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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 (6*R*)-tetrahydrobiopterin (BH_4). It has recently been observed that many PAH mutants associated with BH_4-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 BH_4-responsive c.204A > T (p.R68S) mutant at physiological and superphysiological concentrations of L-Phe and BH_4. 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 BH_4 concentrations. For both wt-PAH and mutant, the apparent affinity for BH_4 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 BH_4-responsiveness in certain PKU mutations.

Keywords: Phenylalanine hydroxylase; Phenylketonuria; Isothermal titration calorimetry; Enzyme activity; Enzyme regulation; Cofactor therapy

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 (6*R*)-L-*erythro*-6,7,8-tetrahydrobiopterin (BH_4) 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:* BH_4, (6*R*)-L-*erythro*-5,6,7,8-tetrahydrobiopterin; ITC, isothermal titration calorimetry; MBP, maltose binding protein; 6M-PH_4, 6-methyl-tetrahydropterin; PAH, phenylalanine hydroxylase; PKU, phenylketonuria; wt, wild-type.

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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 BH_4 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 BH_4-responsiveness [12], the biochemical properties of the PAH mutant proteins and the effect of BH_4 supplementation have been investigated in recent mutational in vitro analyses [7,8]. The BH_4-responsiveness associated mutants analyzed in these studies have been typically associated to mild PKU phenotypes and present high residual activity ($\geqslant 30\%$ 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 μM BH_4). The in vitro analysis with recombinantly expressed enzymes indicated that the responsiveness to BH_4 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 BH_4 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 BH_4 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 BH_4, 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, BH_4 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 recombinant tetrameric human wt-PAH and the p.R68S mutant, a mutation reported as BH_4-responsive [12]. Our results demonstrate the complexity of the PAH *activity landscape*, unravelling some intertwined effects between BH_4 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 BH_4-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 $\varepsilon_{280\text{nm (1mg/ml)}} = 1.63$ [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 μM BH_4, 10–1000 μM L-Phe, 5 mM tris(carboxyethyl)-phosphine (TCEP) as reductant [27], 1 μM 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]:

$$\text{rate} = \frac{\mathrm{d}[\text{L-Tyr}]}{\mathrm{d}t} = \frac{1}{V_{\text{cell}} \times \Delta H_{\text{app}}} \times \frac{\mathrm{d}Q}{\mathrm{d}t}, \qquad (1)$$

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