

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

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Abstract

The activity of phenylalanine hydroxylase (PAH) is regulated by the levels of both the substrate (L-Phe) and the natural cofactor (6R)-tetrahydrobiopterin (BH₄). It has recently been observed that many PAH mutants associated with BH₄-responsive phenylketonuria display abnormal kinetic and regulatory properties as shown by standard kinetic analyses. In this work, we have developed a high-sensitive and high-throughput activity assay based on isothermal titration calorimetry (ITC) in order to study the kinetic properties of wild-type PAH (wt-PAH) and the BH₄-responsive c.204A > T (p.R68S) mutant at physiological and superphysiological concentrations of L-Phe and BH₄. Compared to wt-PAH, the p.R68S mutant showed reduced apparent and equilibrium binding affinity for the natural cofactor and increased affinity and non-cooperative response for L-Phe, together with a strong substrate inhibition that is alleviated at high BH₄ concentrations. For both wt-PAH and mutant, the apparent affinity for BH₄ decreases at increasing L-Phe concentrations, and the affinity for the substrate also depends on the cofactor concentration. Our results indicate that the *activity landscape* for wt and mutant enzymes is more complex than expected from standard kinetic analyses and highlight the applicability of this ITC-based assay to characterize the activity and regulation of PAH at a wide range of substrate and cofactor concentrations. Moreover, the results aid to understand the activity dynamics of wild-type and mutant PAH under physiological and pathological conditions, as well as BH₄-responsiveness in certain PKU mutations.

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Introduction

Phenylalanine hydroxylase (PAH; phenylalanine 4-monooxygenase; EC 1.14.16.1)¹ is a non-heme iron-dependent enzyme that catalyzes the hydroxylation of

L-Phe to L-Tyr in the presence of (6R)-L-erythro-6,7,8-tetrahydrobiopterin (BH₄) using molecular dioxygen as additional substrate. In humans, PAH activity is mainly present in liver, and its impairment leads to phenylketonuria (PKU; [1]). About 500 disease causing mutations have been described for the PAH gene, and the molecular mechanisms responsible for the loss-of-PAH-function have pointed towards decreased conformational stability [2–5] and/or kinetic abnormalities in the PAH enzyme in vitro [2,6–8]. PKU patients have been classically treated with a L-Phe restricted diet, in order to reduce L-Phe plasma levels and avoid cognitive

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¹ Abbreviations used: BH₄, (6R)-L-erythro-5,6,7,8-tetrahydrobiopterin; ITC, isothermal titration calorimetry; MBP, maltose binding protein; 6M-PH₄, 6-methyl-tetrahydropterin; PAH, phenylalanine hydroxylase; PKU, phenylketonuria; wt, wild-type.

dysfunction [1]. Although successful, this treatment has to be continued “for life” and presents a heavy burden to patients and their families. Recently, the administration of high doses of the natural cofactor BH₄ has been described as a realistic alternative in the treatment of some PKU patients, and evidence for the viability of long-term treatment has been reported [9–11]. In order to understand the molecular mechanism of the BH₄-responsiveness [12], the biochemical properties of the PAH mutant proteins and the effect of BH₄ supplementation have been investigated in recent mutational in vitro analyses [7,8]. The BH₄-responsiveness associated mutants analyzed in these studies have been typically associated to mild PKU phenotypes and present high residual activity ($\geq 30\%$ of wt-PAH). Interestingly, most of these mutant proteins showed abnormal kinetic behavior when they were analyzed at standard (saturating) conditions (1 mM L-Phe, 75 μ M BH₄). The in vitro analysis with recombinantly expressed enzymes indicated that the responsiveness to BH₄ has a multifactorial basis. Notably, and in addition to the expected increase in activity [13], cofactor supplementation seems to (i) correct/compensate the decreased affinity for BH₄ shown by some mutants, (ii) protect towards catalytic inactivation, and (iii) protect towards the increased conformational instability induced by some mutations [7,8]. In addition, it has been shown that BH₄ has a chaperon-like effect on PAH also in vivo, protecting the enzyme against auto-inactivation and degradation, without affecting gene expression or Pah-mRNA stability [14].

To maintain L-Phe homeostasis in vivo, PAH activity must be tightly regulated by different molecular mechanisms, being specially relevant the regulatory effects observed for the substrate, the natural cofactor, and phosphorylation/dephosphorylation [1,15]. In order to exhibit maximal activity in the presence of BH₄, PAH must be preincubated with L-Phe, which induces an activating conformational change and binds with positive cooperativity to the enzyme (*Hill coefficient, h*, ≈ 2 ; [16,17]). Also, BH₄ binding to PAH seems to induce a conformational change that keeps the enzyme in a state characterized by low activity and low affinity (for L-Phe) [18], as well as higher stability [8,14,19,20], ready for the activation at increased intracellular L-Phe concentrations in hepatocytes [21]. Many of these regulatory events have been studied by enzyme kinetic analysis typically performed only at standard (saturating, superphysiological) concentrations of either substrate and/or cofactor (higher than those expected in human liver or plasma), and by equilibrium binding analyses [16,17,20,22–25]. In order to analyze the PAH activity dynamics under a wide range of substrate and cofactor concentrations, we have developed in this work a high-sensitive and high-throughput isothermal titration calorimetry (ITC)-based activity assay. This assay has been applied in comparative activity measurements of recom-

binant tetrameric human wt-PAH and the p.R68S mutant, a mutation reported as BH₄-responsive [12]. Our results demonstrate the complexity of the PAH *activity landscape*, unravelling some intertwined effects between BH₄ and L-Phe in a wide concentration range. Also, it appears that some kinetic/binding properties like cooperativity and substrate inhibition may be relevant at physiological and superphysiological (pathological) conditions, and might lead to unexpected effects on the PAH residual activity in vivo. The results presented and the strategy developed here may help to get further understanding on the mutant activity dynamics in PKU and BH₄-responsiveness.

Materials and methods

Expression and purification of recombinant PAH enzymes

Growth of *Escherichia coli* transformed with the pMAL vectors for expression of wild-type (wt) and c.204A > T (p.R68S) mutant PAH proteins and purification of the maltose binding protein (MBP)-PAH fusion proteins were performed as described [7,26]. The tetrameric fusion proteins were isolated by size-exclusion chromatography, concentrated using Centricon 30 Microconcentrators (Amicon), and stored in liquid nitrogen. Protein concentration was measured spectrophotometrically using $\epsilon_{280\text{nm}} (1\text{mg/ml}) = 1.63$ [26].

Isothermal titration calorimetry: kinetic measurements

The experiments were performed in a VP-ITC titration calorimeter (MicroCal) under reducing conditions at 25 °C in 100 mM Na-Hepes, pH 7.0, 5–75 μ M BH₄, 10–1000 μ M L-Phe, 5 mM tris(carboxyethyl)-phosphine (TCEP) as reductant [27], 1 μ M ferrous ammonium sulfate, 0.5 mg/ml bovine serum albumin, and 0.04 mg/ml catalase. Except in the determination of ΔH_{app} (see below) tetrameric fusion protein (MBP-PAH) was typically used in the assays at a final concentration of 3–25 nM subunit. As otherwise indicated, the activity was linear respect to the amount of enzyme and reaction time used.

Enzyme reaction rates were determined by measuring the change in instrumental thermal power supplied to the sample cell as a consequence of the enzymatic reaction, represented by a shift in the calorimetric baseline ([28,29], see also Fig. 1A). The calorimetrically measured steady-state rate, or initial velocity, was determined from Eq. (1) [28]:

$$\text{rate} = \frac{d[\text{L-Tyr}]}{dt} = \frac{1}{V_{\text{cell}} \times \Delta H_{\text{app}}} \times \frac{dQ}{dt}, \quad (1)$$

where V_{cell} is the volume of the reaction cell (1.414 ml), ΔH_{app} is the apparent molar enthalpy of the reaction un-

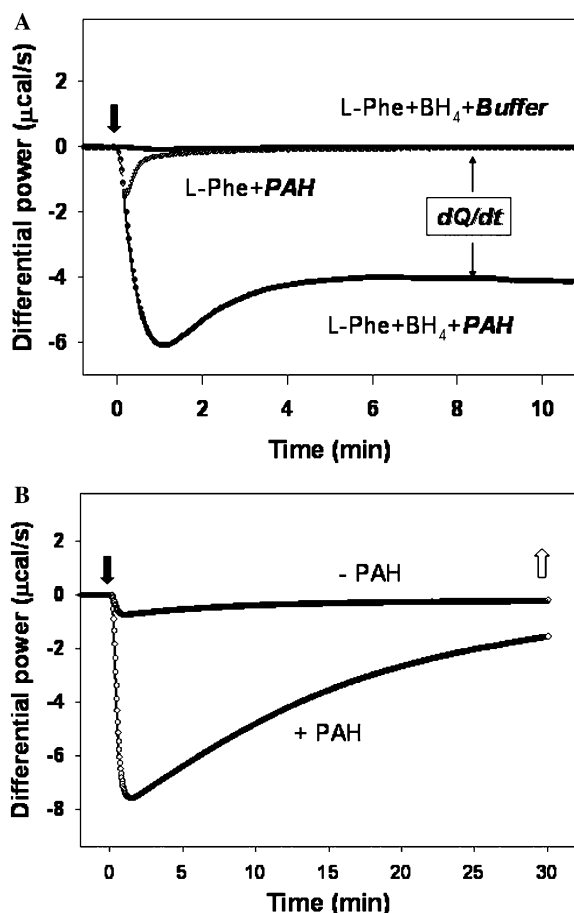


Fig. 1. Calorimetric rate measurements. (A) Representative enthalpograms obtained upon injection (indicated by the black arrow) of 6 nM (final subunit concentration in the cell) tetrameric fusion protein of wt-PAH in the presence of 1 mM L-Phe to the sample cell containing 75 μ M BH₄ and 1 mM L-Phe. Control reactions in the absence of enzyme (injecting buffer) and absence of BH₄ (injecting PAH without BH₄ in the cell sample) are shown, displaying only transient (dilution) thermal effects and negligible contribution to the steady-state signal (dQ/dt). (B) Measurement of the ΔH_{app} for the PAH catalyzed reaction. BH₄ (5 μ M final concentration) was injected (black arrow) to the sample cell containing 1 mM L-Phe and 100 nM PAH subunit (+ PAH) or no PAH (- PAH). The reaction was allowed to proceed for 30 min, and then an aliquot of the reaction mixture was withdrawn (white arrow) and analyzed by HPLC and fluorimetric detection to determine the concentration of L-Tyr formed during the assay. After blank subtraction, a $\Delta H_{app} = -131 \pm 11$ kcal/mol was estimated (means \pm SD of six independent experiments).

der these experimental conditions, and dQ/dt is the shift in the calorimetric baseline (Fig. 1A). The ΔH_{app} was determined by adding BH₄ (5 μ M final concentration) to the PAH enzyme (100 nM subunit) and L-Phe (1 mM) in the sample cell. The total heat released was recorded for 30–60 min (Fig. 1B), and then, 50 μ l of the reaction mixture was taken, mixed with 50 μ l of 2% acetic acid in ethanol, and analyzed for L-Tyr concentration by HPLC and fluorimetric detection ([30] and see below). Blank reactions in the absence of enzyme in the syringe were also performed, and the heat

measured subtracted, in order to account for thermal (dilution) effects and non-enzymatical baseline drifts. By this procedure, a $\Delta H_{app} = -131 \pm 11$ kcal/mol was estimated. This value for ΔH_{app} was not significantly affected by the concentration of enzyme, substrate, and cofactor used or by the time of the reaction.

The measurement of steady-state activity after L-Phe preincubation conditions was performed by injecting 14 μ l of a mixture containing L-Phe (100–1000 μ M) and PAH (0.3–2.4 μ M subunit; the final concentration of PAH will be then 3–24 nM subunit in the calorimetric sample cell) to the cell containing identical L-Phe concentration as injected and 5–75 μ M BH₄. After this injection, the reaction rate was measured for 30 min. The assay was linear in this protein range, and blanks without enzyme were routinely run under identical conditions and subtracted.

For the measurement of PAH activity after BH₄ preincubation conditions, 10–30 nM enzyme subunit and 5–75 μ M BH₄ were subjected to eight injections (2.8–14 μ l per injection), 3 min spaced, of L-Phe (5–25 mM) in the presence of identical BH₄ concentrations as in the cell. Blanks in the absence of enzyme were also routinely performed and subtracted. For the activity calculation, a correction for enzyme dilution upon substrate injection was performed, using the equation [29]:

$$[E]_i = [E]_{i-1} \times \exp(-V_{inj}/V_{cell}), \quad (2)$$

where $[E]_i$ and $[E]_{i-1}$ are the enzyme concentrations after and before the i th injection, respectively, V_{inj} is the injected volume in i th injection, and V_{cell} is the volume of the sample cell (1.414 ml). No corrections were made for substrate dilution or depletion during the assay, because under these experimental conditions, L-Phe dilution was less than 5% of the non-corrected values (as calculated using the expressions described in [29]) and the amount of substrate consumed at the end of the experiments was never more than 4% of the initial concentration (as estimated by HPLC and fluorimetric detection and/or using the total heat measured calorimetrically at the end of the experiments and the ΔH_{app} for the reaction).

The PAH activity dependence on BH₄ concentration was routinely fitted to a single hyperbolic function (Michaelis–Menten kinetic model), and the dependence on L-Phe concentration was adequately fitted to a sigmoidal function (Hill kinetic model). When a significant L-Phe induced inhibition at high L-Phe concentrations is observed, a modified Hill equation with additional terms for substrate inhibition was used [31]

$$v = V_{max} + V_i \left(\frac{[L-Phe]^x}{K_{I(L-Phe)}^x} \right) / \left(1 + (S_{0,5}^h/[L-Phe]^h) + ([L-Phe]^x/K_{I(L-Phe)}^x) \right). \quad (3)$$

Best fits (as measured by χ^2 value) as well as more physically realistic values for the fitting parameters were

obtained when fixed values for V_i ($V_i = 0$) and x ($x = 2$) were used, as recommended [31]. The fitting procedures were performed by non-linear regression analysis using Sigma Plot 2000 (SPSS) and Origin 5.0 (MicroCal).

Isothermal titration calorimetry: equilibrium binding measurements

BH₄ and 6-methyl-tetrahydropterin (6M-PH₄) equilibrium binding measurements to wt-PAH and p.R68S in the absence or presence of 1 mM L-Phe were performed under anoxic conditions (using the glucose/glucose oxidase/catalase system) in 20 mM Na-Hepes, 200 mM NaCl, pH 7.0 at 5–35 °C essentially as previously described [25], using 30–50 μM enzyme subunit. The thermodynamic parameters were estimated by fitting the raw data, after subtraction of heat dilution, to a one-type-of-sites binding model, and ΔC_p was estimated from the slope of the linear fitting of the temperature dependency of ΔH as previously described [25]. For data analysis, MicroCal Origin 5.0 (MicroCal) and SigmaPlot 2000 (SPSS) were routinely used.

L-Tyr quantification by HPLC and fluorimetric detection

At selected end-time points, samples from ITC-based PAH activity measurements were analyzed for L-Tyr content essentially as described [30]. Acetic acid in ethanol (2% V/V) was added to the reaction mixtures, which were incubated at –20 °C for 1 h, centrifuged for 10 min, and 10 μl of the supernatant was applied to an ionic exchange column (Partisphere SCX 125 × 4.6 mm *strong cation exchanger*, Whatman) equilibrated in 10 mM sodium acetate, pH 3.4, 1% propanol (V/V) and controlled by a Shimadzu VP HPLC system (Shimadzu). Chromatographic runs were performed at 1 ml/min flow rate for 4 min, observing a ~3.3 min retention time for L-Tyr. Detection and quantification were performed by fluorimetric detection ($\lambda_{\text{ex}} = 274$ nm and $\lambda_{\text{em}} = 304$ nm) by comparing the fluorescence signals to those obtained for L-Tyr standards (2–50 μM linear range).

Results

ITC-based PAH assay

In order to perform kinetic and equilibrium binding studies on wt-PAH and the p.R68S mutant by ITC, we have utilized the enzymes as MBP-PAH fusion proteins. It is well known that the fusion partner MBP has the potential of stabilizing recombinantly expressed proteins, specially if they are unstable like most of the PKU mutant proteins analyzed so far [2,5,40]. Nevertheless, the fusion proteins appear to display a kinetic behavior similar to that of the isolated PAH, as previously ob-

served for both wt and PKU mutant proteins [2,26]. Thus, we selected the fusion proteins in this ITC study since artifacts arising from protein aggregation and denaturation during the experiments should be minimized in calorimetric studies.

As observed in Fig. 1A, the addition of a small amount of PAH (6 nM final subunit concentration; ~1 μg fusion protein) to the sample cell containing L-Phe and BH₄ generates a large and negative steady-state signal, that is stable up to 30 min. This steady-state signal is proportional to the amount of product (L-Tyr) formed during the PAH catalyzed reaction, as determined by HPLC and fluorimetric quantification of L-Tyr after the calorimetric experiments are completed (see Materials and methods). The independent measurement of the product allows the calculation of ΔH_{app} for the enzymatic reaction, as proposed for reactions that do not proceed to completion [28]. Thus, by dividing the heat released during the reaction (Fig. 1B) by the amount of L-Tyr formed, a $\Delta H_{\text{app}} = -131 \pm 11$ kcal/mol is obtained. This value was used in the consequent experiments to calculate the steady-state rates using Eq. (1). The high sensitivity for this calorimetric assay is originated by the large and negative ΔH_{app} for the reaction, allowing the use of very low amounts of enzyme and preventing substrate depletion during the activity measurements.

PAH activity at variable concentrations of BH₄ under L-Phe preincubation conditions (activated enzyme)

In another set of experiments, wt-PAH was incubated at different concentrations of L-Phe and the reaction was triggered by its injection into the sample cell containing BH₄ and the corresponding concentration of L-Phe in order to estimate the activity dependence on the concentration of BH₄ at different concentrations of L-Phe. Representative kinetic traces for these experiments are shown in Fig. 2A. The results obtained at each L-Phe concentration were fitted to a single hyperbolic model (Fig. 2B), and the kinetic parameters were estimated (Table 1). At 1 mM L-Phe, the $K_m(\text{BH}_4)$ and V_{max} (Table 1) were similar to those reported previously for recombinant human fusion protein MBP-PAH and isolated PAH by HPLC and fluorimetric detection at standard conditions [6,26,32]. Interestingly, when the L-Phe concentration is increased, a significant reduction in the apparent affinity of the cofactor is observed (2.6-fold increase in the $K_m(\text{BH}_4)$ from 100 μM to 1 mM L-Phe) (Table 1).

Wt-PAH activity at different concentrations of L-Phe under BH₄ preincubation conditions (non-activated enzyme)

In order to apply ITC as a high-throughput technique to characterize PAH enzyme kinetics, we have measured

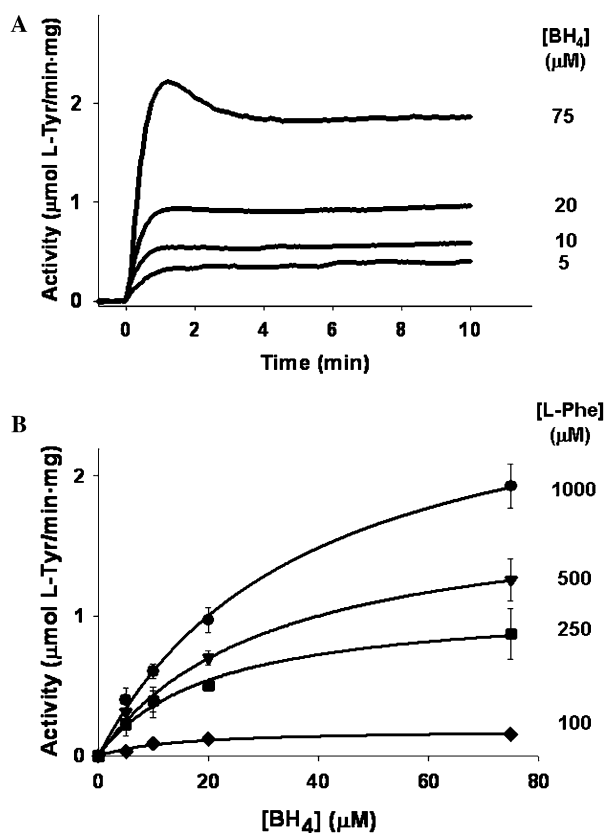


Fig. 2. PAH enzyme kinetics at variable concentrations of BH₄, under L-Phe preincubation (activated enzyme) conditions. (A) Representative kinetic traces obtained upon injection at time $t = 0$ of 3 nM (final subunit concentration in the cell) tetrameric fusion protein of wt-PAH in the presence of 1 mM L-Phe, to the sample cell containing the indicated concentration of BH₄ and 1 mM L-Phe. The original kinetic traces were recorded and, after subtraction of the blank reaction, were converted to enzyme activity units using $\Delta H_{\text{app}} = -131 \pm 11$ kcal/mol. (B) PAH activity dependence on the concentration of BH₄ at different concentrations of L-Phe. Similar experiments as those shown in (A) were performed at the indicated concentration of L-Phe, and the steady-state rates were determined after 10 min of reaction. The data are presented as means \pm SD of three to seven independent experiments, and were fitted to a hyperbolic Michaelis–Menten kinetic model.

Table 1

Steady-state kinetic parameters determined for the BH₄ dependency of the L-Phe-preincubated (activated) wt-PAH activity at different concentrations of L-Phe as determined by ITC

[L-Phe] (μM)	$K_m(\text{BH}_4)$ (μM) ^a	V_{max} ^a ($\mu\text{mol}/\text{min mg}$)
1000	37 ± 4	2780 ± 140
500	31 ± 5	1770 ± 140
250	20 ± 3	1090 ± 60
100	14 ± 5	190 ± 20

^a The values are means \pm SE obtained from the fitting of three to seven independent experiments to a Michaelis–Menten model by non-linear regression analysis (see Fig. 2B).

the steady-state rates after sequential injections of L-Phe to the reaction cell containing BH₄ and PAH. These conditions were also selected because they may mimic

more realistically the intracellular conditions, as the complex [PAH · BH₄] has been proposed as the resting state of the enzyme in vivo [21]. Also, increasing L-Phe concentration through the assay reflects the shift in the PAH activity from lower to higher L-Phe concentrations occurring in vivo and allows to record the activity shift *directly*. Representative enthalpograms for wt-PAH at different BH₄ concentrations in the sample cell are shown in Fig. 3A. After blank subtraction and correction of the data for enzyme dilution during the assay, the steady state rate at the different L-Phe concentrations was calculated, and fitted to a Hill kinetic model (Fig. 3B). No substrate inhibition was observed under these conditions, except at the lowest assayed BH₄ concentration (5 μM), where a slight but reproducible inhibition is observed (data not shown). The kinetic parameters obtained are summarized in Table 2.

At all BH₄ concentrations tested, wt-PAH activity showed cooperative response (Hill coefficient (h) = 1.6–1.8). Also, a progressive decrease in the apparent affinity for L-Phe is observed at increasing BH₄ concentrations (1.8-fold increase in $S_{0.5}(\text{L-Phe})$ from 5 to 30 μM BH₄). At the typical standard conditions (75 μM BH₄), a $S_{0.5} = 308 \pm 17$ μM and a $h = 1.6 \pm 0.1$ were calculated, in agreement with the values previously determined by HPLC and fluorimetric detection for MBP-wt-PAH under non-L-Phe preincubating conditions ($S_{0.5} = 318$ μM and $h = 1.5$) [32].

A further processing and fitting to a Michaelis–Menten model of the activity of wt-PAH at six increasing concentrations of BH₄ and eight fixed concentrations of L-Phe (Fig. 3 and Table 2) allow to determine the kinetic parameters for the BH₄-preincubated (non-activated) enzyme at constant concentrations of L-Phe (Table 3). As observed under L-Phe activating conditions (Table 1), the apparent affinity for BH₄ is reduced at increasing L-Phe concentrations (4.9-fold increase in $K_m(\text{BH}_4)$ from 50 to 1000 μM L-Phe), but the apparent affinities for BH₄ are higher for the non-activated enzyme.

p.R68S mutant PAH activity at different concentrations of L-Phe under BH₄ preincubation conditions

We selected the c.204A > T (p.R68S) PAH mutant to analyze its catalytic and binding properties comparative to wt-PAH by ITC. This mutation is related to mild PKU phenotypes in patients, probably because it displays very mild effects on protein folding and/or stability [7,8,33]. This relatively high stability makes it suitable to be analyzed by ITC without considering possible artifacts arising from protein denaturation during prolonged experiments. Functionally, p.R68S has been described as a *preactivated* mutant, presenting high apparent affinity for L-Phe, and lacking the L-Phe preincubation dependence to exhibit maximal activity

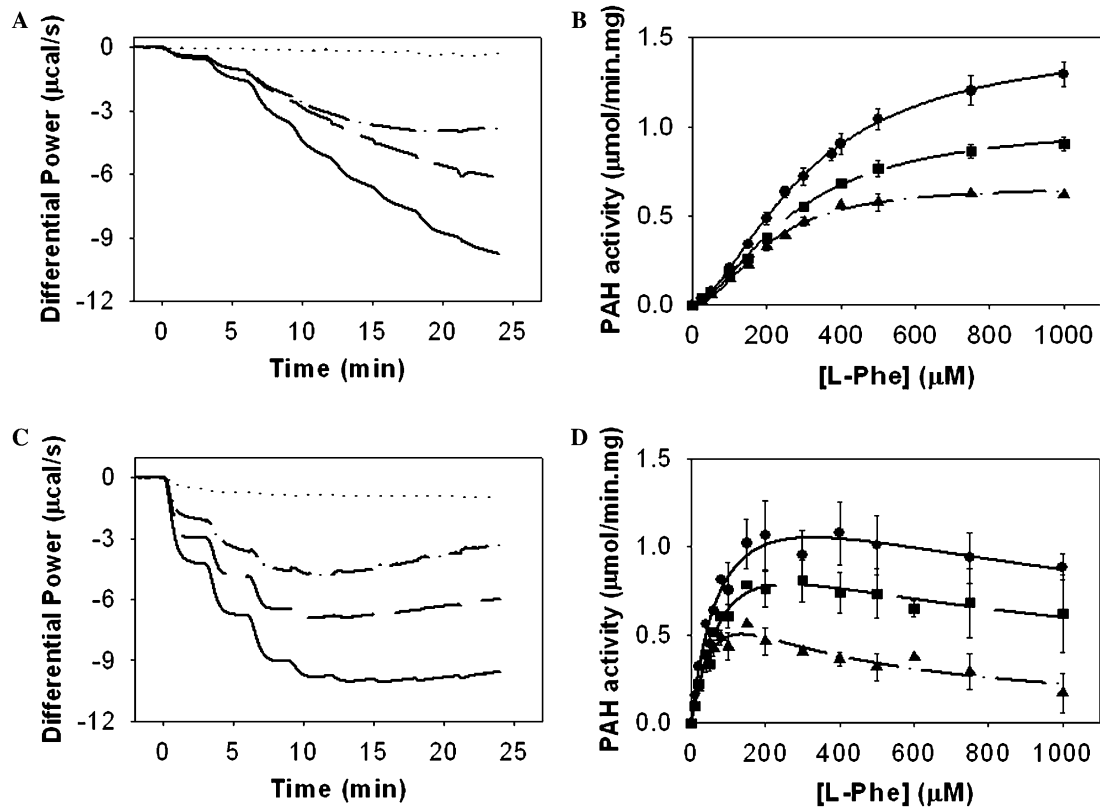


Fig. 3. PAH steady-state enzyme kinetics determined at successive L-Phe injections for non-activated enzyme at variable concentrations of BH₄. Representative enthalpograms obtained for the successive injections of L-Phe (first injection at time $t = 0$, and 3 min spaced) to 25 nM (final subunit concentration in the cell) tetrameric fusion protein of wt-PAH (A) and p.R68S (C) in the presence of the indicated concentrations of BH₄. Control experiments were performed in the absence of enzyme (blanks, dotted lines). Steady-state rates versus the concentration of L-Phe (B, wt; D, p.R68S mutant) for replicate experiments, which allow to determine steady-state kinetic parameters by fitting to a Hill equation (wt) or Hill-modified equation including substrate inhibition (p.R68S), at 10 μM (\blacktriangle), 20 μM (\blacksquare) and 75 (\bullet) μM BH₄.

Table 2

Steady-state kinetic parameters determined for the L-Phe dependency of BH₄-preincubated (non-activated) wt-PAH and mutant p.R68S activity at different concentrations of BH₄ as determined by ITC

Enzyme	[BH ₄] (μM)	V_{max}^a ($\mu\text{mol}/\text{min mg}$)	$S_{0.5(\text{L-Phe})}^a$ (μM)	h^a	$K_{\text{I}(\text{L-Phe})}^a$ (μM)
Wt	75	1.49 ± 0.05	308 ± 17	1.6 ± 0.1	n.a.
	30	1.20 ± 0.06	316 ± 22	1.7 ± 0.1	n.a.
	20	1.03 ± 0.03	274 ± 14	1.6 ± 0.2	n.a.
	15	0.83 ± 0.02	243 ± 10	1.7 ± 0.1	n.a.
	10	0.67 ± 0.02	201 ± 9	1.8 ± 0.1	n.a.
	5	0.46 ± 0.02	173 ± 14	1.7 ± 0.2	≥ 4000
p.R68S	75	1.27 ± 0.07	57 ± 9	1.1 ± 0.2	1525 ± 277
	20	0.92 ± 0.05	54 ± 8	1.2 ± 0.2	1306 ± 192
	10	0.60 ± 0.05	35 ± 11	1.1 ± 0.2	635 ± 86

n.a., not applicable.

^a The values are means \pm SE obtained from the fitting of three to five (wt) or two to four (p.R68S) independent experiments to a Hill or a Hill-modified equation to account for substrate inhibition (Eq. (3)).

[7]. Structurally, Arg68 is located at the loop 68–75 in the regulatory domain of PAH, that interacts with residues at the catalytic domain of the adjacent subunit in the dimer, and notably with Cys237 [17]. Mutation of Arg68 to a smaller and uncharged serine appears to perturb enzyme regulation leading to a *preactivated state* similar to that previously reported for the R68A mutant

[17]. The mutation p.R68S has been classified as potentially associated to BH₄-responsive PKU [12], being the characterization of its *activity landscape* relevant to understand the activity dynamics underlying BH₄-responsiveness.

Kinetic analysis using the p.R68S mutant was performed at the same conditions as for BH₄-preincubated

Table 3
Steady-state kinetic parameters determined for the BH₄ dependency of BH₄-preincubated (non-activated) wt-PAH and mutant p.R68S activity at different concentrations of L-Phe as determined by ITC

Enzyme	[L-Phe] (μM)	V_{\max}^a (μmol/min mg)	$K_m(\text{BH}_4)^a$ (μM)
Wt	1000	1.59 ± 0.03	16.1 ± 0.9
	750	1.41 ± 0.03	13.3 ± 0.7
	500	1.18 ± 0.02	10.8 ± 0.5
	400	0.96 ± 0.04	8.1 ± 1.1
	300	0.73 ± 0.04	6.4 ± 1.4
	200	0.47 ± 0.03	5.2 ± 1.5
	100	0.21 ± 0.01	3.6 ± 0.9
	50	0.08 ± 0.01	3.3 ± 0.8
p.R68S	1000	1.30 ± 0.41	32.2 ± 20.4
	400	1.42 ± 0.18	22.6 ± 7.1
	200	1.31 ± 0.07	16.3 ± 2.6
	100	0.85 ± 0.03	9.0 ± 1.0

^a The values are means ± SE obtained from the fitting of three to five (wt) or two to four (p.R68S) independent experiments to a Michaelis–Menten equation by non-linear regression analysis. The large standard errors associated to the values for p.R68S are probably due to fitting uncertainties because of the low number of experimental points used in the non-linear fitting procedure and/or the strong substrate inhibition displayed by this mutant.

wt-PAH (non-activated enzyme). Representative enthalpograms at different BH₄ concentrations in the sample cell are shown in Fig. 3C. Substrate inhibition was observed for the p.R68S mutant at all BH₄ concentrations tested (Fig. 3D) and in order to determine the kinetic parameters (Table 2), the dependencies of the mutant activity on the concentration of L-Phe at each concentration of BH₄ were fitted to a Hill kinetic model modified for substrate inhibition. The kinetic properties of the p.R68S mutant differ largely from those observed for wt-PAH, displaying no significant cooperativity ($h = 1.1$ – 1.2) and a higher apparent affinity for L-Phe ($S_{0.5}$ in the 35–57 μM range versus 173–316 μM in wt-PAH). The mutant also showed a decrease in the apparent affinity for the substrate at higher BH₄ concentrations (1.6-fold). At the typical standard conditions (75 μM BH₄), $S_{0.5} = 57 \pm 9$ μM and $h = 1.1 \pm 0.1$ were estimated, in agreement with the values previously determined by HPLC and fluorimetric detection under L-Phe preincubation conditions ($S_{0.5} = 73 \pm 6$ μM and $h = 1.4$) [7]. Interestingly, substrate inhibition is stronger for the p.R68S mutant than for wt-PAH, and is strengthened at low BH₄ concentrations, probably due to the higher apparent affinity of the mutant for L-Phe (Fig. 3 and Table 2). We also estimated the $K_m(\text{BH}_4)$ values for the mutant at several L-Phe concentrations (Table 3). The apparent affinity for BH₄ was about 2-fold lower for the p.R68S mutant than for wt-PAH at all concentrations of L-Phe tested but, as observed for wt-PAH, the $K_m(\text{BH}_4)$ for the mutant increased with increasing concentrations of L-Phe, varying from 9 ± 1 to 32 ± 20 μM at 100 μM and 1 mM L-Phe, respectively.

Equilibrium binding of BH₄ in the absence or presence of L-Phe

The incubation of wt-PAH with L-Phe in the 1–5 mM range induces an activating conformational change that decreases the binding affinity for the natural cofactor BH₄, but not for synthetic and non-regulatory cofactors such as 6M-PH₄ [25,30]. In order to deepen into the effect of PAH activation on the affinity for BH₄, we have performed additional equilibrium binding analyses by ITC on the wt-PAH and the p.R68S mutant, and the results are summarized in Table 4. These experiments have revealed a reduced affinity (3.3-fold increase in K_d compared to wt-PAH) for the natural cofactor in the *preactivated* p.R68S mutant in the absence of L-Phe. In contrast to wt-PAH, which showed a 2.6-fold reduction in the affinity for BH₄ in the presence of 1 mM L-Phe, the substrate did not significantly affect the affinity of the p.R68S mutant for the natural cofactor. The thermodynamic origin of the reduced affinity for BH₄ in this mutant seems to be the large and unfavorable entropic contribution to the binding, that is partially compensated by an increase in the favorable enthalpy change [7]. When these analyses are performed at different temperatures, the absolute ΔC_p value calculated for the p.R68S mutant is smaller than for wt-PAH (Table 4). ΔC_p is a parameter very sensitive to differences in the surface buried upon ligand binding [34,35]. We have earlier shown that this parameter is specially indicative for the regulatory conformational change induced on the enzyme upon BH₄ binding [25], and our results thus suggest that in the mutant protein this conformational change is affected. Interestingly, the affinity for the synthetic cofactor 6M-PH₄ is similar for both proteins. Altogether, our results indicate that the reduced kinetic affinity for BH₄ measured for the p.R68S mutant is related to a decreased binding affinity, probably caused by a structural perturbation upon mutation related to its *preactivated* state.

Discussion

One of the main goals of the present work has been the quantification of PAH activity at physiological concentrations of the substrate and cofactor, in addition to the standard conditions typically used in PAH activity assays. Different *physiological* values have been reported for L-Phe and BH₄, mainly from human or rat hepatocytes and liver tissues. L-Phe levels are typically in the range 50–100 μM [21,36], close to the normal plasma concentrations [1], whereas BH₄ concentrations have been reported in the range 5–10 μM [14,21,37–39]. PAH levels in the 3–9 μM range have been estimated [14,15,21], indicating substoichiometric (~0.5) [14] to stoichiometric (~1) [21] BH₄:PAH ratios in vivo. Based

Table 4

Thermodynamic parameters obtained for equilibrium binding of BH₄ and 6M-PH₄ to wt-PAH and p.R68S in the absence or presence of 1 mM L-Phe in 20 mM Na-Hepes, 200 mM NaCl, pH 7.0 at 25 °C under anoxic conditions using ITC

	L-Phe	<i>n</i>	<i>K_d</i> (μM)	<i>K_a</i> × 10 ⁻⁴ (M ⁻¹)	Δ <i>G</i> (kcal/mol)	Δ <i>H</i> (kcal/mol)	-TΔ <i>S</i> (kcal/mol)	Δ <i>C_p</i> (kcal/mol K) ^c
<i>BH₄</i>								
Wt	- ^a	1.2 ± 0.1	2.7 ± 0.1	37.6 ± 1.7	-7.6 ± 0.1	-10.7 ± 0.1	3.1 ± 0.1	-0.36 ± 0.03 ^b
	+	0.8 ± 0.1	6.9 ± 0.5	14.6 ± 0.8	-7.1 ± 0.1	-7.3 ± 0.2	0.2 ± 0.1	ND
p.R68S	- ^a	0.8 ± 0.1	9.0 ± 1.0	11.0 ± 0.9	-6.9 ± 0.1	-13.3 ± 0.5	1.1 ± 0.1	-0.26 ± 0.04
	+	1.1 ± 0.1	6.8 ± 0.7	14.8 ± 0.9	-7.1 ± 0.1	-5.8 ± 0.1	-1.2 ± 0.1	N.D
<i>6-MPH₄</i>								
Wt	- ^b	0.9 ± 0.1	16.5 ± 1.9	6.0 ± 0.7	-6.5 ± 0.1	-3.3 ± 0.3	-3.2 ± 0.3	ND
	+ ^b	0.9 ± 0.1	14.7 ± 2.6	6.8 ± 1.1	-6.6 ± 0.1	-1.9 ± 0.2	-4.7 ± 0.2	ND
p.R68S	-	1.0 ± 0.1	14.6 ± 3.0	6.9 ± 1.5	-6.6 ± 0.1	-3.4 ± 0.4	-3.2 ± 0.4	ND
	+				ND			

Data are presented as means ± SE from non-linear regression fitting. ND, not determined.

^a Taken from [7].

^b Taken from [25]; data are presented as means ± SD from two to three independent measurements.

^c Determined at different temperatures in the range 5–35 °C as described [25].

on these physiologically relevant values, Fig. 4 displays the activity of both wt-PAH and p.R68S mutant measured in this work from *physiological* (e.g., 100 μM L-Phe and BH₄ 10 μM) to *superphysiological* concentrations (up to 1 mM L-Phe and 75 μM BH₄). For L-Phe, these superphysiological levels may represent those present in PKU patients, because L-Phe is supposed to accumulate in hepatocytes when present at high levels in plasma [1], whereas the BH₄ superphysiological levels may be expected after BH₄ supplementation [39]. It has been observed that in hepatocytes the [BH₄ · PAH] *low-activity* complex represents a resting state of the enzyme that is progressively dissociated and shifted to a more catalytically active form in response to a rise in the extracellular L-Phe levels [21]. Thus, measuring PAH activity under BH₄ preincubation conditions (as in our

study) may reflect more realistically the physiological situation *in vivo*.

At healthy physiological concentrations of L-Phe (i.e., ~100 μM), PAH activity is almost no sensitive to the concentration of BH₄ varying from physiological (5–10 μM) to superphysiological (up to 75 μM) levels, whereas at increased pathological concentrations of L-Phe (>500 μM), the activity becomes more sensitive to BH₄ levels (Fig. 4), mainly due to the 5-fold increase in *K_m*(BH₄) (Table 3). The kinetic analysis performed in this work indicates a remarkable regulation of wt activity by both L-Phe and BH₄ through L-Phe-dependent cooperative response and intertwined changes in apparent affinity for substrate and cofactor, leading to a complex *activity landscape* (Fig. 5A). These intertwined changes in apparent kinetic affinity are in agreement with the effect of L-Phe on the affinity for BH₄ measured at equilibrium binding conditions (Table 4), and are also consistent with results in other multi-substrate enzymes [41].

In contrast to the results with the wt-PAH, the activity of p.R68S at physiological concentrations of L-Phe (i.e., ~100 μM) is remarkably dependent on the concentration of BH₄ (Figs. 4B and 5B) due to an apparently higher *K_m*(BH₄) than for wt-PAH. Also, as observed for the wt enzyme, the affinity of the mutant for BH₄ increases at increasing concentration of L-Phe. A lower equilibrium binding affinity for this mutant has been previously detected at equilibrium conditions using ITC, but not by standard kinetic activity analysis [7]. The origin of these discrepancies may be related to differences in the activity measurement; measurements in Pey et al. [7] were initiated by the simultaneous addition of BH₄ and L-Phe to enzyme not preincubated with BH₄, and were measured for 1 min reaction. Interestingly, the equilibrium binding affinities for BH₄ (*K_d* values) measured by ITC for wt-PAH and p.R68S are similar to the apparent kinetic

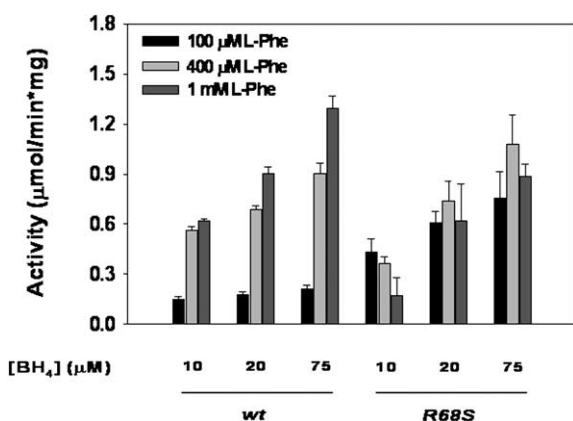


Fig. 4. The activity of wt-PAH and p.R68S mutant at representative physiological/superphysiological concentrations of substrate and natural cofactor. The enzyme activities were measured preincubating the enzymes with BH₄ and triggering the reaction by the injection of L-Phe as shown in Fig. 3. Data are presented as means ± SD from three to five (wt) or two to four (p.R68S mutant) independent experiments.

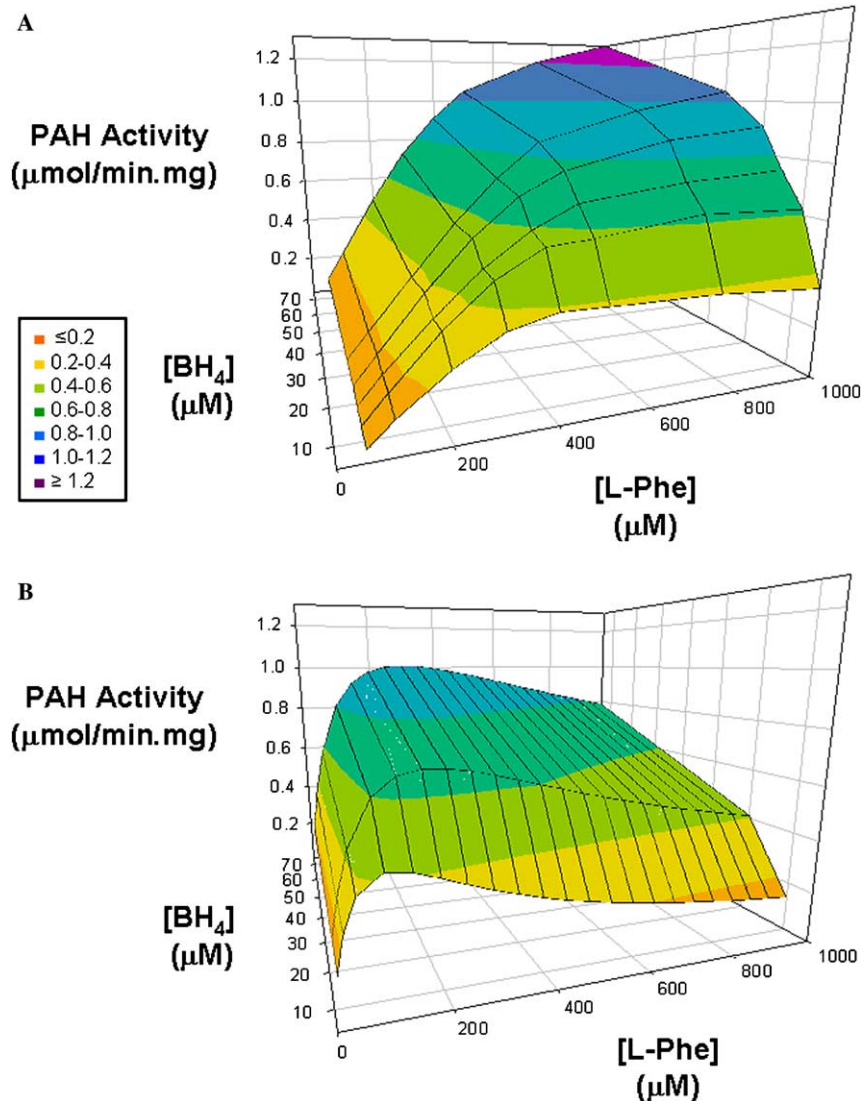


Fig. 5. Activity landscapes for wt-PAH and the p.R68S mutant. The coloured-surfaces display the activities experimentally determined by the ITC-based method at a wide-range of relevant physiological/superphysiological substrate and cofactor concentrations.

affinities (K_m values), suggesting that under catalytic turnover conditions at low concentration of L-Phe, the apparent affinity approaches the binding affinity for the natural cofactor. When L-Phe levels are increased at low concentration of BH_4 , a reduction in the activity of p.R68S is observed due to a strong substrate inhibition, an effect that is reduced if the BH_4 concentration is raised (Fig. 4). This feature is probably related to the higher apparent affinity displayed by this *preactivated* mutant.

The composite results shown in Figs. 4 and 5 predict minor effects of BH_4 -supplementation therapy in the activity of wt-PAH at low hepatic/plasma concentration of L-Phe. On the other hand, these results also imply that increases in BH_4 levels (e.g., after a BH_4 overload by therapeutic supplementation) may contribute to raise the residual mutant activity alleviating sub-

strate inhibition for some mutant proteins (e.g., other *preactivated* mutants associated to BH_4 -responsiveness, like p.F39L, p.I65T and p.R68S ([8] and this work), and p.R408Q ([42] and unpublished results). Taken together, our results highlight the applicability of the ITC assay to characterize the complex *activity landscape* of wt and mutant PAH in a wide range of substrate and cofactor concentrations. The kinetic abnormalities observed for many PKU-(and BH_4 -responsive) mutants appear to lead to unexpected changes in the *activity landscape*, with important implications to understand the dynamics of the PAH mutant residual activity in PKU. Moreover, some remarkable features of this abnormal kinetic behavior like increased substrate inhibition may also contribute to understand the genotype–phenotype correlation and BH_4 -responsiveness in PKU.

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