). The positions of molecular-weight markers are indicated (in kDa). Under denaturing conditions, all proteins migrate predominantly as a 56-kDa band. Under nondenaturing conditions, the wild-type protein migrates almost entirely as a dimer, whereas some of the mutant proteins represent several molecular forms.

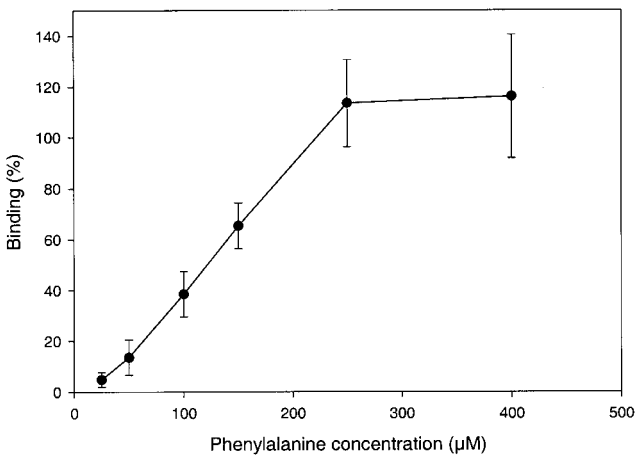

Figure 4 Phenylalanine binding to wild-type MBP-PAHRD fusion protein given in percent of saturation (mean of triplicates ± standard deviation). The binding was calculated under the assumption of one phenylalanine-binding site per protein monomer.

with interest that among the PDH mutants examined by Pohnert et al. (1999), the A310V and L311S variants (homologous to the naturally occurring A47V and L48S variants of human PAH) significantly reduced phenylalanine binding. Furthermore, a single–amino acid substitution of G309 (homologous to G46 in human PAH) was found among the induced *pheA* mutations that cause resistance of the P-protein to phenylalanine-mediated feedback inhibition (Nelms et al. 1992).

Recently, Eiken et al. (1996) and Waters et al. (1998*a*, 1999, 2000) provided evidence that N-terminal *PAH* mutations affect the stability of PAH and increase the protein degradation rate in vitro. Therefore, it could be argued that the observed reduction in the phenylalanine-binding capacity of mutant PAH regulatory sites can be ascribed to protein misfolding. Analysis of the oligomeric composition of the regulatory domain of PAH expressed as a fusion protein showed some higher–molecular-weight forms for some mutations, presumably representing aggregated proteins, and we cannot exclude the possibility that this tendency towards aggregation may be even stronger in the multidomain holoenzyme. However, for all mutant fusion proteins, the ratio between aggregates and dimer was less than one-third higher than that measured for the wild-type protein, and there was no correlation between the amount of dimer in the protein preparation and the ability to bind phenylalanine. Therefore, it seems reasonable to suggest that aberrant phenylalanine binding, leading to lack of subsequent activation, and improper folding, leading to protein instability, are mechanisms acting in concert for some mutant PAH proteins. In support of this notion, Eiken et al. (1996) showed lack of positive cooperativity of phenylalanine binding for PAH carrying the G46S mutation, suggesting loss of phenylalanine-dependent activation.

Although there is now substantial evidence that the *PAH* mutation genotype is the major determinant of the metabolic phenotype in PAH deficiency, a series of recent reports has provided documentation of cases where patients with identical *PAH* mutation genotypes exhibit different degrees of PAH deficiency (Scriver and Waters 1999). Notably, the F39L, L48S, I65T, and R68S mutations, all of which are located in the N-terminal domain of PAH, have been associated with a wide range of metabolic phenotypes, from classical PKU to mild PKU (Kayaalp et al. 1997; Guldberg et al. 1998). The existence of a mechanism by which some *PAH* mutations produce desensitization of the enzyme to the activating effect of phenylalanine, as suggested by the present data, may explain some of these discordant genotype-phenotype associations. One attractive hypothesis would be that the activity of these mutant enzymes are subject to regulation by substrate concentrations and, accordingly, depend on the concentration of phenylalanine in the liver. We are presently investigating the degree to which phenylalanine tolerance depends on target plasma phenylalanine levels during treatment in patients with mutations in the regulatory domain of PAH.

PAH belongs to a family of aromatic amino acid hydroxylases, including TH and TRH, which share many structural and physical properties and catalyze key steps in the synthesis of neurotransmitters. On the basis of the sequences of the PAH, TH, and TRH genes and recent X-ray crystallographic studies of PAH and TH (Erlandsen et al. 1997; Goodwill et al. 1997; Fusetti et al. 1998; Kobe et al. 1999), it has been established that the three hydroxylases share significant homology in the C-terminal two-thirds of the proteins—that is, the region comprising the catalytic core. The N-terminal domain shares less homology among the vertebrate and invertebrate hydroxylases and is entirely absent in bac-

Table 1

Binding Saturation of Wild-Type and Mutant Forms of MBP-PAHRD

MBP-PAHRD Allele	Mean ± SD Binding Saturation (%) at Phenylalanine Concentration	
	100 μM	400 μM
Wild-type	39 ± 9	116 ± 24
G46S	6.0 ± 1.1	19 ± 6
A47V	7.2 ± 2.6	38 ± 2
T63P/H64N	0[a]	0[a]
I65T	0[a]	0[a]
R68S	13 ± 5	51 ± 9

Note.—The values were calculated under the assumption of one phenylalanine-binding site per monomer (mean of triplicates ± standard deviation).

[a] Not distinguishable from background.

terial PAH, which is not subject to allosteric regulation by phenylalanine. Neckameyer and White (1992) showed that PAH and TRH activities are encoded by a single gene in *Drosophila melanogaster* and suggested the existence of an ancestral gene encoding a hydroxylase that most strongly resembles PAH in structure and function. An attractive model is that this gene, after an initial duplication event, diverged to form TH and TRH during the evolution of the vertebrate nervous system. On the basis of the data presented here, we propose that the common progenitor for the three vertebrate aromatic amino acid hydroxylases was formed by juxtaposition of bacterial PAH with the regulatory domain of PDH, and that evolutionary changes in this domain have conferred at least some of the regulatory properties of the different hydroxylases. On the basis of the crystal structures of rat and human PAH, Kobe et al. (1999), Erlandsen and Stevens (1999), and Jennings et al. (2000) suggested that naturally occurring *PAH* missense mutations may be assigned to three main categories according to the residues affected: (*i*) residues in the active site, (*ii*) residues in key structural domains anywhere in the protein, and (*iii*) residues in the domain interfaces. The different lines of evidence presented here would suggest a fourth category: mutations affecting residues directly involved in the binding of phenylalanine to the regulatory domain of PAH. Although such categorization may be useful from a functional point of view, there is now strong in vitro evidence that most *PAH* mutations exert their pathogenic effect through the concerted action of different mechanisms. A major challenge for future research is to determine the predominant mechanisms of individual *PAH* mutations in vivo.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for PAH deficiency [MIM 261600])

PAHdb, http://www.mcgill.ca/pahdb/ (for naturally occurring *PAH* missense mutations)

SWISS-PROT, annotated protein sequence database, http://www.expasy.ch/sprot/sprot-top.html (accession names are listed in parentheses in the legend of fig. 1)

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