RESEARCH ARTICLE

Mechanisms Underlying Responsiveness to Tetrahydrobiopterin in Mild Phenylketonuria Mutations

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A subtype of phenylalanine hydroxylase (PAH) deficiency that responds to cofactor (tetrahydrobiopterin, BH_4) supplementation has been associated with phenylketonuria (PKU) mutations. The underlying molecular mechanism of this responsiveness is as yet unknown and requires a detailed in vitro expression analysis of the associated mutations. With this aim, we optimized the analysis of the kinetic and cofactor binding properties in recombinant human PAH and in seven mild PKU mutations, i.e., c.194T>C (p.I65T), c.204A>T (p.R68S), c.731C>T (p.P244L), c.782G>A (p.R261Q), c.926C>T (p.A309V), c.1162G>A (p.V388M), and c.1162G>A (p.Y414C) expressed in *E. coli*. For p.I65T, p.R68S, and p.R261Q, we could in addition study the equilibrium binding of BH_4 to the tetrameric forms by isothermal titration calorimetry (ITC). All the mutations resulted in catalytic defects, and p.I65T, p.R68S, p.P244L, and most probably p.A309V, showed reduced binding affinity for BH_4. The possible stabilizing effect of the cofactor was explored using a cell-free in vitro synthesis assay combined with pulse-chase methodology. BH_4 prevents the degradation of the proteins of folding variants p.A309V, p.V388M, and p.Y414C, acting as a chemical chaperone. In addition, for wild-type PAH and all mild PKU mutants analyzed in this study, BH_4 increases the PAH activity of the synthesized protein and protects from the rapid inactivation observed in vitro. Catalase and superoxide dismutase partially mimic this protection. All together, our results indicate that the response to BH_4 substitution therapy by PKU mutations may have a multifactorial basis. Both effects of BH_4 on PAH, i.e., the chemical chaperone effect preventing protein misfolding and the protection from inactivation, may be relevant mechanisms of the responsive phenotype. Hum Mutat 24:388–399, 2004. © 2004 Wiley-Liss, Inc.

KEY WORDS: phenylketonuria, mild; PKU; PAH; BH_4-responsiveness; tetrahydrobiopterin; cofactor replacement therapy

DATABASES:
PAH – OMIM: 261600; GenBank: U49897.1 (cDNA), NM_000277 (cDNA), AF404777 (gDNA); Swiss-Prot: P090439; www.pahdb.mcgill.ca (*PAHdb*); www.bh4.org (Tetrahydrobiopterin Home Page)

INTRODUCTION

(6R)-L-erythro-5,6,7,8-tetrahydrobiopterin (BH_4) is the essential cofactor in the hydroxylation of L-Phe catalyzed by the nonheme iron-dependent phenylalanine hydroxylase (PAH), using dioxygen as additional substrate. A deficiency in any of the components of the PAH system results in hyperphenylalaninemia (HPA), and the majority of these deficiencies are caused by mutations in the PAH gene (MIM# 261600). According to patient phenotypes, the HPAs can be categorized as classical phenylketonuria (PKU), moderate PKU, mild PKU, and mild HPA [Scriver and Kaufman, 2001]. More infrequent forms of HPA are due to defects in the biosynthesis or regeneration of BH_4 [Blau et al., 2001]. A standardized BH_4 loading test allows the distinction between PAH and BH_4 defects. No decline of plasma phenylalanine levels is expected in PAH deficiency, whereas in defects of enzymes involved in either the

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synthesis or regeneration of BH_4, the phenylalanine levels are normalized within 4–8 hr after BH_4 loading [Blau et al., 1994]. Kure et al. [1999] reported for the first time several patients with a PAH deficiency showing a normalization of plasma phenylalanine concentration after oral administration of BH_4. Since then, there have been an ever increasing number of reports of BH_4 responsiveness in PKU patients [for reviews see Erlandsen and Stevens, 2001; Spaapen and Rubio-Gozalbo, 2003; Blau et al., 2004]. This has lead to the definition of a novel subtype of PAH deficiency, and suggests a therapeutic potential for BH_4 that may overcome the psychosocial burden of a restricted dietary therapy in a subset of PKU patients. Some mutations, characterized by their high residual activity when analyzed in vitro [Spaapen and Rubio-Gozalbo, 2003], are repeatedly found to be associated with this type of PAH deficiency, leading to the suggestion that responsiveness is determined by the nature of the mutations. However, there are patients with identical genotypes showing a discordant response to a BH_4 loading test, suggesting that other factors (e.g., intragenic polymorphisms, modifier genes, or nongenetic factors) also determine the response to cofactor supplementation [Lindner et al., 2003].

The tetrameric PAH enzyme belongs to the family of aromatic amino acid hydroxylases sharing a similar enzyme mechanism and a common structural pattern. The 3D-structure of different truncated forms of human and rat PAH has been determined, providing essential information on the active-site structure and the residues involved in binding of the substrate L-Phe and the cofactor BH_4 [Andersen et al., 2002; Teigen et al., 1999], and offering a framework in which to interpret the structural effects of the disease-causing mutations [Erlandsen et al., 1997; Fusetti et al., 1998; Kobe et al., 1999]. PAH has a three-domain structure consisting of a regulatory domain containing an N-terminal autoregulatory sequence stretching over the active site, a catalytic domain including the active site iron and the substrate and BH_4 binding sites, and a C-terminal oligomerization domain [Erlandsen et al., 1997; Fusetti et al., 1998; Kobe et al., 1999]. The substrate L-Phe, which activates the enzyme, is proposed to induce a conformational change that displaces the autoregulatory sequence from the active site [Kobe et al., 1999; Thórólfsson et al., 2003]. BH_4, on the contrary, acts as a negative regulator, inducing a low-activity conformational state, blocking L-Phe activation and disfavoring phosphorylation at Ser16 [Jennings et al., 2001; Kaufman, 1993; Teigen and Martinez, 2003; Solstad et al., 2003].

The need for a more correct classification of potential BH_4-responsive patients according to their genotype and biochemical phenotype, as well as for the elucidation of the molecular mechanism underlying BH_4 responsiveness, has been stressed [Ponzone et al., 2003]. Several possibilities have been put forward, alone or in combination, to explain the response to BH_4 in mild PKU [Erlandsen and Stevens, 2001; Muntau et al., 2002; Spaapen and Rubio-Gozalbo, 2003; Steinfeld et al., 2003]: 1) correction of a catalytic defect of the mutant proteins (decreased affinity for BH_4); 2) stabilization of the mutant proteins, i.e., BH_4 could act as a chemical chaperone, protecting the active tetramer/dimer forms from proteolytic cleavage; 3) upregulation of PAH gene expression levels, as recently shown for the hph1 mouse [Hyland and Munk-Martin, 2001]; or 4) PAH mRNA stabilization, similarly to the effect of BH_4 on inducible nitric oxide synthase mRNA [Linscheid et al., 1998]. The most generalized assumption is that mutations associated with BH_4 responsiveness result in K_m variants (decreased BH_4 binding affinity) of the PAH enzyme, and BH_4 therapy of mild PKU would then join the list of high-dose vitamin treatments that are available to correct mutation induced–decreased binding affinity for certain coenzymes [Ames et al., 2002]. Many of these BH_4-responsive PKU mutations map to the catalytic domain in regions that interact with secondary elements involved in cofactor binding [Erlandsen and Stevens, 2001]. However, without expression analysis and kinetic measurements of the mutant proteins, the current understanding of the mechanism of BH_4 responsiveness is incomplete. In this work, we analyzed the kinetic behavior of recombinantly expressed PAH proteins corresponding to frequent PKU mutations present in BH_4-responsive patients (i.e., c.194T>C (p.I65T), c.204A>T (p.R68S), c.782G>A (p.R261Q), c.1162G>A (p.V388M), and c.1241A>G (p.Y414C)) [Lassker et al., 2002; Muntau et al., 2002; Spaapen and Rubio-Gozalbo, 2003], or to rare mutations selected from previous expression studies that suggested they could correspond to defects in cofactor binding (i.e., c.731C>T (p.P244L) and c.926C>T (p.A309V)) [Pey et al., 2003] and that are also potentially associated with BH_4-responsiveness (L.R. Desviat et al., in press). In addition, for p.R68S, p.I65T, and p.R261Q, the thermodynamic parameters for BH_4 binding could be determined by isothermal titration calorimetry (ITC), gaining insights into the energetics of cofactor binding. The possible stabilizer effect of BH_4 was analyzed in the mutant proteins synthesized in vitro, using a coupled transcription–translation (TnT) assay combined with pulse-chase methodology. This is the first detailed analysis probing the mechanism of BH_4 responsiveness that could be included in this novel subtype of PAH deficiency; it may help as a reference for further studies and classification of mild PKU mutations.

MATERIALS AND METHODS

Expression and Purification of Recombinant PAH Enzymes

Mutations were introduced on the human PAH cDNA (Genbank U49897.1) cloned in the pMAL expression vector as described [Gamez et al., 2000; Pey et al., 2003]. Growth of *E. coli* transformed with the pMAL vectors for expression of wild-type (wt) and mutant forms of PAH and purification of the fusion proteins of PAH and maltose binding protein was performed as described [Martínez et al., 1995; Pey et al., 2003]. The tetrameric enzyme forms were purified using size-exclusion chromatography, concentrated with Centricon 30 and stored in liquid nitrogen.

Protein concentration was measured spectrophotometrically using $\epsilon_{280\ nm}$ (1 mg/ml) = 1.63 [Martínez et al., 1995].

PAH Activity Assay

PAH activity was measured for 1 min at 25°C as described [Bjørgo et al., 1998; Martínez et al., 1995]. Two types of reaction were performed: 1) the enzyme was preincubated for 4 min at 25°C in a mixture containing 100 mM NaHepes (pH 7.0), 1 mM L-Phe, and 0.04 mg/ml catalase; 100 μM ferrous ammonium sulfate was subsequently added, and after another 1-min incubation, the reaction was started with the addition of 75 μM BH_4 and 5 mM dithiothreitol (DTT) (L-Phe activated); 2) the reaction was performed as above, except that L-Phe was added together with BH_4 after the 5 min preincubation (non–L-Phe activated). The amount of L-Tyr was measured by HPLC and fluorimetric detection. The kinetic parameters were calculated by nonlinear regression analysis of the data using Michaelis-Menten kinetics (for BH_4 saturation curves) or the Hill equation (for L-Phe saturation curves).

ITC

The experiments were performed using a VP-ITC titration calorimeter (MicroCal, www.microcalorimetry.com) under anoxic and reducing conditions. At anoxic conditions, measurements were carried out using 20 mM NaHepes, 200 mM NaCl (pH 7.0) with the glucose-glucose oxidase-catalase system as described, i.e., 0.5 U glucose oxidase (type II, Sigma, www.sigma-aldrich.com), 750 U Catalase (Sigma), and 20 mM D-Glucose [Rajagopalan and Pei, 1998]. This buffer was incubated at room temperature for 15 min before BH_4 (stocks prepared in 10 mM HCl) or the PAH enzymes (10–50 μM subunit of tetrameric fusion protein with approximately 0.5 mol ferrous ammonium sulfate/subunit) were added to prepare the solutions used in the syringe and the sample cell, respectively. Alternatively, we performed the experiments under reducing conditions, where PAH and BH_4 were prepared in 20 mM NaHepes, 0.2 M NaCl, 1 mM DTT, 7.5 μM Fe(II) (pH 7.0). All the solutions were filtered and degassed prior to titration. In each run, up to 90 injections of 1.2–3.0 μl 1 mM BH_4 were added to the cell sample (one injection every 240 sec) and mixed via the rotating (300 rpm) stirrer syringe. The final ratio [BH_4]/[enzyme subunit] was typically ≥ 4; the mean of the last 5–10 injections was used as experimental heat dilution, and routinely subtracted from the raw data. Under the anoxic conditions, baseline was stable up to 10 hr in the calorimetric cell. Processing of the data by fitting and calculation of the number of sites, equilibrium association constant (K_a), ΔH, ΔG, and ΔS was performed using the MicroCal Origin v.5.0 software (MicroCal).

PAH Activity and Pulse-Chase Analysis After In Vitro Synthesis

PAH protein was synthesized in vitro using the TnT-T7 TnT system from Promega (www.promega.com). Wild-type (GenBank U49897.1) and mutant PAH cDNAs cloned in the pRC/CMV vector [Pey et al., 2003] were amplified using a sense primer that introduces the T7 promoter and the consensus Kozak sequences close to the ATG initiation codon [Gamez et al., 2000]. TnT of PAH cDNA was carried out in the presence of 40 μM L-methionine. A total of 500 μM BH_4, kept reduced with 5 mM DTT, was added in the cases indicated and DTT (5 mM) was also present in the synthesis assays carried out in the absence of BH_4. After 35 min at 30°C, the synthesis reaction was stopped with ribonuclease A (1 mg/ml) and desoxyribonuclease I (1 mg/ml), and frozen immediately at −20°C. For PAH activity analysis, the samples (20 μl) were thawed at room temperature for 1 min and diluted in 300 μl freshly prepared and filtered cold 20 mM Hepes and 200 mM NaCl (pH 7.0). Free amino acids were eliminated after two dilution–concentration steps at 4°C using an ultrafree-MC NMWL-10000 microconcentration filter (Millipore, www.millipore.com). PAH activity measurement was performed using 20 μl of the amino acid free extract in each assay and was normalized using total protein determined by the Bradford assay. To perform the inactivation curves, the normal and mutant proteins were synthesized at 30°C during 35 min in the absence and presence of 500 μM BH_4. The pulse was stopped, with ribonuclease A (1 mg/ml) and desoxyribonuclease I (1 mg/ml), and subsequently incubated at 37°C. PAH activity was measured at standard conditions in the aliquots removed at different times up to 1 hr.

For pulse-chase analysis, PAH synthesis was carried out for 35 min at 30°C, as described above, with or without BH_4, in the presence of [^{35}S]-methionine+[^{35}S]-cysteine (*Promix* L-[^{35}S] in vitro cell labeling mix, 14.3 mCi/ml; Amersham Biosciences (www.amershambiosciences.com). The pulse was stopped, with ribonuclease A (1 mg/ml) and desoxyribonuclease I (1 mg/ml) and cold methionine. The reaction was further incubated at 37°C, aliquots were removed at different times up to 7 hr, and these were subjected to denaturing PAGE. The labeled gels were fluorographed using Amplify (Amersham) and the bands were quantified by laser densitometry [Gamez et al., 2000].

Size-Exclusion Chromatography

Size-exclusion chromatography was performed at 4°C using a HiLoad Superdex 200 column (1.6 × 360 cm), prepacked from Pharmacia and calibrated as described [Martínez et al., 1995]. On chromatography of [^{35}S]methionine-labeled PAH, obtained by the coupled in vitro TnT system, 0.76-ml fractions were collected, and radioactivity was determined by liquid-scintillation counting.

RESULTS

Expression in *E. coli* and Steady-State Kinetic Analysis

All the mutations analyzed in this study have been expressed previously using different systems, including COS cells, and were found to exhibit a considerable residual activity ($\gtrsim$30% of that of wt-PAH when analyzed at standard conditions) [Gamez et al., 2000; Pey et al., 2003]. Transient expression in COS or other mammalian cell lines provides small amounts of protein not readily amenable for purification and proper characterization of the molecular and kinetic properties of the PAH enzymes. These enzymatic characterizations are usually performed with the recombinant PAH enzyme expressed in prokaryote systems as a fusion protein [Waters et al., 1998]. Although the mutants show folding defects when expressed both in *E. coli* and in COS cells [Gamez et al., 2000; Pey et al., 2003], none appeared to result in extreme folding variants. Only p.I65T, p.A309V, and p.V388M resulted in significant amounts of aggregated protein when expressed in *E. coli*, but large amounts of isolated tetrameric forms were obtained for all mutants, allowing their kinetic characterization (Table 1). The half-denaturation temperatures were also similar in wt-PAH and most of the mutants (T_m = 55–59°C) except for p.A309V and p.V388M (T_m = 50°C) [Gamez et al., 2000; Pey et al., 2003].

In order to exhibit maximal specific activity with the natural cofactor BH_4, PAH must be preincubated with its substrate L-Phe, which induces a regulatory activating conformational change and binds with positive cooperativity (Hill coeficient, $h \sim 2$) [Kaufman, 1993; Thórólfs-

TABLE 1. Steady State Kinetic Parameters of the Wild-Type (wt) and Mutant PAH Tetrameric Fusion Proteins Expressed in *E. coli*[*]

Enzyme	Specific activity (nmol Tyr/min · mg)			$S_{0.5}$ (L-Phe)[c] (μM)	K_m(BH4)[d] (μM)	*h*(L-Phe)[c]	Substrate inhibition[c,e]
	Non L-Phe activated[a]	L-Phe activated[b]	V^c_{max} (nmol Tyr/min · mg)				
Wt	700 ± 20	1905 ± 12	1890 ± 50	135 ± 5	27 ± 2	2.0	+
c.194T>C (p.I65T)	1600 ± 30	2300 ± 50	2470 ± 100	80 ± 10	40 ± 3	1.0	+
c.204A>T (p.R68S)	1650 ± 150	1770 ± 130	1950 ± 90	73 ± 6	30 ± 3	1.5	+
c.731C>T (p.P244L)	450 ± 20	381 ± 1	440 ± 20	160 ± 25	40 ± 4	1.0	+
c.782G>A (p.R261Q)	1450 ± 56	1500 ± 12	1920 ± 20	610 ± 60	25 ± 2	1.1	−
c.926C>T (p.A309V)	790 ± 12	850 ± 2	1160 ± 60	150 ± 20	38 ± 3	1.1	+
c.1162G>A (pV388M)	280 ± 4	440 ± 7	1238 ± 111	1200 ± 110	24 ± 3	1.0	−
c.1241A>G (p.Y414C)	650 ± 136	1170 ± 150	2400 ± 150	110 ± 20	22 ± 3	1.5	+

[*]The data include the specific activity with and without prior incubation with L-Phe (activated and non-L-Phe activated), maximum velocity (V_{max}) for L-Phe, apparent affinity for L-Phe ($S_{0.5}$ (L-Phe)), and for BH_4 (K_m) and Hill constant (*h*) as a measure of positive cooperativity. GenBank reference sequence for PAH cDNA: U49897.1, nucleotide numbering starts at the ATG translation initiation codon.
[a]Measured at standard conditions (1 mM L-Phe and 75 mM BH_4) with non-L-phe preincubated enzyme.
[b]Measured at standard conditions (1 mM L-Phe and 75 mM BH_4) with L-phe preincubated enzyme.
[c]Obtained with L-Phe preincubated enzyme, assayed with 75 μM BH_4 and 0–4 mM L-Phe.
[d]Obtained with non-L-Phe preincubated enzyme, assayed with 1 mM L-Phe and 0–200 μM BH_4.
[e]Manifested at [L-Phe] > 2 mM.

son et al., 2003]. The specific activities of the enzyme forms prior to and after preincubation with L-Phe are summarized in Table 1. All mutant forms except p.I65T showed lower activity after preincubation with L-Phe compared with wt-PAH, while p.I65T, p.R68S, and p.R261Q show a higher basal activity than wt-PAH in the absence of L-Phe preincubation, i.e., they seem to be activated as isolated. The steady state kinetic parameters (Table 1) revealed the effects of the mutations on the affinity for L-Phe ($S_{0.5} > 500$ μM for p.R261Q and p.V388M), the *h* for cooperative substrate binding (no mutant showed full cooperativity, $h \lesssim 1.5$), and on the affinity for BH_4 (modest increase in K_m for p.I65T, p.P244L, and p.A309V), in addition to their effect on the activity and activation by L-Phe. The decreased affinity for L-Phe shown by p.R261Q and p.V388M is also accompanied by the absence of the characteristic substrate inhibition displayed by PAH at high L-Phe concentrations (Table 1). The defect in the positive cooperativity for L-Phe reflects a disturbance in the regulatory properties for all the mutants.

BH_4 Binding Studied by ITC

In order to obtain further information on the binding of BH_4 to wt-PAH and its mild-PKU associated mutants, we studied the thermodynamic properties of BH_4 binding to the enzymes at pH 7.0 using ITC. Aggregation was observed for the p.A309V, p.V388M, and p.Y414C mutant proteins and the binding of BH_4 to these mutants could not be analyzed by ITC. On the contrary, wt-PAH and the mutants p.R68S, p.I65T, p.P244L, and p.R261Q did not show any aggregation or loss of activity after incubations of up to 2-hr at 10–20°C (pH 7.0), allowing the analysis of their interaction with BH_4 by ITC.

BH_4 rapidly oxidizes at pH 7.0 under aerobic conditions in the absence of reductants like DTT [Davis et al., 1988; Pey et al., 2004] and ITC measurements were performed using 1 mM DTT to keep BH_4 reduced. At these conditions the cofactor was not oxidized when experiments were performed at temperatures <10°C. At 10°C in the presence of DTT, binding was found to be exothermic and the thermodynamic parameters could be estimated (Table 2). Nevertheless, with DTT as reductant the enzyme seems to bind substoichiometric amounts of the cofactor per subunit ($n < 1$). We then tested an alternative system based on the glucose-glucose oxidase-catalase coupled reaction [Bou-Abdallah et al., 2002; Rajagopalan and Pei, 1998] to create an "anoxic" environment. Best results were obtained with the system of Rajagopalan and Pei [1998], which totally prevented oxidation of the cofactor, as seen by the lack of formation of oxidized derivatives with absorbance at 330 nm [Pey et al., 2004]. This system was selected for further ITC studies, and calorimetric control experiments demonstrated the stability of BH_4 in this anoxic environment for several hours at pH 7.0 at 25°C (data not shown). At these conditions, the interaction of BH_4 with wt-PAH is also exothermic (Fig. 1A) and analysis of the thermo-

TABLE 2. Thermodynamic Parameters for the Formation of the PAH-BH_4 Complex as Determined by ITC*

Enzyme	$K_a(10^{-5})$ (M)	K_d (μM)	ΔG (kcal/mol)	ΔH (kcal/mol)	ΔS (cal/mol·K)
Wt[a]	3.8 ± 0.2	2.7 ± 0.1	−7.6	−10.7 ± 0.1	−10.4
p.I65T[a]	2.6 ± 0.2	3.9 ± 0.4	−7.4	−14.8 ± 0.6	−24.8
p.R68S[a]	1.1 ± 0.1	9.0 ± 1.0	−6.9	−13.3 ± 0.4	−21.4
Wt[b]	15 ± 1	0.7 ± 0.1	−8.0 ± 0.1	−20.4 ± 0.1	−44 ± 1
p.I65T[b]	9 ± 2	1.1 ± 0.2	−7.8 ± 0.1	−34.1 ± 6.4	−93 ± 23
p.R261Q[b]	14 ± 6	0.8 ± 0.3	−8.0 ± 0.3	−18.9 ± 3.4	−39 ± 11

*Data include the binding affinity (K_a), the dissociation constant (K_d), the free energy (ΔG), the enthalpy (ΔH) and the entropy (ΔS) of the binding of BH_4 to PAH.
[a]BH_4 was kept reduced by maintaining anoxic conditions with the glucose-glucose oxidase-catalase system [Rajagopalan and Pei, 1998]. Measurements performed at 25°C.
[b]BH_4 was kept reduced by 1 mM DTT. Measurements performed at 10°C.

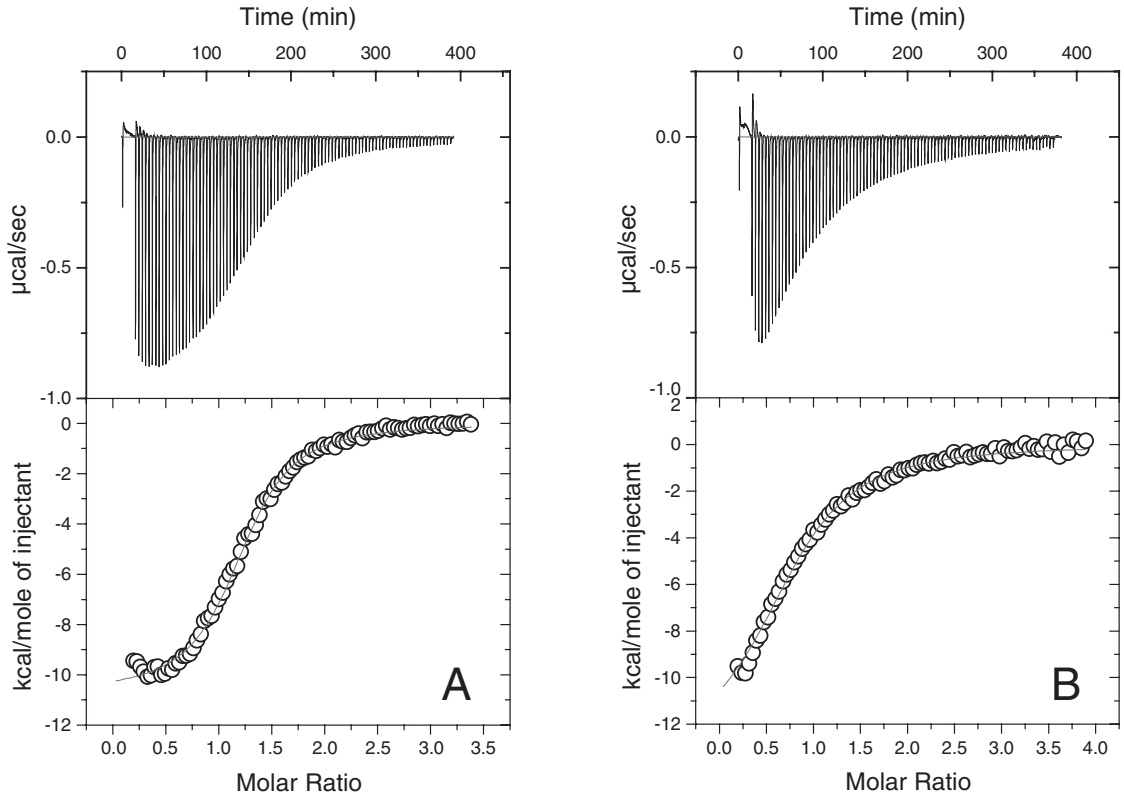

FIGURE 1. **Binding of BH_4 studied by ITC. Representative thermograms (upper panels) for the binding of BH_4 at 25°C in anoxic 20 mM Na-Hepes, 200 mM NaCl (pH 7.0) to wt-PAH (A) and p.R68S (B). Tetrameric enzymes at 40–50 μM subunit were titrated with 0.75 mM BH_4 (90 injections, 3 μl each injection). The lower panels show the binding isotherms, in which each point represents the integrated area of the corresponding peak in the thermograms and the solid line represents the best fit to the data.**

dynamic data for PAH-BH_4 complex formation indicates that the full-length tetrameric enzymes bind stoichiometric amounts of the cofactor per subunit ($n \sim 1$). The thermodynamic parameters for the binding of BH_4 to wild-type PAH obtained after mean heat dilution subtraction and fitting to a one-set-of-sites binding model are shown in Table 2. Binding analyses with the mutants p.I65T, p.R68S, and p.R261Q also provided exothermic thermograms (Fig. 1B; Table 2; and data not shown). The absolute K_d-values measured in this anoxic environment at 25°C are lower than when DTT is used to keep BH_4 reduced at 10°C, but the relative affinities for BH_4 for the mutants with respect to wt-PAH were similar in both type of measurements (shown in Table 2 for I65T). The cofactor binds to wild-type PAH with high affinity; a $K_d = 2.7 \pm 0.1$ μM was measured at 25°C, about 10-fold lower than the K_m value measured by steady state kinetics at the same temperature (Table 1). The binding is enthalpically driven, with unfavorable entropy change, as also inferred from the complete temperature dependence of the binding (data not shown). Similar experiments were carried out with the p.P244L tetrameric enzyme but a specific titration signal could not be detected at the standard conditions applied for wt and the other mutants, indicative of a considerable reduction of the binding affinity for this form. Accurate

determination of binding parameters from ITC for low-affinity interactions pose specific experimental challenges, including the need for very large amounts of protein [Wiseman et al., 1989].

The p.R261Q mutant shows similar parameters for the binding of BH_4 as wt-PAH (Table 2). The other mutants studied by ITC, i.e., p.I65T and p.R68S, showed reduced binding affinity ($K_d = 3.9$ μM for p.I65T and 9.0 μM for p.R68S, vs. 2.7 μM for wt at anoxic conditions) and a disfavorable entropy change upon binding of BH_4, i.e., about 2.0–2.5-fold larger than for binding to the wt at the respective conditions (anoxic or DTT). Interestingly, the concomitant enthalpic compensation of the entropic change [Leavitt and Freire, 2001] leads to a more modest decrease of the affinity compared to wt (Table 2). This is a common thermodynamic behavior in protein–ligand binding processes and it is usually manifested by a correlation between the enthalpy and entropy of a binding reaction obtained from temperature-dependence data. The compensation is also manifested when comparing the binding of different ligand analogs to the same protein or the effect of particular mutations, as in this work, especially in associations with large deltaCp, and results in small changes in the free energy of binding, and consequently of affinity [Dunitz, 1995].

Synthesis of PAH and Its Mutants in the Coupled In Vitro TnT System and Effect of BH_4 on Their Activity and Stability

We initiated the analysis of the possible stabilizing effect of BH_4 on the conformation and/or the activity of the wt and mutant PAH enzymes using a cell-free coupled TnT system in which BH_4 can easily be added to the synthesis extract. In vitro expression of the mutant cDNAs in the TnT system produced labeled protein in comparable amounts to the wild-type, both in the absence or the presence of BH_4 (Fig. 2). The characteristic double band of PAH expressed either in eukaryote hosts or in the TnT system was observed, with the bands with apparent M_r of approximately 51 and 50 kDa, corresponding to the enzyme phosphorylated on Ser16 and to the nonphosphorylated enzyme, respectively [Bjørgo et al., 1998]. The enzyme activities for the different PAH enzymes are summarized in Table 3. Interestingly, the activity of all the enzymes at synthesis stop (35 min) was higher when synthesis occurred in the presence of the cofactor (Table 3).

In order to investigate if the increase in PAH activity in the TnT extracts with added BH_4 was due to an increased conformational stability, we measured the half-lives of the enzymes after synthesis during a 7-hr incubation period at 37°C, in which further synthesis and labeling was prevented, i.e., half-lives for protein degradation ($t_{d1/2}$). All mutant proteins were proteolytically degraded more rapidly than the wt-PAH in absence of BH_4 (Fig. 2; Table 4). As has previously been shown for other PAH mutants [Bjørgo et al., 1998; Gamez et al., 2000], decreased $t_{d1/2}$ for the proteins is indicative of folding defects that are also manifested in increased aggregated forms for the enzyme expressed in *E. coli* and reduce the stability of the mutants expressed in COS cells [Pey et al., 2003]. The presence of BH_4 had a significant protective effect on the degradation rate of the mutants p.A309V, p.V388M, and p.Y414C (Table 4), which reach $t_{d1/2}$ values similar to that for wt-PAH.

Activity measurements showed that wt-PAH and its mutants synthesized in the absence of BH_4 were rapidly inactivated, actually at a much faster rate than that inferred from proteolytic degradation. Thus, in another

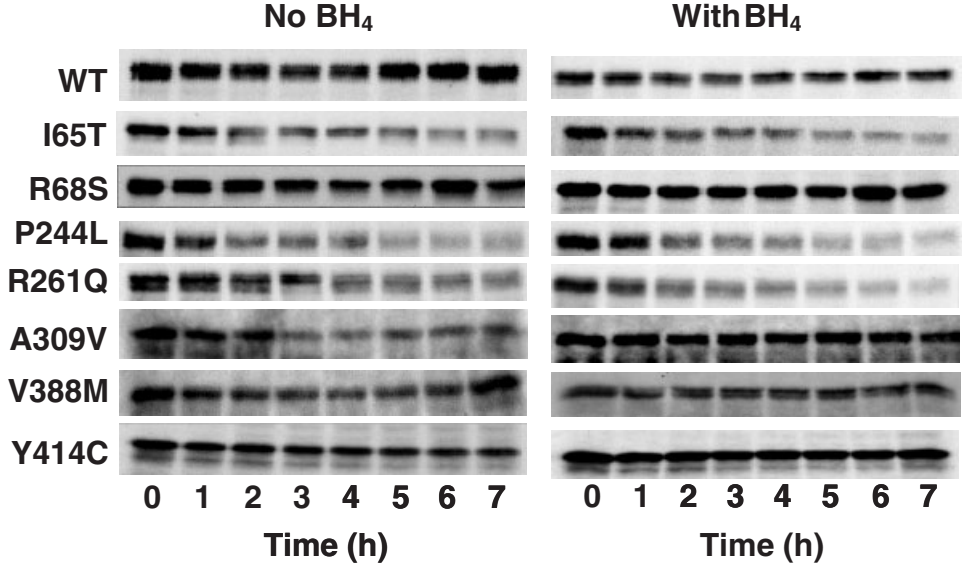

FIGURE 2. **SDS-PAGE analysis of the conformational stability of wild-type and mutant PAH proteins after synthesis by the coupled in vitro TnT system in the absence (left) or the presence (right) of BH_4. Remaining labeled PAH protein was quantitated by laser densitometry after fluorography of the gels in order to calculate the half-lives for protein degradation ($t_{d1/2}$) of the enzymes by semilogarithmic plots of the degradation time courses.**

TABLE 3. Relative Activities From Expression of wt-PAH and Its Mutants in the Coupled In Vitro Transcription-Translation (TnT) System*

Enzyme	Activity at the end of synthesis[a]		Activity 1 hr after end of synthesis[b]	
	Without BH_4 (%)	With BH_4 (%)	Without BH_4 (%)	With BH_4 (%)
Wt	100	140	9	114
p.I65T	118	134	13	64
p.R68S	44	61	5	63
p.P244L	32	43	2	21
p.R261Q	57	61	3	10
p.A309V	9	14	2	11
p.V388M	12	16	2	7
p.Y414C	9	18	3	11
Wt+catalase+SOD[c]	170	–	59	–

*The activity of the TnT extracts was measured at standard conditions (1 mM L-Phe, 75 μM BH_4, 5 min preincubation with L-Phe at 25°C). The activity is given relative to the activity for wt-PAH synthesized in the absence of BH_4, i.e., 20.8 ± 15.4 nmol Tyr/min · mg protein (100%). The values are the average of four experiments.

[a]Activity of PAH enzymes expressed in vitro in the TnT system at 30°C for 35 min in the absence or the presence of BH_4 (500 μM).

[b]Activity of PAH enzymes incubated for 1 hr at 37°C after the stop of the synthesis by adding ribonuclease A (1 mg/ml) and desoxiribonuclease I (1 mg/ml).

[c]The synthesis of wt-PAH was carried out in the presence of catalase (1 mg/ml) and superoxide dismutase (SOD) (1 mg/ml).

TABLE 4. Half-lives for PAH Protein and Activity of Wild-Type and Mutants Expressed in the Coupled In Vitro Transcription Translation (TnT) System in the Absence or the Presence of BH_4 (500 μM)

	$t^a_{d1/2}$		$t^b_{i1/2}$	
	Without BH_4 (min)	With BH_4 (min)	Without BH_4 (min)	With BH_4 (min)
Wt	522	474	9.0	128
p.I65T	198	192	10.8	48.2
p.R68S	486	516	7.1	>500
p.P244L	174	156	3.8	44.4
p.R261Q	258	192	3.6	9.5
p.A309V	222	516	16.6	133
p.V388M	330	420	6.9	31.2
p.Y414C	348	528	18.6	43.9

[a]Half-lives for protein degradation ($t_{d1/2}$) obtained from the semilogarithmic plots of the degradation time courses. After pulse-chase, labeled PAH protein was quantitated by laser densitometry after SDS-PAGE and fluorography.

[b]Half-lives for PAH inactivation ($t_{i1/2}$) obtained from semilogarithmic plots of activity vs. time (same data as in Fig. 3).

set of experiments, the in vitro synthesized enzymes were incubated for 1 hr at 37°C, and samples were withdrawn at discrete time points for PAH activity analysis. These experiments provided the $t_{1/2}$ values for inactivation ($t_{i1/2}$), which were in fact much shorter than those for protein degradation (Table 4). As seen in Figure 3 and Table 4, all the mutants appear to be significantly protected from the rapid inactivation of enzyme activity by the presence of BH_4, with the highest effect for p.R68S and the lowest for p.R261Q. To our knowledge, this is the first time that the quick inactivation of wt-PAH synthesized in the TnT-system in the usual manner, i.e., without the presence of BH_4, has been described.

In spite of the similar amounts of protein apparently present at the end of the synthesis periods (Fig. 2; lane corresponding to time 0) or the inability of BH_4 in increasing the $t_{d1/2}$ for some of the proteins (Table 4), the quick inactivation of the enzymes in the TnT systems and the protection exerted by BH_4 might be related to a rapid aggregation of the protein in the absence of the cofactor. It has been shown that aggregation of certain PAH forms expressed in the TnT systems certainly occurs, and the aggregated protein still contributes to the detected band in the SDS-autoradiograms [Bjørgo et al., 1998]. We therefore investigated the oligomeric distribution of the wt-PAH in the TnT extracts 60 min after the stop of the synthesis, which according to the inactivation curves (Fig. 3A) mostly correspond to inactive enzyme (absence of BH_4 during synthesis) or to enzyme with 70% remaining activity (presence of BH_4 during synthesis). Aggregated forms were not observed for enzyme synthesized in the absence of BH_4, in which the normal tetrameric and dimeric forms dominated (Fig. 4). Except for a somewhat higher proportion of the dimeric with respect to the tetrameric form, a similar elution profile was in fact observed for the enzyme synthesized in the presence of BH_4, indicating that quick aggregation does not seem to cause the inactivation.

We then hypothesized that oxidative modification of residues involved in substrate or cofactor binding and/or catalysis might contribute to the inactivation, and BH_4 binding to the newly synthesized PAH could protect the active site from oxidation. We tested the effect of catalase and superoxide dismutase addition to the TnT expression system on the activity at the end of the 35-min synthesis and 60 min after synthesis stop, and found

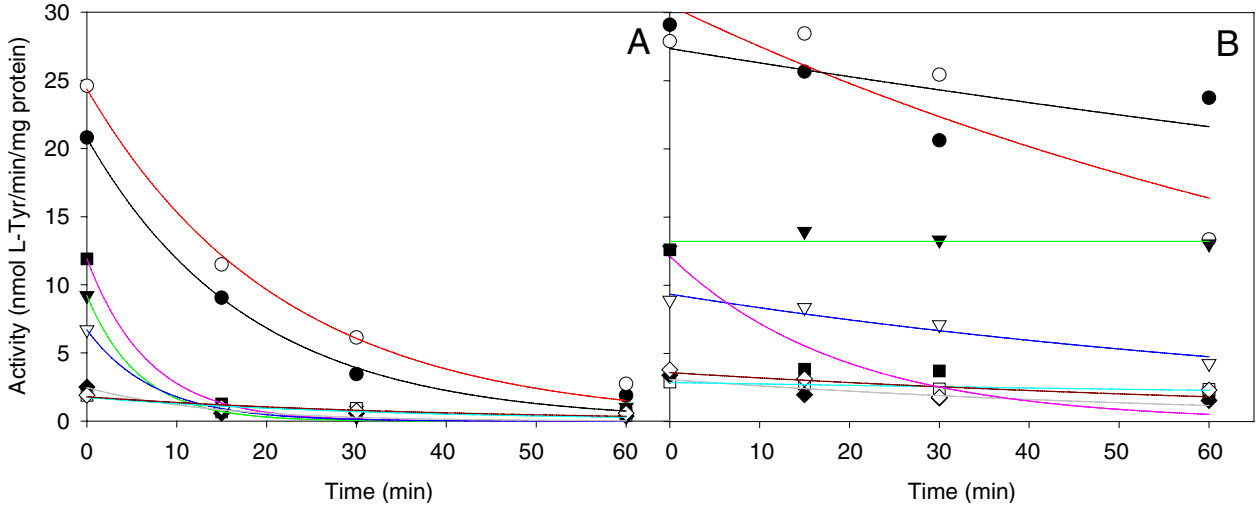

FIGURE 3. **Inactivation curves obtained for the wild-type and mutant PAH enzymes synthesized by the coupled in vitro TnT system. Wild-type (●, black), and mutant p.I65T (○, red), p.R68S (▼, green), p.P244L (▽, blue), p.R261Q (■, pink), p.A309V (□, cyan), p.V388M (◆, gray), and p.Y414C (◇, brown) PAH enzymes were synthesized for 35 min at 30°C in the absence (A) or the presence (B) of 500 μM BH_4, and subsequently incubated at 37°C; the remaining activity at the indicated time points was measured at standard conditions (1 mM L-Phe, 75 μM BH_4 at 25°C). These time courses provided the half-lives for protein inactivation ($t_{i1/2}$).**

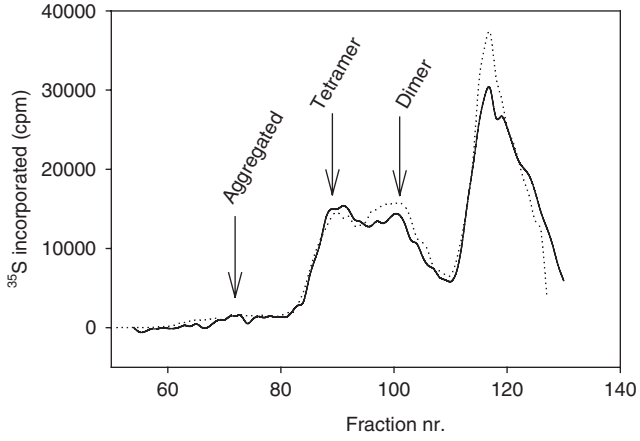

FIGURE 4. **Size-exclusion chromatography of PAH synthesized in the coupled in vitro TnT system. A total of 200 μl of the reaction mixture of the wt-PAH synthesized in the absence (—) or the presence (- - -) of 500 μM BH_4, were applied to a High Load Superdex 200 HR column equilibrated with 20 mM NaHepes and 0.2 M NaCl (pH 7.0), and eluted at a flow rate of 0.38 ml/min. Peak positions of the main components of wt-PAH: fractions 72–75, 114 min, aggregated form; fractions 87–92, 156 min, tetrameric form (209 kDa); and fractions 100–103, 170 min, dimeric form (104 kDa). The fraction eluted at about 225–230 min at least partly represents a monomeric form of the enzyme [Bjørgo et al., 1998].**

that these additions partially mimic the protective effect of BH_4 preventing inactivation (Table 3).

DISCUSSION

Facing the increasing clinical interest in the possibility to treat mild PKU patients with high doses of BH_4, caution has been requested to avoid being premature in overestimating both the benefits of this treatment and the number of patients that may profit from cofactor replacement therapy (see, for instance, Ponzone et al. [2003]). It is therefore important to concentrate efforts to understand the molecular basis and the genotype–phenotype relationships for the responsiveness, since leaving the phenylalanine-free diet to enroll in a substitution therapy with high doses of BH_4 is both very expensive and potentially dangerous if the response is not elevated.

The 3D-structural location of the residues involved in the mild PKU, BH4-responsive mutations selected in this study is shown in Figure 5. Ile65 and Arg68 are located at the regulatory domain. Ile65 is at the start of β2, and contributes to a cluster of hydrophobic residues. The introduction of a polar residue in the mutation p.I65T will certainly disturb the hydrophobic packing of the regulatory domain. Arg68 is located at the loop 68–75 that interacts with the catalytic subunit of the other subunit in the dimer, notably with Cys237, and its mutation to a smaller and noncharged serine will potentially disturb this interaction. Pro244 is located at the end of β1 in the catalytic domain. The mutation p.P244L will destabilize the loop between β1 and α6, and may affect the local structure of the active site. Arg261 is at a loop connecting α6 and β2 in the catalytic domain. Its mutation to a Gln residue may result in the disruption of the hydrogen bonding network to Glu304 and Thr238, and would certainly affect the geometry of the active site. In addition, the guanidinium group of Arg261 stacks with the ring of Tyr417 in the adjacent subunit in the dimer, indicating an important role of Arg261 in the regulation of the enzyme. The mutation thus might impair the transmission of L-Phe–induced activating conformational changes between the dimers forming the tetramer. Ala309 is at the end of α8 at the catalytic domain, and mutation to a larger valine residue may affect the orientation of the neighboring α12 and the

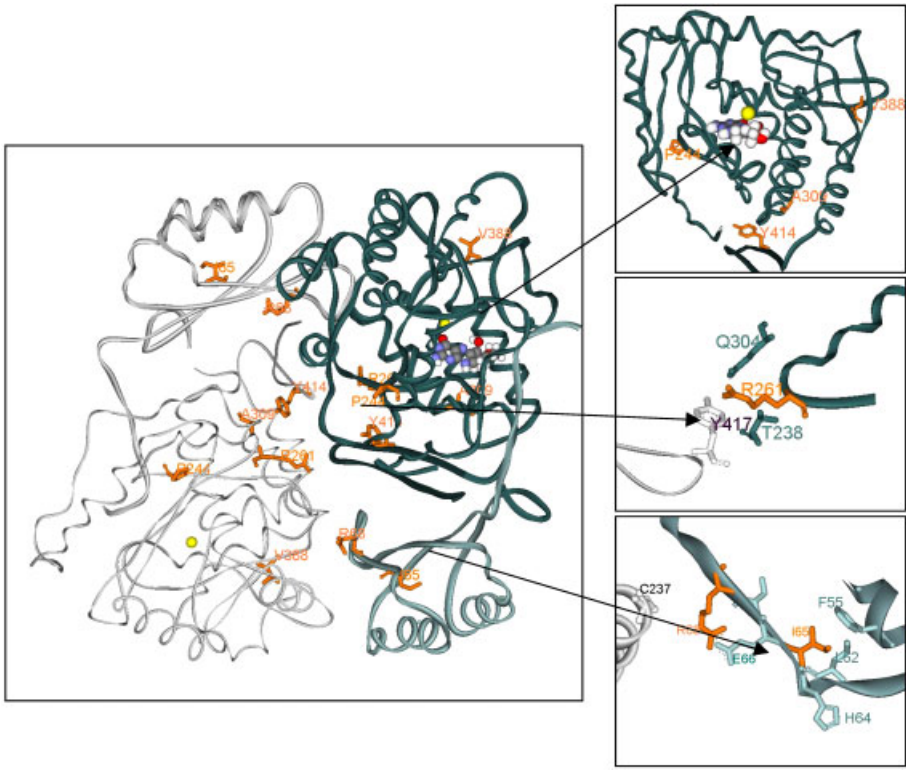

FIGURE 5. **Localization of the PAH mutations analyzed in this study. The side chains of the mutated residues are shown as orange sticks in the structure of dimeric rat PAH (PDB code 1PHZ) [Kobe et al., 1999], Fe is shown in yellow, and BH_4 bound according to the catalytic domain-BH_4 complex (PDB code 1KWO) [Andersen et al., 2002] is shown in ball and stick representation. Insets, detailed views of the mutation sites.**

geometry of the active site. Val388 is on the catalytic domain at a loop leading to the L-Phe binding site, while Tyr414 is located at the dimerization motif (411–427). Mutations in these important positions are therefore expected to result in both catalytic and regulatory defects.

The detailed kinetic analysis of the purified tetrameric recombinant proteins show that all the mutations either lack or have diminished activation by L-Phe preincubation, lack or have diminished positive cooperativity by L-Phe, and (specifically for p.R261Q and p.V388M) have highly decreased affinity for L-Phe (Table 1). Regarding the BH_4 binding affinity in kinetic assays, p.I65T, p.P244L, and p.A309V showed a moderate increase (1.5-fold) in K_m, which may be an apparent kinetic effect resulting from an increased affinity for L-Phe in this multisubstrate enzyme. This does not seem to be the reason for these mutations, since some decrease in the $S_{0.5}$(L-Phe)-value is encountered only for the mutations p.I65T and p.R68S (Table 1), and the direct equilibrium binding measurements of the affinity in the absence of L-Phe by ITC also corroborated a decreased affinity for the cofactor in the mutants p.I65T and p.R68S. For the mutant p.P244L, the absence of calorimetric response to BH_4 could be attributed to a strong binding defect at our experimental conditions, and the instability of p.A309A hindered its characterization by ITC. For the other mutants, no reduction in the affinity for the cofactor could be measured by either kinetic measurements (p.R261Q, p.V388M, and p.Y414C) or by ITC (p.R261Q). It is probable that for p.R261Q and p.V388M, the decreased affinity for L-Phe, in combination with their folding defect, are the most deleterious functional effects in vivo. In particular for p.V388M, its very low affinity for L-Phe and consequent low catalytic efficiency is in agreement with the location of Val388 at a loop leading to the L-Phe binding site.

Both p.R68S, p.I65T, and p.R261Q are activated in the basal state, as isolated, prior to activation by preincubation with L-Phe (Table 1). The activation shown by p.R68S and p.I65T, both located at the regulatory domain, might be related to a partial unfolding of this domain in the mutant enzymes, which imposes a regulatory inhibiting effect on the catalytic domain [Kobe et al., 1999]. The effect of the mutation p.R68S is as expected from results with other mutations at the same position and from the role of the loop 68–75 in the transmission of activating conformational changes from one subunit to the others [Thórólfsson et al., 2003]. The mutation p.R261Q is also interesting in this context. The substitution of the charged arginine guanidinium group will damage the polar-stacking interaction with Tyr417 at the dimerization motif in the adjacent subunit. This finding might indicate that while the interactions between the regulatory domain from one subunit and both the catalytic and oligomerization domain from the

neighboring subunit in the dimer seem to be strengthened upon cooperative activation by L-Phe [Thórólfsson et al., 2003], the interaction between the catalytic domain and the dimerization motif from adjacent subunits is weakened, in order to establish adequate interactions with the tetramerization domain and transmit the conformational changes to the other dimer in the tetramer. Interestingly, the mutations p.Y417N (c.1249T>A) and p.Y417H (c.1249T>C) are also associated to mild PKU, and the latter was recently classified as probably associated with responsiveness to BH_4 [Muntau et al., 2002]. Final proof for the structural activating effect in these three mutations (p.I65T, p.R68S, and p.R261Q) must await the elucidation of their crystal structure in the full-length tetrameric form, a task which has been unattainable so far even for the wild-type form of PAH.

Further studies on the selected mild PKU mutants were performed using the expression of the enzyme forms in an in vitro coupled TnT cell-free system. Opposite to the expression of the enzyme in *E. coli* or in eukaryote cells, expression in this system is very amenable and easily run in the presence of BH_4, and although the physiological relevance of comparative activity measurements is questionable in this system, these analysis provide insights on the effects of BH_4 on the conformational and kinetic stability of the mutants. The cofactor appears to have a significant role as a chemical chaperone, preventing misfolding for three of the mutants considered in this study, i.e., p.A309V, p.V388M, and p.Y414C. Our results, nevertheless, point to a role of the cofactor in increasing the specific activity by preventing a chemical inactivation for essentially all the enzymes, including wt-PAH. It has been shown in hepatocytes that the newly synthesized PAH and BH_4 form a complex (PAH-BH_4) that sequesters the metabolic availability of the cofactor [Mitnaul and Shiman, 1995]. The PAH-BH_4 complex seems thus to represent an enzyme with low activity but high stability [Teigen and Martinez, 2003; Xia et al., 1994]. At high concentrations of the cofactor, most of the enzyme will be present as a PAH-BH_4 complex in which the protective effect of BH_4 might be exerted by blocking the solvent accessibility of the active site nonheme iron and preventing the generation of destructive oxygen species. PAH is known to be quickly and irreversibly inactivated by hydrogen peroxide [Kappock and Caradonna, 1996; Milstien et al., 1990], which can be formed at the iron site by uncoupled reactions and by oxidation of common biochemicals [Kappock and Caradonna, 1996; Kemsley et al., 2003]. The binding of BH_4 at saturating concentrations might prevent peroxide formation and protect the right configuration of the active site. Our results on the partially protective effect exerted by catalase and superoxide dismutase agree with this hypothesis. In fact, this protective effect of BH_4 toward inactivation of PAH may be of primary significance in understanding the mechanism by which cofactor supplementation also increases enzyme activity in vivo. The protective effect was found for all the mutants distributed around the 3D structure (Fig. 5), without any specific preference to one of the domains. The highest protective effect of BH_4 was found for p.R68S, a mutation clearly associated to BH_4 responsiveness, and the lowest effect was found for p.R261Q, a mutation that, although positively associated to the BH_4 responsive phenotype [Spaapen and Rubio-Gozalbo, 2003], has also been found to be inconsistently associated with responsiveness [Muntau et al., 2002]. In this case, in which this residue is implicated in the interaction of two neighboring subunits, the second PKU allelic variant in the patient could have a high significance in determining the responsiveness to BH_4.

Our in vitro expression studies further identify an additional factor that may contribute to increase the PAH activity of certain mild-PKU mutants in vivo: i.e., the chaperone-like activity of the cofactor for some mutations (shown for p.A309V, p.V388M, and p.Y414C in this study). These residues are located at the dimerization motif (Y414) and near this motif (A309, V388). The binding of the cofactor may favor the proper dimer/tetramer formation, resulting in reduced protein instability and degradation as suggested by Spaapen and Rubio-Gozalbo [2003]. Both mutations have been found in responsive patients [Spaapen and Rubio-Gozalbo, 2003], although p.Y414C has also been described as inconsistently associated with the responsive phenotype [Muntau et al., 2002]. Finally, an additional factor that may contribute to the BH_4 responsiveness in some mutants is the obvious increased activity with increased cofactor concentration. This effect would be more relevant for the mutations with low affinity for the cofactor, i.e., p.R68S and p.I65T (both located at the regulatory domain), and p.A309V and p.P244L (located at the cofactor binding site in the catalytic domain). Recent results show that mutations p.A309V and p.P244L are also potentially associated with BH_4-responsiveness (L.R. Desviat et al., in press). Mutations with high residual activity and higher $K_m(BH_4)$ than wt-PAH would be potentially responsive, as it has been proven for p.R68S and p.I65T [Muntau et al., 2002; Spaapen and Rubio-Gozalbo, 2003]. All these mechanisms would aid some patients to increase the amount of active enzyme, and its activity and reach the lower-limit value of hydroxylation, allowing the catabolism of the L-Phe amounts present in normal diets.

In this study, we have used two different expression systems, i.e., a bacterial approach and a rabbit reticulocyte lysate, providing relevant information of the functional effect of these mutations. Mammalian cell expression of human PAH wild-type and mutants is assumed to provide a more reliable description of the in vivo biochemical phenotype than other expression systems, and the most frequently used mammalian host for comparative expression analyses is COS (a monkey kidney cell line) [Waters et al., 1998]. While expression in *E. coli* or eukaryote cell systems like COS cells is not readily amenable to be performed in the presence of defined concentrations of BH_4, expression in the TnT

system is advantageous in this respect, since BH_4 can easily be added to the cell-free extract. Moreover, transient expression in COS cells provides small amounts of protein that is not readily amenable for purification and characterization of the molecular and proper kinetic properties of the PAH enzymes. This is usually undertaken with the large scale expression in *E. coli* of the fusion proteins of the PAH enzymes with maltose binding protein [Martínez et al., 1995]. The specific activity measured at standard conditions of the purified enzymes is, in some mutations, moderately correlated to the activity in crude extracts of COS cells expressing the corresponding mutants. This is mostly due to the fact that the different deleterious effects of the mutations, mainly kinetic or folding, are manifested and contribute differently to the final biochemical phenotype in each expression system. For instance, folding variants result in a high content of aggregates in *E. coli* and lower detectable amount of protein in eukaryote cells (the high activity mutant p.I65T is a clear example of this type of mutant), and for some mutations associated with classical PKU, the purified protein contains hardly any tetrameric and dimeric forms [Bjørgo et al., 1998].

In conclusion, the set of expression and kinetic and binding analyses outlined and developed in this work contributes to the characterization of the effects of BH_4 on the activity and stability of PAH. Our results suggest that the response to BH_4 supplementation therapy may be multifactorial. This study contributes to the understanding of the molecular basis of the responsiveness to BH_4 supplementation in PKU patients and may contribute to a more accurate selection of genotypes predictably associated with a positive response to cofactor treatment. Nevertheless, the in vivo response to BH_4 in heterozygotic patients containing these mutations would also depend on the second mutant allele, mostly for mutants with low residual activity. The second mutation, in turn, would determine the molecular composition of the PAH mutant subunits present in the tetrameric form of the enzyme in vivo [Erlandsen and Stevens, 2001].

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