J Inherit Metab Dis (2009) 32:10–21
DOI 10.1007/s10545-008-0942-6

Biochemical characterization of mutant phenylalanine hydroxylase enzymes and correlation with clinical presentation in hyperphenylalaninaemic patients

S. F. Dobrowolski · A. L. Pey · R. Koch · H. Levy · C. C. Ellingson · E. W. Naylor · A. Martinez

Received: 5 May 2008 / Submitted in revised form: 29 July 2008 / Accepted: 31 July 2008 / Published online: 21 October 2008

Summary The biochemical properties of mutant phenylalanine hydroxylase (PAH) enzymes and clinical characteristics of hyperphenylalaninaemic patients who bear these mutant enzymes were investigated. Biochemical characterization of mutant PAH enzymes p.D143G, p.R155H, p.L348V, p.R408W and p.P416Q included determination of specific activity, substrate activation, V_{max}, K_m for (6*R*)-L-*erythro*-5,6,7,8-tetrahydrobiopterin (BH_4), K_d for BH_4, and protein stabilization by BH_4. Clinical data from 22 patients either homozygous, functionally hemizygous, or compound heterozygous for the mutant enzymes of interest were correlated with biochemical parameters of the mutant enzymes. The p.L348V and p.P416Q enzymes retain significant catalytic activity yet were observed in classic and moderate PKU patients. Biochemical studies demonstrated that BH_4 rectified the stability defects in p.L348V and p.P416Q; additionally, patients with these variants responded to BH_4 therapy. The p.R155H mutant displayed low PAH activity and decreased apparent affinity for L-Phe yet was observed in mild hyperphenylalaninaemia. The p.R155H mutant does not display kinetic instability, as it is stabilized by BH_4 similarly to wild-type PAH; thus the residual activity is available under physiological conditions. The p.R408W enzyme is dysfunctional in nearly all biochemical parameters, as evidenced by disease severity in homozygous and hemizygous patients. Biochemical assessment of mutant PAH proteins, especially parameters involving interaction with BH_4 that impact protein folding, appear useful in clinical correlation. As additional patients and mutant proteins are assessed, the utility of this approach will become apparent.

Communicating editor: Nenad Blau

Competing interests: None declared

References to electronic databases: Online Mendelian Inheritance in Man: http://www.ncbi.nlm.nih.gov/sites/entrez?db = omim. Enzyme Commission: http://www.chem.qmul.ac.uk/iubmb/enzyme/. PAH Mutation database: http://www.pahdb.mcgill.ca/.

S. F. Dobrowolski (✉) · C. C. Ellingson
Idaho Technology, 390 Wakara Way,
Salt Lake City, Utah 84098, USA
e-mail: steven_dobrowolski@idahotech.com

A. L. Pey · A. Martinez
Department of Biomedicine, University of Bergen,
Bergen, Norway

R. Koch
University of Southern California,
Los Angeles, California, USA

H. Levy
Children's Hospital Boston,
Boston, Massachusetts, USA

E. W. Naylor
NGS-Research and Development-GmbH,
Medical University of South Carolina,
Charleston, South Carolina, USA

Abbreviations

BH_4	(6*R*)-L-*erythro*-5,6,7,8-tetrahydrobiopterin
Phe	phenylalanine
Tyr	tyrosine
Trp	tryptophan
CD	circular dichroism
PAH	phenylalanine hydroxylase
PKU	phenylketonuria
MHP	mild hyperphenylalaninaemia
wt	wild-type
DTT	dithiothreitol

Introduction

Phenylketonuria (PKU, MIM261600, EC 1.14.16.1) results from defects in the liver enzyme phenylalanine hydroxylase (PAH). As PKU is the paradigm of a treatable genetic disease, newborn screening has prospectively identified affected newborns by an increased concentration of phenylalanine (normal 50–120 μmol/L) (Chace et al 2003; Guthrie and Susi 1963). An observed increase in phenylalanine concentration identifies a spectrum of disease ranging from mild hyperphenylalaninaemia (MHP), in which the blood phenylalanine concentration is 180–600 μmol/L, to classical PKU, in which the phenylalanine concentration is >1200 μmol/L. The treatment goal for an affected patient is to maintain the blood phenylalanine concentration between 120–360 μmol/L, which has traditionally been achieved with a phenylalanine-restricted diet (Levy 1999). In a subset of patients, the phenylalanine concentration can be managed with pharmacological doses of the PAH cofactor BH_4 with either limited or no dietary restriction (Blau and Erlandsen 2004; Kure et al 1999).

Genotype–phenotype relationships in most inborn errors of metabolism have at best been partially realized. In PKU, the genotype–phenotype relationship is a more reliable predictive tool as PAH genotyping has utility for diagnosis, predicting disease category (classical PKU, mild PKU, MHP, etc.), and predicting the potential for response to BH_4 therapy (Guldberg et al 1998; Guttler and Guldberg 2000; Kayaalp et al 1997; Koch et al 2002). Given the relatively strong genotype–phenotype relationship observed in PKU, genotype analysis is a standard component of the diagnostic regimen.

To better understand relationships between mutant PAH protein biochemistry and PKU disease presentation, detailed biochemical studies of the mutant PAH enzymes p.D143G (c.428A>G), p.R155H (c.464G>A), p.L348V (c.1042C>G), p.R408W (c.1222C>T), and p.P416Q (c.1247C>A) were performed. Analysis included (i) steady-state enzyme kinetic analysis, (ii) the apparent binding affinity for BH_4, and (iii) the conformational stability of the mutant proteins, studied by kinetic circular dichroism (CD) experiments. Biochemical parameters of the mutant proteins were correlated with disease presentation in patients harbouring these variants.

Materials and methods

Patients and sample collection

Hyperphenylalaninaemic patients were followed at the Children's Hospital Boston, University of Southern California Medical Center, or Emory University Medical Center. Blood was obtained by heel prick or finger prick and applied to a newborn screening filter paper blood card. Dried blood samples were given a numeric designation to protect patient privacy. Informed consent was obtained for each patient and the University of Utah Institutional Review Board approved this study.

Assessment of the *PAH* gene

Dried blood samples were used to assess the *PAH* gene. DNA was prepared from dried blood as described (Heath et al 1999). The *PAH* gene was assessed using a modification of the high-resolution melt profiling assay as described (Dobrowolski et al 2007). Primers for the *PAH* exon scanning assays were modified on their 5′ end with M13 universal DNA sequencing tails to streamline follow-on DNA sequence analysis when required.

Patient inclusion criteria

Patients with the missense mutations p.D143G, p.R155H, p.L348V, p.R408W or p.P416Q were selected. To better understand the impact of mutant protein biochemistry on disease presentation, the set of patients was narrowed to include only those homozygous for a mutation of interest, compound heterozygous for two mutations of interest, or functionally hemizygous for a mutation of interest. To be functionally hemizygous, a patient is compound heterozygous for one of the mutations while the other allele is defective at the level of the mRNA and does not produce a protein product. Mutations impacting the mRNA that will not create a protein product include nonsense mutations, variants altering the reading frame, and splice-site mutations that lead to exon skipping and disruption of the reading frame. Mutations of these types upregulate the nonsense-mediated mRNA decay pathway, which degrades the defective mRNA (Muhlemann 2008).

Clinical and biochemical data were collected for patients who met the inclusion criteria. Clinical data collected include: disease phenotype (MHP, classical PKU, etc.), IQ, basal serum phenylalanine concentration, phenylalanine tolerance, response to challenge with BH_4, and other relevant data.

Creating mutant PAH enzymes by site-directed mutagenesis

The mutations p.D143G, p.R155H, p.L348V, p.R408W and p.P416Q were introduced in the human PAH

cDNA on the pMALc2 expression vector (Martínez et al 1995) by polymerase chain reaction-based site-directed mutagenesis using the QuikChange kit (Stratagene, La Jolla, CA, USA). The primers used for mutagenesis were provided by MWG Biotech AG (Ebersberg, Germany). Mutagenesis was verified by DNA sequencing (Eiken et al 1996; Knappskog et al 1996).

Expression of recombinant PAH in *E. coli*

Tetrameric full-length wild-type PAH (wt-PAH) and mutant PAH human enzymes were expressed in *E. coli* as fusion proteins with maltose-binding protein (MBP) as described (Martínez et al 1995), but using 28°C after induction. Tetrameric PAH proteins were obtained after cleavage with factor Xa (300:1 fusion protein:protease at 4°C for 16 h) and isolated by size-exclusion chromatography using a HiLoad 16/60 Superdex 200 prep grade column (Amersham, Uppsala, Sweden) (Martínez et al 1995).

Enzyme kinetic analysis

PAH enzyme activity was measured at 25°C for 1 min as described (Bjørgo et al 1998). At standard assay conditions, tetrameric wt-PAH and mutant proteins (1–2 μg) were incubated in 100 mmol/L Na-Hepes, pH 7.0, containing catalase (0.04 mg/ml) and 1 mmol/L L-Phe. After 4 min incubation at 25°C, ferrous ammonium sulfate (100 μmol/L) was added, and the reaction was triggered after 1 min by adding BH_4 (75 μmol/L) in the presence of 5 mmol/L dithiothreitol (DTT). In some experiments, the preincubation step with L-Phe was omitted and L-Phe was added together with BH_4 to analyse the l-Phe induced activation of the enzyme. To determine the steady-state kinetic parameters for BH_4 and L-Phe, BH_4 was used at 0–200 μmol/L (at 1 mmol/L L-Phe) and L-Phe at 0–1 mmol/L (at 75 μmol/L BH_4). L-Tyr formed was quantified by HPLC and fluorimetric detection (Døskeland et al 1984). The saturation curves were fitted to hyperbolic (for BH_4) or sigmoidal (for L-Phe) models with Sigma Plot v.9.0. (SPSS Inc., Chicago, IL, USA).

Circular dichroism (CD)

CD measurements were performed in a Jasco J-810 spectropolarimeter equipped with a Peltier element for temperature control. Kinetic unfolding experiments were performed by equilibrating samples containing 20 mmol/L potassium phosphate, 200 mmol/L KCl, pH 7.5, 5 mmol/L DTT and 0–250 μmol/L BH_4 at a 37°C for 5 min and then adding a concentrated protein solution containing Fe(II) (the final protein and Fe(II) concentration were 5 μmol/L). CD signal at 222 nm was monitored for 1 h. Dead times in kinetic experiments were 10–20 s. Blanks were subtracted and kinetic data were analysed by fitting to single exponential decay curves, including a term for non-zero asymptotic values at $t=\infty$ using Sigma Plot 9.0 (SPSS Inc.).

Fluorescence measurements

Tryptophan-emission (Trp-emission) fluorescence was measured in a Perkin-Elmer LS50 spectrofluorimeter with a constant temperature cell holder and 1 cm path length cuvettes. Tetrameric PAH proteins (1 μmol/L subunit) were prepared in 20 mmol/L Na-Hepes, 200 mmol/L NaCl, pH 7.0 in the presence of 10-fold excess Fe(II) and 0.5 mmol/L DTT. Titrations were performed by stepwise addition of small volumes (0.5–5 μl) of 2 mmol/L BH_4 under constant stirring. One minute after each addition, emission spectra were recorded with excitation at 295 nm (3 n m slit) and emission between 300 and 400 nm (5 nm slit). Blanks in the absence of protein were acquired and subtracted. BH_4 quenching of Trp-emission fluorescence was monitored at 340 nm as previously described (Knappskog and Haavik 1995). The normalized fluorescence as the ratio of the fluorescence intensity in the presence/absence of BH_4 (F) was fitted to a one-independent-binding-site model which, using Eq. 1, includes a term for inner filter effects of BH_4 (Cremades et al 2005):

$$F = F_0 + \frac{(F_c - F_0)}{2C_p} \bullet \left(C_p + C_L + K_d - \sqrt{(C_p + C_L + K_d)^2 - 4C_pC_L} \right) + BC_L \quad (1)$$

where F_0 and F_c stand for the fitting values for the normalized fluorescence of PAH alone and the 1:1 BH_4:PAH complex, respectively, C_p is the total protein concentration (1 μmol/L subunit), K_d is the dissociation constant of the complex (in μmol/L), C_L is the total BH_4 concentration, and B is a constant to

take into account a linear dependence of the inner filter effect on BH_4 concentration.

Results

Clinical and biochemical characteristics of hyperphenylalaninaemic patients

Table 1 provides clinical and biochemical data for 22 patients who reached inclusion criteria. Patients 15–18 are siblings, but no others are from a common pedigree. The patient population includes 15 classical PKU, 2 moderate PKU, 1 mild PKU and 4 MPH. Six patients are homozygous for one of the mutations (four p.R408W homozygotes, two p.L348V homozygotes), five patients are compound heterozygotes for two mutations (four p.D143G/p.R155H, one p.L348V/p.R408W), and the remaining 11 specimens are compound heterozygotes for a mutation and an mRNA processing mutation that does not generate a protein product.

Expression of wild-type and mutant PAH enzymes in *E. coli* and determination of steady-state enzyme kinetics

While the mutations p.D143G, p.L348V and p.R408W have previously been analysed in prokaryote and eukaryote expression systems (http://www.pahdb.mcgill.ca/) (Gámez et al 2000; Gjetting et al 2001; Knappskog et al 1996), p.R155H and p.P416Q are novel mutations. Except p.D143G, a mutant with a mild misfolding defect, and whose enzyme kinetic parameters have previously been characterized (Knappskog et al 1996), these PAH mutants presented considerable folding defects that lead to aggregation when expressed in *E. coli*. The misfolding was very severe for the mutation p.R408W, as previously reported (Bjørgo et al 2001; Gjetting et al 2001; Pey et al 2003). Expression of mutants in *E. coli* was nevertheless successful and each was purified in milligram quantities necessary for detailed characterization.

wt-PAH displays regulatory properties stimulated by the substrate, as seen in both the 3.5-fold increase in activity by preincubation with 1 mmol/L L-Phe, and the positive cooperativity for L-Phe (Hill coefficient (h) ~2; Table 2) (Knappskog et al 1996). The non-L-Phe-preincubated and L-Phe-preincubated PAH correspond to two different metabolic scenarios for the enzyme, which displays low activity at the non-L-Phe-activated basal state when L-Phe levels in plasma are low, and is activated by increased L-Phe around the $S_{0.5}$ value providing the L-Phe-activated state (Kaufman 1993; Mitnaul and Shiman 1995). The purified mutants show relatively high activity, i.e. ≥25% and ≥65% of wt-PAH in the L-Phe-activated and non-L-Phe activated basal states, respectively (Table 2). This is in agreement with previous studies on BH_4-responsive PKU mutations that showed substantial residual activity in the purified mutant proteins (Aguado et al 2006; Erlandsen et al 2004; Pey et al 2004). Regarding the kinetic behaviour towards the substrate, only three of the mutants (p.D143G, p.L348V and p.P416Q) are significantly activated upon incubation with L-Phe (2.9-, 1.8- and 2.9-fold, respectively) and display positive cooperativity (h≥1.4; Table 2). Remarkably, p.R408W was devoid of regulatory properties for the substrate and could be activated. On the other hand, p.D143G, p.R155H and p.R408W show reduced apparent affinity ($S_{0.5}$) for L-Phe, (i.e. 2.1-, 1.9- and 4.9-fold increase in $S_{0.5}$ for L-Phe compared to wt-PAH). Regarding the steady-state kinetic properties for BH_4, only p.D143G showed reduced apparent affinity for the cofactor (1.6-fold increase in the K_m value with respect to wt-PAH) (Knappskog et al 1996). Overall, the mutants display significant specific activity and defective kinetic and regulatory properties towards the substrate, notably p.R408W.

Equilibrium binding of BH_4 to wild-type- and mutant-PAH enzymes studied by tryptophan fluorescence

The apparent affinity of tetrameric wild-type and mutant PAH for BH_4 was estimated spectroscopically by quenching of Trp-emission fluorescence. As seen in Fig. 1A, emission spectra for wild-type protein (excitation at 295 nm) show a maximum at ~338 nm, corresponding to Trp residues partially buried in the protein structure (Knappskog and Haavik 1995). As previously shown for the human and rat enzymes (Knappskog and Haavik 1995; Phillips et al 1984), Trp-emission fluorescence is quenched in the presence of BH_4 (Fig. 1A) in a concentration-dependent manner, showing a steep decrease in the fluorescence intensity at low BH_4: protein ratios. This effect is likely caused by a Forster energy transfer from Trp120 to the BH_4 bound to the active site (Knappskog and Haavik 1995). At higher BH_4 concentrations, a linear decrease in intensity is observed due to an inner filter effect of BH_4 (at 10 μmol/L, BH_4 absorption at 295 nm is about 0.1 absorbance units; Fig. 1B). We thus estimated the apparent affinity for BH_4 based on a 1:1 interaction model (Pey et al 2004) and considering a linear dependence of the inner filter effect on BH_4 concentration

Table 1 Clinical and biochemical data of selected patients

Patient	Disease category	PAH genotype	IQ	Basal Phe	Phe tolerance (mg/kg per day)	BH_4 response	Other data
1	PKU	p.R408W/ c.806delT	Unstable		250	Non-responsive	Schizophrenic age 21 years
2	PKU	p.R408W/ c.1315+1G>A	70		NA	ND	Deceased age 35 years
3	PKU	p.R408W homozygous	10		250	ND	Severe mental retardation
4	PKU	p.R408W homozygous	106		250	Non-responsive	
5	PKU	p.R408W/ c.1066-11G>A	104		200–300	Non-responsive	Concentration difficulty
6	PKU	p.R408W/ c.60+5G>T	94		200–300	Non-responsive	Psychiatric hospitalization when off diet.
7	PKU	p.R408W/ c.60+5G>T	70		200–300	Non-responsive	
8	Mod PKU	p.R408W/ c.1315+1G>A	80	1200 μmol/L	600	ND	Severe mental retardation
9	Mild PKU	p.R408W homozygous	115	950 μmol/L	900	ND	
10	PKU	p.R408W homozygous	103	1980 μmol/L	250	ND	
11	PKU	p.R408W/ p.R243X	Unstable	1500 μmol/L	250	ND	
12	PKU	p.R408W/ c.1315+1G>A	85	1200 μmol/L	200	ND	
13	PKU	p.R408W/ p.R111X	135	1090 μmol/L	230	ND	
14	PKU	p.P416Q/ c.664-665delGA	110	NA	450	Responsive	
15	MHP	p.D143G/ p.R155H	ND	200 μmol/L	Unrestricted	ND	
16	MHP	p.D143G/ p.R155H	121	200 μmol/L	Unrestricted	ND	
17	MHP	p.D143G/ p.R155H	88	228 μmol/L	Unrestricted	ND	
18	MHP	p.D143G/ p.R155H	ND	210 μmol/L	Unrestricted	ND	
19	Mod PKU	p.L348V homozygous	84	1061 μmol/L	NA	Pending	Possible celiac disease
20	PKU	p.L348V/ c.442-2A>C	116	1350 μmol/L	265	ND	
21	PKU	p.L348V/ p.R408W	ND	1255 μmol/L	300	ND	Pyloric stenosis, possible celiac disease
22	PKU	p.L348V homozygous	116		200	Responsive	Phe tolerance 2500 mg/kg per day on BH_4 therapy at 20 mg/kg per day

BH_4 response defined as a greater than 30% decrease in phenylalanine concentration within 24 hours of an oral challenge (20 mg/kg per day) with BH_4.
ND, not determined.

Table 2 Steady-state kinetic parameters for wild-type and mutant tetrameric PAH proteins

Enzyme	Specific activity[a]		V_{max}[b]	$S_{0.5}$ (L-Phe)[b] (μmol/L)	h[b]	K_m (BH_4)[c] (μmol/L)
	+L-Phe	−L-Phe				
Wt	2.98 ± 0.13	0.85 ± 0.04	3.49 ± 0.07	144 ± 11	2.0 ± 0.2	33 ± 3
p.D143G	1.64 ± 0.09	0.56 ± 0.04	1.73 ± 0.06	297 ± 22	1.6 ± 0.1	53 ± 4
p.R155H	1.32 ± 0.04	1.15 ± 0.09	3.23 ± 0.42	704 ± 194	1.1 ± 0.1	27 ± 2
p.L348V	2.76 ± 0.14	1.57 ± 0.07	3.57 ± 0.17	177 ± 15	2.2 ± 0.3	40 ± 3
p.R408W	0.75 ± 0.01	0.68 ± 0.07	1.01 ± 0.14	397 ± 93	1.1 ± 0.2	35 ± 4
p.P416Q	3.32 ± 0.12	1.14 ± 0.06	3.88 ± 0.24	187 ± 24	1.4 ± 0.2	30 ± 5

Specific activities and V_{max} are given in μmol L-Tyr/min per mg protein.
[a] Specific activity determined in the presence of 1 mmol/L L-Phe and 75 μmol/L BH_4, after 5 min preincubation in the presence (+L-Phe) or the absence (−L-Phe) of substrate.
[b] Parameters measured at standard conditions with 75 μmol/L BH_4 and 0–1 mmol/L L-Phe, with L-Phe preincubation and fitting to a Hill function.
[c] Parameter measured at standard conditions with 1 mmol/L L-Phe and 0–200 μmol/L BH_4, with L-Phe preincubation and fitting to a Michaelis–Menten function.

(see Materials and Methods). This approach renders an apparent dissociation constant (K_d) of 0.9±0.3 μmol/L for BH_4, which is in agreement with the K_d estimated at low protein concentration by isothermal titration calorimetry (0.75±0.18 μmol/L) (Pey et al 2004). From these fittings, about 37±6% of the total Trp-fluorescence intensity is quenched owing to specific BH_4 binding to wt-PAH.

The same procedure was applied to estimate the binding affinity for BH_4 in the mutant PAH enzymes. All the PKU mutants under study showed similar fluorescence intensity at the maximum (within ±10% of

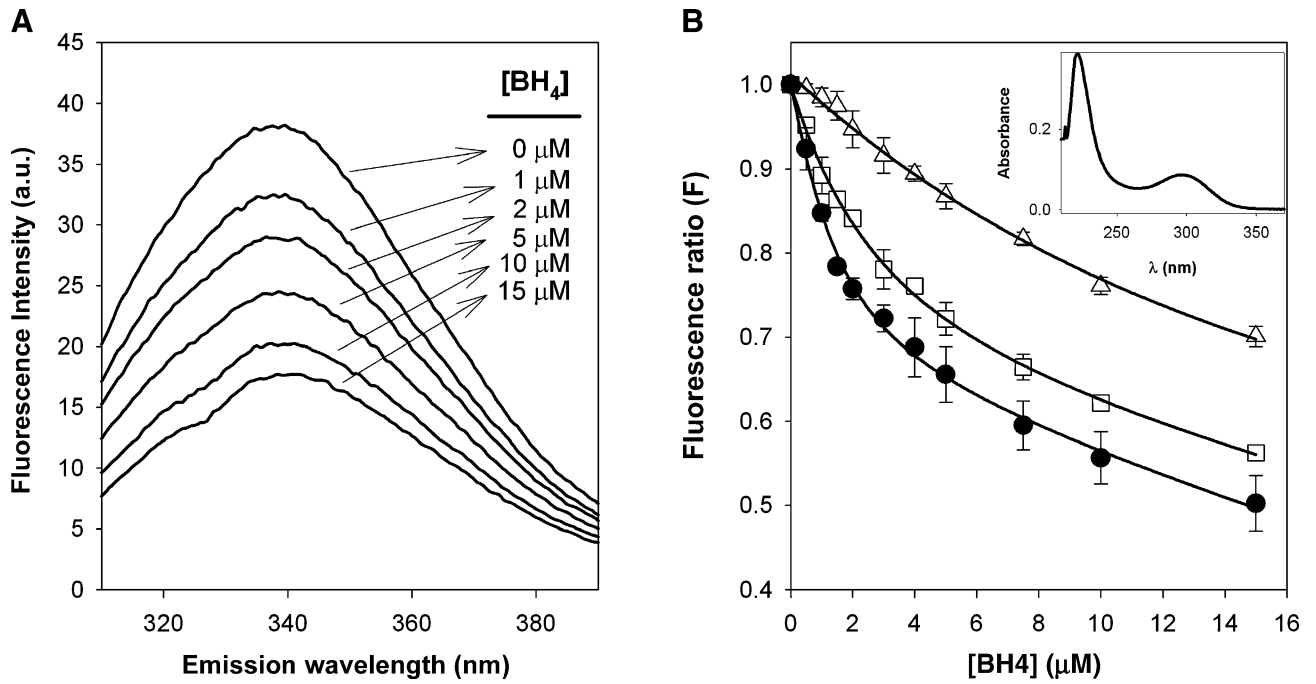

Fig. 1 BH_4 titrations of tetrameric PAH monitored by Trp-emission fluorescence. (A) Representative Trp-emission spectra of wt-PAH with increasing BH_4 concentrations. (B) Dependence of the fraction of fluorescence intensity at 340 nm (F) on the BH_4 concentration for wt (circles) and p.P416Q (squares) and p.L348V (triangles) mutant proteins. Data are mean ± SD of two or three independent experiments. Lines are best fits to a one-to-one equilibrium binding model. Experiments were performed using a PAH concentration of 1 μmol/L subunit in 20 mmol/L Na-Hepes, 200 mmol/L NaCl, pH 7.0 in the presence of 0.5 mmol/L DTT and using an excitation wavelength of 295 nm. Inset: absorption spectra of 10 μmol/L BH_4 under the same experimental conditions

Table 3 Apparent binding dissociation constants for BH_4 determined by quenching of Trp-emission fluorescence spectroscopy using tetrameric wt PAH and mutant proteins

Enzyme	K_d (μmol/L)[a]
Wt	0.9 ± 0.3
p.D143G	1.2 ± 0.5
p.R155H	1.0 ± 0.3
p.L348V	14 ± 6[b]
p.R408W	12 ± 5[b]
p.P416Q	2.2 ± 0.3

[a] K_d values are given as mean ± SD of two or three independent titrations.
[b] Low signal/noise ratio possibly due to low affinity.

the wild-type) except for p.R408W, which displayed about 20% higher fluorescence intensity (due to the additional Trp residue). The maximum of their emission spectra was similar to wild-type in three mutants (p.R155H, p.D143G and p.P416Q; 337–340 nm), but was significantly red-shifted in mutants p.L348V and p.R408W (to ~344 nm) (data not shown). The dependence of Trp-quenching observed at increasing BH_4 concentrations was used to calculate K_d values (see Table 3). p.L348V and p.R408W showed a substantially decreased binding affinity (K_d about 10-fold higher than for the wild-type protein).

Effects of BH_4 on the stability of wt- and mutant-PAH proteins

We have recently shown that wt-PAH is not kinetically stable at physiological temperature, undergoing irreversible denaturation on a timescale of minutes; BH_4 significantly protects it against unfolding in a concentration-dependent manner (Martinez et al, unpublished results). Time-dependent analysis of PAH unfolding by CD spectroscopy, for wild-type and the five mutants, provided information on the intrinsic (kinetic) stability of these proteins and a possible stabilizing effect upon BH_4 binding. Representative kinetic traces are shown in Fig. 2, and fitting to a single-exponential function provided the parameters compiled in Table 4. Three of the mutants (p.D143G, p.R408W and p.P416Q) unfold faster than wt-PAH (from 1.5-fold for p.D143G to 3-fold for p.R408W), indicating that these mutations reduce PAH protein stability (probably by destabilizing the PAH native state; Pey et al 2007). In three of the mutants (p.R155H, p.L348V and p.P416Q), stabilization by BH_4 is observed similar to that measured for wt-PAH, and this stabilization is concentration dependent (Table 4). In the case of p.D143G, moderate stabilization is observed at very high BH_4 concentrations, while the p.R408W mutant does not appear to be stabilized by the cofactor in this concentration range (Table 4).

Correlation of PAH enzyme biochemistry with disease presentation

Patient 14 (see Table 1) is compound heterozygous for two novel mutations, p.P416Q and c.664–665delGA (Dobrowolski et al 2007). The c.664–665delGA deletion alters the reading frame creating downstream termination codons; thus the mRNA is degraded by nonsense-mediated decay (Muhlemann et al 2008). Patient 14 is functionally hemizygous, and hence the source of PAH activity is provided by the p.P416Q mutant. The p.P416Q enzyme has approximately wild-type kinetic parameters (see Table 2), but shows a 2.2-fold increase in the K_d for BH_4 and unfolds at a rate faster than wild-type (see Tables 3 and 4). However, BH_4 stabilizes the mutant protein in a dose-dependent manner similar to wild-type PAH (see Table 4). Patient 14 responded to BH_4 therapy (see Table 1), which may be explained by excess BH_4 overcoming the lower binding affinity, stabilizing the enzyme, and thus enabling the enzyme's near-normal catalytic activity. The PAH p.P416Q mutant is a newly identified BH_4 responsive allele.

Four MPH patients, from a common pedigree, are compound heterozygotes for p.D143G and p.R155H. These siblings are not on dietary therapy, all have apparently normal IQ, and BH_4 challenge was not performed. Both mutations are mild, retaining approximately one-half specific activity (p.D143G, 44%; p.R155H, 55%). The V_{max} of p.D143G is 49.5% of wild-type and the V_{max} of p.R155H is approximately equivalent to wild-type. Both variants are stabilized by BH_4 (p.D143G only at high concentration, see Table 4) and have approximately wild-type K_d for BH_4, and the K_m for BH_4 is approximately wild-type in the case of p.D143G and 1.6-fold higher in the case of p.R155H. Altogether these data indicated that these variants result in mild perturbations of PAH enzyme activity and stability. The reduced activity of the enzymes (~50%) should be available at physiological conditions and the mild patient phenotypes would bear this out.

The p.L348V protein has a lower substrate activation (p.L348V,1.75-fold activation vs wild-type, 3.5 fold) but kinetic properties are similar to wild-type (see Table 2). Interaction between p.L348V and BH_4 is somewhat affected as the K_m for BH_4 is elevated (p.L348V = 40±3 vs wt = 33±3) and the K_d for BH_4 is >10-fold higher than wild-type (wt = 0.9±0.3

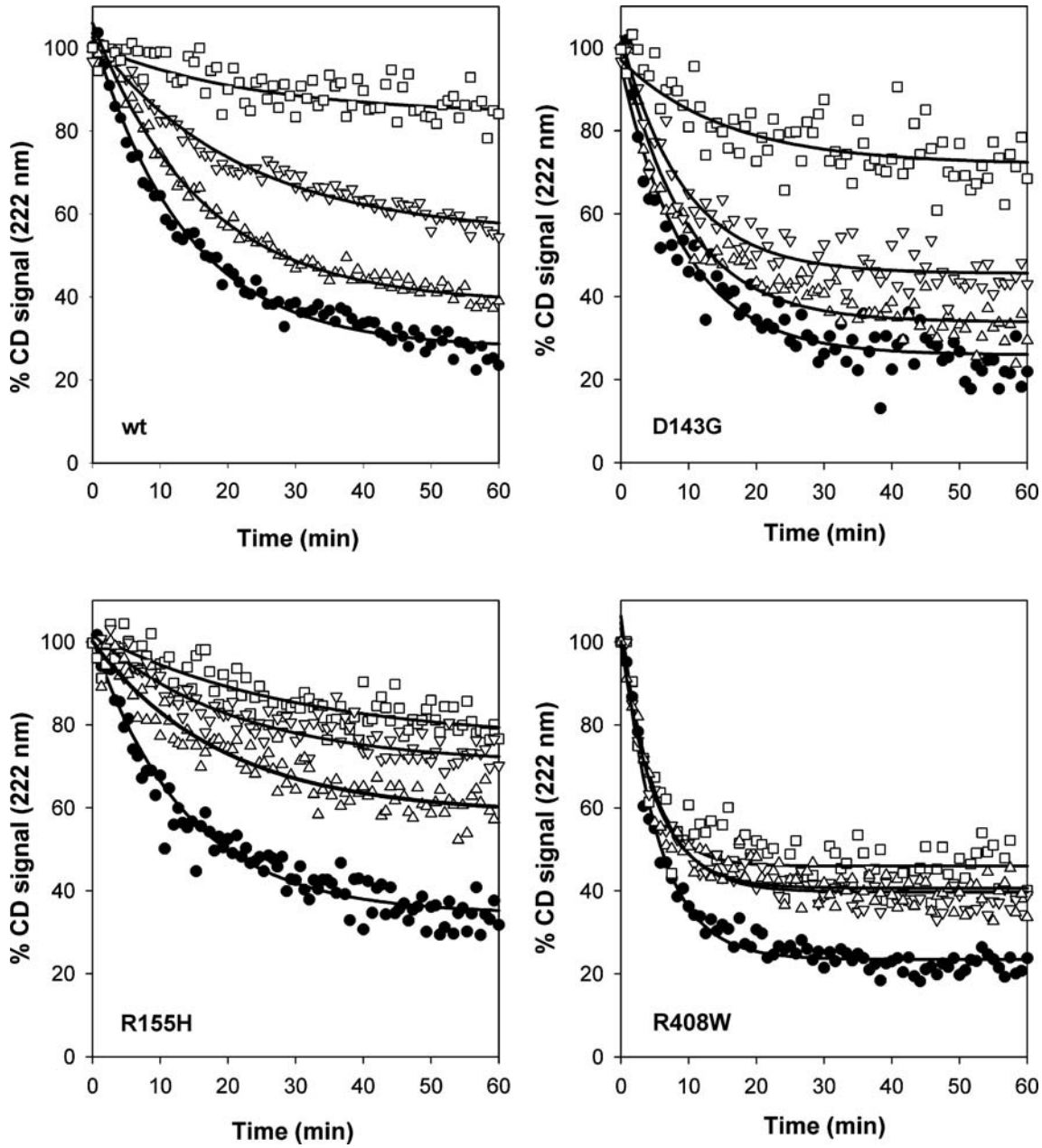

Fig. 2 Time dependence of CD signal at 37°C for isolated tetrameric PAH (wild-type and mutants) in the absence (•) or presence of 5 μmol/L (△), 25 μmol/L (▽) and 250 μmol/L (□) BH_4. Lines are fits to a single exponential decay function. Experiments performed at 5 μmol/L PAH subunit in 20 mmol/L potassium phosphate, 200 mmol/L KCl, pH 7.5, with 5 mmol/L DTT

vs p.L348V = 14 ± 6). Table 1 identifies two patients homozygous for p.L348V (patient 22, PKU and patient 19, moderate PKU) and two additional classical PKU patients who are compound heterozygous: patient 20, p.L348V/c.442–2A>C and patient 21, p.L348V/p.R408W. Disease presentation in the homozygous or hemizygous patients demonstrates that p.L348V may impart a severe phenotype. Patient 22 (homozygous for p.L348V) responded to therapy with BH_4, most profoundly demonstrated by a >10-fold increase in Phe tolerance during treatment. This observation indicates that the p.L348V protein generated significant catalytic activity following BH_4 therapy, consistent with biochemical findings that BH_4 stabilizes the enzyme and in doing so restores significant residual activity. A BH_4 challenge is pending for patient 19 (p.L348V homozygote).

Fourteen patients with p.R408W were identified, of whom 4 are homozygotes, 9 are functionally hemizygous, and 1 is a compound heterozygote with p.L348V. Of the 14 patients with p.R408W, 12 are PKU, 1 is moderate PKU, and 1 is mild PKU. None of the

Table 4 Kinetic unfolding parameters determined for isolated wt and mutant tetrameric PAH from time-dependent CD measurements at 37°C in the absence or presence of BH_4

Enzyme	BH_4 (μmol/L)	Amplitude (%)[a]	k (min^{-1})(10^2)[a]
Wt	0	75.9 ± 0.4	7.27 ± 0.09
	5	67.6 ± 0.3	6.27 ± 0.06
	25	47.6 ± 0.3	4.66 ± 0.10
	250	16.7 ± 0.6	4.23 ± 0.45
p.D143G	0	69.9 ± 0.9	10.7 ± 0.2
	5	66.7 ± 0.7	10.6 ± 0.2
	25	57.1 ± 0.9	10.9 ± 0.3
	250	25.1 ± 0.9	6.41 ± 0.63
p.R155H	0	66.4 ± 1.2	7.29 ± 0.31
	5	41.7 ± 1.0	5.26 ± 0.40
	25	31.3 ± 0.9	4.78 ± 0.47
	250	25.6 ± 1.5	3.42 ± 0.56
p.L348V	0	69.6 ± 0.5	8.00 ± 0.13
	5	62.8 ± 0.4	7.13 ± 0.11
	25	54.2 ± 0.3	6.82 ± 0.14
	250	28.4 ± 0.5	5.57 ± 0.34
p.R408W	0	81.2 ± 0.8	18.6 ± 0.3
	5	59.5 ± 1.2	19.7 ± 0.7
	25	63.6 ± 1.1	19.2 ± 0.6
	250	60.2 ± 2.1	24.7 ± 1.4
p.P416Q	0	73.7 ± 0.6	13.5 ± 0.3
	5	53.6 ± 0.5	9.33 ± 0.24
	25	34.6 ± 0.4	6.82 ± 0.44
	250	20.2 ± 4.2	3.10 ± 0.94

[a] From fitting to a single-exponential decay function.

patients homozygous or functionally hemizygous for p.R408W (Table 1, patients 1 and 4–7) responded to challenge with BH_4. Disease severity associated with p.R408W is borne out by its biochemical characterization as it has low specific activity, loss of substrate activation, low affinity for the substrate, a greater rate of protein unfolding, and absent stabilization by BH_4. The p.R408W/p.L348V compound heterozygote is classical PKU with other systemic complications (pyloric stenosis, possible celiac disease). The manifest biochemical dysfunction of the p.R408W protein underlies disease severity associated with homozygosity or hemizygosity for the mutation.

Discussion

Five mutant PAH enzymes (p.D143G, p.R155H, p.L348V, p.R408W, p.P416Q) were selected for intensive biochemical characterization which included their interaction with BH_4. These mutations are localized within the catalytic domain, with Asp143 and Leu348 situated in the active-site crevice (Fig. 3). The structural importance of Arg408, holding the catalytic domain with the tetramerization domain, has been highlighted previously (Erlandsen and Stevens 1999). The mutation p.R408W, the recurrent single mutation, produces the most deleterious enzyme kinetic and folding defects of the five mutations studied, in accordance with the severe phenotype of the homozygous patients.

Early analyses of the genotype–phenotype correlations and BH_4-responsiveness have suggested that patients carrying two mild mutations (or mutations displaying significant residual activity) will likely be associated with BH_4-responsiveness, while the opposite was proposed for those patients carrying two 'null' alleles (Blau and Erlandsen 2004). In the case of functional hemizygotes (one mild/one null allele), responsiveness would depend on the properties of the mild allele. On a similar basis, mild phenotypes were expected to be responsive while severe phenotypes would not. A recent update on these relationships has highlighted the association of mild phenotypes

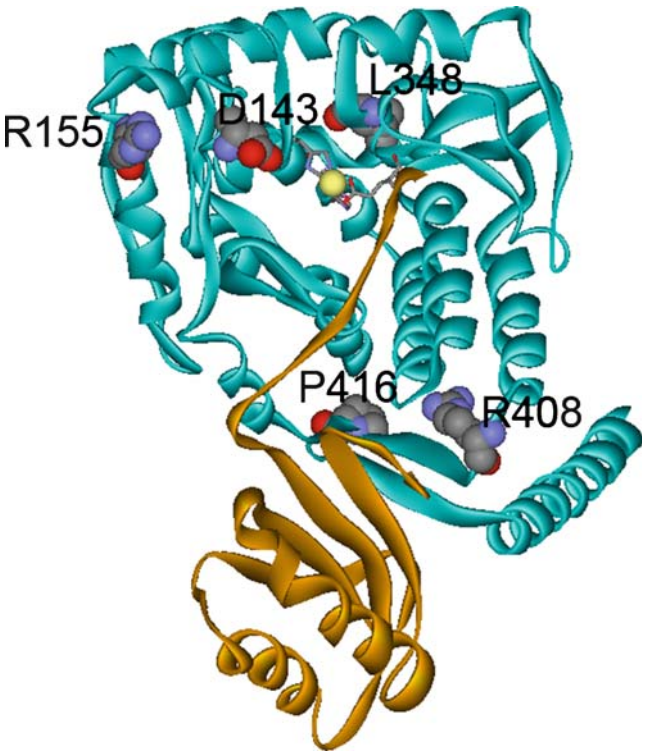

Fig. 3 Structure of human PAH composite model of the full-length subunit. The model was generated using the structures of human catalytic/tetramerization domains (PDB 2PAH) and rat regulatory/catalytic domains (PDB 1PHZ). The regulatory domain is shown in ochre and the catalytic and tetramerization domains in light blue. The iron is shown as a yellow sphere and the coordinating 2-His-carboxylate facial triad as wires. The residues D143, R155, L348, R408, and P416 are shown in CPK representation

(and mild mutations) with responsiveness, as many as 80% of all the mild phenotypes, while in some cases severe phenotype is also responsive (Zurfluh et al 2008). At least five different mechanisms were originally invoked as possibly associated with BH_4-responsiveness, as follows (Blau and Erlandsen 2004): (1) K_m mutants displaying low affinity for BH_4; (2) stabilization of PAH mutant by BH_4 (chaperone effect); (3) BH_4-induced alterations in BH_4 biosynthesis; (4) upregulation of *PAH* gene expression; (5) stabilization of PAH mRNA by BH_4. Experimental support for low-affinity mutants for BH_4 (Erlandsen et al 2004) and BH_4-induced stabilization of wild-type and mutant proteins (Thony et al 2004) has been provided, and also indicates that mutants associated with the responsiveness generally display significant specific activity (more than 30% of residual activity (Aguado et al 2007; Erlandsen et al 2004). Effects on gene expression or mRNA stability have been ruled out (Aguado et al 2006; Scavelli et al 2005; Thony et al 2004). In addition, it is likely that BH_4 administration increases the suboptimal physiological cofactor concentration and therefore enhances PAH activity. Accordingly, administration of BH_4 to normal subjects (Okano et al 2007) and mice (Kure et al 2004) increases L-Phe oxidation rates, but only when they are loaded with high L-Phe concentrations prior to BH_4 administration. Analysis on the complex activity landscape of wild-type and mutant PAH proteins has also shown that under high L-Phe concentrations (i.e. pathological or L-Phe loaded), a modest increase in BH_4 concentration is able to induce a substantial enhancement in PAH activity and also increases the K_m value for BH_4 (Pey and Martinez 2005), explaining why L-Phe oxidation rates increase only when L-Phe concentrations are raised (Kure et al 2004; Okano et al 2007). This alternative mechanism is expected to operate generally in PAH mutants with substantial residual activity (as observed in all the BH_4-responsive mutations characterized *in vitro*) and could be relevant for mutations not stabilized by BH_4.

Interesting results were observed with the novel p.P416Q mutation, identified in a moderate PKU patient (Table 1 patient 14). While the p.P416Q protein retained near-normal kinetic characteristics (except for reduced positive cooperativity for L-Phe, see Table 2), the K_d for BH_4 was 2.4-fold higher than with wild-type, and the protein also unfolded faster than wild-type. This suggests the p.P416Q mutation leads to a protein folding defect. These experiments also indicated that high BH_4 concentration would stabilize the protein, allowing for increased residual activity. Patient 14 responded to BH_4 challenge (20 mg/kg per day) as would be anticipated from characteristics of the mutant enzyme. When this variant is observed in additional patients, their disease presentation and response to BH_4 challenge will be of interest.

Comparisons between the properties of the p.L348V enzyme and patient characteristics were intriguing. The p.L348V mutant has near-normal kinetic characteristics (see Table 2). However, the K_d for BH_4 is at least 10-fold higher than wild-type, but enzyme stabilization by BH_4 is similar to wild-type. This may suggest a protein folding defect that would respond to BH_4 challenge. Patient 22 is homozygous for p.L348V and showed a strong response to BH_4 challenge, demonstrated in the 10-fold increase in phenylalanine tolerance when receiving BH_4 therapy.

The p.R408W variant has long been considered a severe mutation associated with classical disease when in homozygous form or in heterozygous form with another severe mutation. Essentially every biochemical parameter measured in this study (see Tables 2–4 and Fig. 2) shows the p.R408W mutation to have profound deleterious impact. The clinical presentation of the 13 patients homozygous or functionally hemizygous for p.R408W was generally severe (11 classical PKU, 1 moderate PKU, 1 mild PKU), and of the five patients challenged with BH_4 none was responsive.

Genotype–phenotype relationships in inborn errors of metabolism have been poorly realized, but the PAH genotype in PKU provide greater predictive value. In-depth biochemical assessment of PAH mutant enzymes and in particular the enzyme interaction with BH_4 may suggest response to therapy with BH_4. As the extensive regimen of biochemical assessments is carried out on additional mutant PAH enzymes, greater opportunity will be available to make comparisons with disease presentation. These comparisons may result in an increased ability to make accurate genotype–phenotype predictions in PKU patients.

Acknowledgements We are very grateful to Randi Svebak and Ali Javier Sepulveda for expert technical help. A. L. P. and A. M. are supported by the Research Council of Norway and Helse-Vest. This work was supported by NIH grant 2R44HD075156-02 to S. F. D.

References

Aguado C, Perez B, Ugarte M, Desviat LR (2006) Analysis of the effect of tetrahydrobiopterin on PAH gene expression in hepatoma cells. *FEBS Lett* **580**(7): 1697–1701. doi:10.1016/j.febslet.2006.02.005.

Aguado C, Perez B, Garcia MJ, et al (2007) BH4 responsiveness associated to a PKU mutation with decreased binding affinity for the cofactor. *Clin Chim Acta* **380**(1–2): 8–12. doi:10.1016/j.cca.2007.02.034.

Bjørgo E, de Carvalho RM, Flatmark T (2001) A comparison of kinetic and regulatory properties of the tetrameric and dimeric forms of wild-type and Thr427→pro mutant human phenylalanine hydroxylase: contribution of the flexible hinge region Asp425-Gln429 to the tetramerization and cooperative substrate binding. *Eur J Biochem* **268**(4): 997–1005. doi:10.1046/j.1432-1327.2001.01958.x.

Bjørgo E, Knappskog PM, Martínez A, Stevens RC, Flatmark T (1998) Partial characterization and three-dimensional-structural localization of eight mutations in exon 7 of the human phenylalanine hydroxylase gene associated with phenylketonuria. *Eur J Biochem* **257**(1): 1–10. doi:10.1046/j.1432-1327.1998.2570001.x.

Blau N, Erlandsen H (2004) The metabolic and molecular bases of tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency. *Mol Genet Metab* **82**(2): 101–111. doi:10.1016/j.ymgme.2004.03.006.

Chace DH, Kalas TA, Naylor EW (2003) Use of tandem mass spectrometry for multianalyte screening of dried blood specimens from newborns. *Clin Chem* **49**(11): 1797–1817. doi:10.1373/clinchem.2003.022178.

Cremades N, Bueno M, Toja M, Sancho J (2005) Towards a new therapeutic target: *Helicobacter pylori* flavodoxin. *Biophys Chem* **115**(2–3): 267–276. doi:10.1016/j.bpc.2004.12.045.

Dobrowolski SF, Ellingson C, Coyne T, et al (2007) Mutations in the phenylalanine hydroxylase gene identified in 95 patients with phenylketonuria using novel systems of mutation scanning and specific genotyping based upon thermal melt profiles. *Mol Genet Metab* **91**(3): 218–227. doi:10.1016/j.ymgme.2007.03.010.

Døskeland AP, Døskeland SO, Øgreid D, Flatmark T (1984) The effect of ligands of phenylalanine 4-monooxygenase on the cAMP-dependent phosphorylation of the enzyme. *J Biol Chem* **259**: 11242–11248.

Eiken HG, Knappskog PM, Apold J, Flatmark (1996) PKU mutation G46S is associated with increased aggregation and degradation of the phenylalanine hydroxylase enzyme. *Hum Mutat* **7**(3): 228–238. doi:10.1002/(SICI)1098-1004(1996)7:3<228::AID-HUMU7>3.0.CO;2-6.

Erlandsen H, Pey AL, Gamez A, et al (2004) Correction of kinetic and stability defects by tetrahydrobiopterin in phenylketonuria patients with certain phenylalanine hydroxylase mutations. *Proc Natl Acad Sci U S A* **101**: 16903–16908. doi:10.1073/pnas.0407256101.

Erlandsen H, Stevens RC (1999) The structural basis of phenylketonuria. *Molec Genet Metab* **68**(2): 103–125.

Gámez A, Pérez B, Ugarte M, Desviat LR (2000) Expression analysis of phenylketonuria mutations. Effect on folding and stability of the phenylalanine hydroxylase protein. *J Biol Chem* **275**(38): 29737–29742. doi:10.1074/jbc.M003231200.

Gjetting T, Petersen M, Guldberg P, Guttler F (2001) *In vitro* expression of 34 naturally occurring mutant variants of phenylalanine hydroxylase: correlation with metabolic phenotypes and susceptibility toward protein aggregation. *Mol Genet Metab* **72**(2): 132–143. doi:10.1006/mgme.2000.3118.

Guldberg P, Rey F, Zschocke J, et al (1998) A European multicenter study of phenylalanine hydroxylase deficiency: classification of 105 mutations and a general system for genotype-based prediction of metabolic phenotype. *Am J Hum Genet* **63**(1): 71–79. doi:10.1086/301920.

Guthrie R, Susi A (1963) A simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants. *Pediatrics* **32**: 338–343.

Guttler F, Guldberg P (2000) Mutation analysis anticipates dietary requirements in phenylketonuria. *Eur J Pediatr* **159**(Suppl 2): S150–S153. doi:10.1007/PL00014381.

Heath EM, O'Brien DP, Banas R, Naylor EW, Dobrowolski S (1999) Optimization of an automated DNA purification protocol for neonatal screening. *Arch Pathol Lab Med* **123**(12): 1154–1160.

Kaufman S (1993) The phenylalanine hydroxylating system. *Adv Enzymol Relat Areas Mol Biol* **67**: 77–264. doi:10.1002/9780470123133.ch2.

Kayaalp E, Treacy E, Waters PJ, et al (1997) Human phenylalanine hydroxylase mutations and hyperphenylalaninemia phenotypes: a metanalysis of genotype–phenotype correlations. *Am J Hum Genet* **61**(6): 1309–1317. doi:10.1086/301638.

Knappskog PM, Eiken HG, Martínez A, et al (1996a) PKU mutation (D143G) associated with an apparent high residual enzyme activity: expression of a kinetic variant form of phenylalanine hydroxylase in three different systems. *Hum Mutat* **8**: 236–246. doi:10.1002/(SICI)1098-1004(1996)8:3<236::AID-HUMU7>3.0.CO;2-7.

Knappskog PM, Flatmark T, Aarden JM, Haavik J, Martínez A (1996b) Structure/function relationships in human phenylalanine hydroxylase. Effect of terminal deletions on the oligomerization, activation and cooperativity of substrate binding to the enzyme. *Eur J Biochem* **242**(3): 813–821. doi:10.1111/j.1432-1033. 1996.0813r.x.

Knappskog PM, Haavik J (1995) Tryptophan fluorescence of human phenylalanine hydroxylase produced in *Escherichia coli*. *Biochemistry* **34**: 11790–11799. doi:10.1021/bi00037a017.

Koch R, Burton B, Hoganson G, et al (2002) Phenylketonuria in adulthood: a collaborative study. *J Inherit Metab Dis* **25**(5): 333–346. doi:10.1023/A:1020158631102.

Kure S, Hou DC, Ohura T, et al (1999) Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency. *J Pediatr* **135**(3): 375–378. doi:10.1016/S0022-3476(99)70138-1.

Kure S, Sato K, Fujii K, et al (2004) Wild-type phenylalanine hydroxylase activity is enhanced by tetrahydrobiopterin supplementation *in vivo*: an implication for therapeutic basis of tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency. *Mol Genet Metab* **83**(1–2): 150–156. doi:10.1016/j.ymgme.2004.06.016.

Levy HL (1999) Phenylketonuria: old disease, new approach to treatment. *Proc Natl Acad Sci U S A* **96**(5): 1811–1813. doi:10.1073/pnas.96.5.1811.

Martínez A, Knappskog PM, Olafsdottir S, et al (1995) Expression of recombinant human phenylalanine hydroxylase as fusion protein in *Escherichia coli* circumvents proteolytic degradation by host cell proteases. Isolation and characterization of the wild-type enzyme. *Biochem J* **306**: 589–597.

Mitnaul LJ, Shiman R (1995) Coordinate regulation of tetrahydrobiopterin turnover and phenylalanine hydroxylase activity in rat liver cells. *Proc Natl Acad Sci U S A* **92**(3): 885–889. doi:10.1073/pnas.92.3.885.

Muhlemann O, Eberle AB, Stalder L, Zamudio Orozco R (2008) Recognition and elimination of nonsense mRNA. *Biochim Biophys Acta* **1779**(9): 538–549.

Okano Y, Takatori K, Kudo S, et al (2007) Effects of tetrahydrobiopterin and phenylalanine on *in vivo* human phenylalanine hydroxylase by phenylalanine breath test. *Mol Genet Metab* **92**(4): 308–314. doi:10.1016/j.ymgme.2007.07.013.

Pey AL, Desviat LR, Gamez A, Ugarte M, Perez B (2003) Phenylketonuria: genotype–phenotype correlations based on expression analysis of structural and functional mutations in PAH. *Hum Mutat* **21**(4): 370–378. doi:10.1002/humu.10198.

Pey AL, Martinez A (2005) The activity of wild-type and mutant phenylalanine hydroxylase and its regulation by phenylalanine and tetrahydrobiopterin at physiological and

pathological concentrations: an isothermal titration calorimetry study. *Mol Genet Metab* **86**(Suppl 1): S43–S53. doi:10.1016/j.ymgme.2005.04.008.

Pey AL, Perez B, Desviat LR, et al (2004a) Mechanisms underlying responsiveness to tetrahydrobiopterin in mild phenylketonuria mutations. *Hum Mutat* **24**(5): 388–399. doi: 10.1002/humu.20097.

Pey AL, Thórólfsson M, Teigen K, Ugarte M, Martínez A (2004b) Thermodynamic characterization of the binding of tetrahydropterins to phenylalanine hydroxylase. *J Am Chem Soc* **126**: 13670–13678. doi:10.1021/ja047713s.

Pey AL, Stricher F, Serrano L, Martinez A (2007) Predicted effects of missense mutations on native-state stability account for phenotypic outcome in phenylketonuria, a paradigm of misfolding disease. *Am J Hum Genet* **81**(5): 1006–1024.

Phillips RS, Parniak MA, Kaufman S (1984) Spectroscopic investigation of ligand interaction with hepatic phenylalanine hydroxylase: evidence for a conformational change associated with activation. *Biochemistry* **23**: 3836–3842. doi:10. 1021/bi00312a007.

Scavelli R, Ding Z, Blau N, et al (2005) Stimulation of hepatic phenylalanine hydroxylase activity but not Pah-mRNA expression upon oral loading of tetrahydrobiopterin in normal mice. *Mol Genet Metab* **86**(Suppl 1): S153–S155. doi:10.1016/j.ymgme.2005.09.015.

Thony B, Ding Z, Martínez A (2004) Tetrahydrobiopterin protects phenylalanine hydroxylase activity *in vivo*: implications for tetrahydrobiopterin-responsive hyperphenylalaninemia. *FEBS Lett* **577**(3): 507–511. doi:10.1016/j.febslet.2004.10.056.

Zurfluh MR, Zschocke J, Lindner M, et al (2008) Molecular genetics of tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency. *Hum Mutat* **29**(1): 167–175. doi: 10.1002/humu.20637.