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Quantification of phenylalanine hydroxylase activity by isotope-dilution liquid chromatography–electrospray ionization tandem mass spectrometry

Caroline Heintz [a], Heinz Troxler [a], Aurora Martinez [b], Beat Thöny [a,c,d], Nenad Blau [a,c,d,e,*]

[a] Division of Clinical Chemistry and Biochemistry, University Children's Hospital, Zürich, Switzerland
[b] Department of Biomedicine, University of Bergen, Bergen, Norway
[c] Zürich Center for Integrative Human Physiology (ZIHP), Zürich, Switzerland
[d] Research Center for Children (RCC), Zürich, Switzerland
[e] Division of Metabolism, University Children's Hospital, Heidelberg, Germany

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ABSTRACT

Background: Residual phenylalanine hydroxylase (PAH) activity is the key determinant for the phenotype severity in phenylketonuria (PKU) patients and correlates with the patient's genotype. Activity of *in vitro* expressed mutant PAH may predict the patient's phenotype and response to tetrahydrobiopterin (BH_4), the cofactor of PAH.

Methods: A robust LC–ESI-MSMS PAH assay for the quantification of phenylalanine and tyrosine was developed. We measured PAH activity a) of the PAH mutations p.Y417C, p.I65T, p.R261Q, p.E280A, p.R158Q, p.R408W, and p.E390G expressed in eukaryotic COS-1 cells; b) in different cell lines (e.g. Huh-7, Hep3B); and c) in liver, brain, and kidney tissue from wild-type and PKU mice.

Results: The PAH assay was linear for phenylalanine and tyrosine ($r^2 \geq 0.99$), with a detection limit of 105 nmol/L for Phe and 398 nmol/L for Tyr. Intra-assay and inter-assay coefficients of variation were <5.3% and <6.2%, respectively, for the p.R158Q variant in lower tyrosine range. Recovery of tyrosine was 100%. Compared to the wild-type enzyme, the highest PAH activity at standard conditions (1 mmol/L L-Phe; 200 μmol/L BH_4) was found for the mutant p.Y417C (76%), followed by p.E390G (54%), p.R261Q (43%), p.I65T (33%), p.E280A (15%), p.R158Q (5%), and p.R408W (2%). A relative high PAH activity was found in kidney (33% of the liver activity), but none in brain.

Conclusions: This novel method is highly sensitive, specific, reproducible, and efficient, allowing the quantification of PAH activity in different cells or tissue extracts using minimum amounts of samples under standardized conditions.

1. Introduction

Deficiency of phenylalanine hydroxylase (PAH, EC 1.14.16.1) is causing phenylketonuria (PKU, OMIM 261600), an autosomal recessively inherited disease presenting with elevated blood phenylalanine (Phe) levels [1,2]. The phenotypic severity of PKU is characterized by the type of mutation, and thus by residual PAH enzyme activity. The fully functional homotetrameric PAH catalyzes hydroxylation of Phe to tyrosine (Tyr) in the presence of cofactor (*6R*)-L-*erythro*-5,6,7,8-tetrahydrobiopterin (BH_4) and molecular oxygen [3,4]. According to the Locus Knowledgebase (PAHdb, www.pahdb.mcgill.ca), about 60% of mutations in the *PAH* gene are missense mutations, which may lead to a misfolding of the protein [5,6], disturbing the complex enzyme regulation and changes in kinetics, due to altered affinities for the Phe substrate and the BH_4 cofactor.

The incidence of PKU is about 1 in 10,000 newborns in Caucasian populations [2]. For most patients, therapy consists in a life-long dietary restriction of Phe to prevent neurological impairment. Recently, it has been reported that a subgroup of PKU patients (mild to moderate phenotype) can benefit from a pharmacological therapy with BH_4 (sapropterin dihydrochloride; Kuvan®) [7,8]. Newborn screening program for PKU, initially based on the Guthrie test [9], has been established for the early detection of PKU patients. Today, electrospray ionization tandem mass-spectrometry (ESI-MSMS or TMS) is the method of choice for fast screening and monitoring of Phe and Tyr levels in dried blood spots (DBS) [10].

Of the over 550 disease-causing mutations listed in the PAHdb [11], 88 were expressed in different *in vitro* cell systems to estimate the residual PAH activity. Expression systems like *Escherichia coli*, eukaryotic cell lines, or cell-free systems were most commonly used

Abbreviations: LC–ESI-MSMS, liquid chromatography electrospray ionization tandem mass spectrometry; Phe, phenylalanine; Tyr, tyrosine; PAH, phenylalanine hydroxylase; PKU, phenylketonuria; BH_4, tetrahydrobiopterin.

* Corresponding author at: Division of Metabolism, University Children's Hospital, Steinwiesstrasse 75, CH-8032 Zürich, Switzerland. Fax: +41 44 266 7169.
E-mail address: nenad.blau@kispi.uzh.ch (N. Blau).

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systems [12–14]. In addition to cell systems, PAH activity was studied in rat liver biopsy samples [15]. Expression of recombinant PAH in bacteria was applied for characterization of physical and chemical properties of the enzyme [16].

Previous methods for PAH activity measurement were based on the determination of ^{14}C-labeled Tyr produced [3] or release of ^{3}H [17]. Other methods are based on detection of Tyr by fluorescence coupled to HPLC [18], colorimetric assays [19], or fluorescence monitoring [20]. Recently described method by Gersting et al. [20] was developed for characterization of purified mutant PAH proteins at different Phe and BH_4 concentrations.

In our novel assay, we applied liquid chromatography (LC) with ESI-MSMS for the quantification of Tyr produced from Phe. Prior to analysis, the amino acids are derivatized to propyl chloroformate derivatives, using the commercially available Phenomenex EZ:faast™ kit. Our method allows for short analysis times and lower limit of detection and is optimized for determination of PAH enzyme activity of recombinantly expressed mutant proteins in COS-1 and other cell lines, as well as in mice liver, kidney, and brain. Thus, it allows comparison between different mutant proteins at standard conditions.

2. Materials and methods

2.1. Materials

The Phenomenex EZ:faast™ kit for LC with ESI-MSMS amino acid analysis was purchased from Phenomenex (Torrance, CA, USA). L-phenylalanine-d_5 and L-tyrosine-d_4 standard reagents were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA) whereas L-phenylalanine-d_8 was obtained from C/D/N Isotopes Inc. (Pointe Claire, Quebec, Canada). L-Phenylalanine and L-Tyrosine, as well as the DMEM cell culture medium were purchased from Sigma Aldrich (St. Louis, MO, USA). RPMI 1640 medium was from Invitrogen (San Diego, CA, USA). BH_4 dihydrochloride was obtained from Schircks Laboratories (Jona, Switzerland). Mouse tissues were extracted from C57BL/6 (wild-type) or C57Bl/6-*Pah*^{enu2} (PKU) [21] mice strains.

2.2. Expression plasmid preparation

The expression plasmid pCMV-FLAG-PAH (Promoter-N-Fusion-PAH) was received as courtesy gift from L. R. Desviat [22]. Mutations in the human *PAH-cDNA* sequence were introduced by site-directed mutagenesis, using QuikChange XL kit from Agilent Technologies (Santa Clara, CA, USA) and confirmed by sequencing analysis using BigDye Terminator Cycle sequencing v1.1 (Applied Biosystems) on an ABI Prism 3100 Sequencer.

2.3. Transfection of cells and preparation of mouse tissue

Cell lines (COS-1, Hep3B, HuH-7) were cultured either in DMEM or RPMI1640 (HaCat, lymphoblasts) medium, with appropriate additives, at 37 °C under 5% CO_2. One day prior to transfection, COS-1 cells were seeded at 2×10^5 cells/mL in 10-cm dishes. Transfection experiments were performed using Fugene 6 (Roche Diagnostics, Mannheim, Germany) according to manufacturer's recommendations. Hereby, 13 μg of the pCMV-FLAG-PAH plasmid (either wild-type or mutant) were co-transfected with 2 μg of pSV-βgal reporter plasmid (Promega, Madison, USA). Transfected cells were harvested after 48 h for determination of PAH activity or flash-frozen in liquid N_2 for storage at −80 °C.

Transfection efficiency was verified by determining β-galactosidase activity in 5 μl lysate in PAH assay cell lysis buffer (1× PBS pH 7.4, 0.25 M sucrose, complete protease inhibitors cocktail (Roche)) using in-situ β-galactosidase enzyme assay system (Promega, Madison, USA). PAH activities of wild-type and mutants were normalized according to transfection efficiency.

Frozen mouse tissues were lysed in homogenization buffer (10 μl/mg tissue), as described in [23], and homogenized using Qiagen TissueLyser II at 4 °C. After centrifugation at 13,000 *g* and 4 °C for 30 min, supernatants were kept frozen at −80 °C.

2.4. PAH activity assay

Cell lysates were prepared and enzyme activity was determined using previously described methods [24]. Briefly, assay conditions included pre-incubation at 25 °C with L-Phe (1 mmol/L) for 4 min, then $Fe(NH_4)_2(SO_4)_2$ (100 μmol/L) was added, and incubation was continued for one more minute. After 5 min total pre-incubation time, BH_4 (200 μmol/L for cell extracts or 75 μmol/L for mouse tissue) was added to start the reaction. Between 2.5 and 20 μl (2–165 μg) of total protein lysate extracted from cells or mouse tissue was used for activity measurements. The applied total protein amount depended on lysate type, transfected, non-transfected, or mouse tissue samples. It was generally higher for sample types with low activity where only little amounts of Tyr were produced. Initially, this was determined empirically by measuring a series of increasing total protein amounts for each sample type to determine the measurable linear range. Reaction time was 2 min for mouse tissue lysates and 15 min for cell lysates. Short incubation time reduces any possible chaperone-like effect of BH_4. The amount of Tyr produced was determined by LC with ESI-MSMS (see below).

Protein concentrations of all sample types were determined using Pyrogallol Red protein dye binding assay [25] after completion of PAH assay due to low stability of protein lysates. Specific PAH activities are expressed in mU per mg total protein to account for differences in total protein amount and with mU equal to nmol L-Tyr produced per minute.

2.5. Stock solution preparation and calibration

Labeled internal standard stock solutions (10 mmol/L Phe-d_5 and 10 mmol/L Tyr-d_4) were prepared in 50 mmol/L HCl, stored at −20 °C, and freshly diluted to working concentrations (see sample preparation). Stock solutions of non-labeled Phe (50 mmol/L) and Tyr (8 mmol/L) were prepared for calibration curves in 50 mmol/L HCl and stored at −20 °C.

Working solutions for calibration curves were freshly prepared from the non-labeled stock solutions in H_2O from 100 to 700 μmol/L Phe and 4 to 350 μmol/L Tyr (Table 1). In order to include the matrix effect to the calibration curves, 20 μl of COS-1 non-transfected cell lysate was added to the calibration samples.

2.6. Sample preparation and derivatization

Samples were prepared according to the Phenomenex EZ:faast™ kit's manual [26], with the following modifications: prior to amino acid extraction and derivatization, 20 μL of each internal standard solution containing 100 μmol/L Phe-d_5 and 20 μmol/L Tyr-d_4 (in 50 mmol/L HCl) were added to 20 μL of sample lysate. Using the kit's reagents, the amino acids are derivatized with propyl chloroformate resulting in the addition of a propyl formate at the amine moiety and a propyl group at the carboxylic end of the amino acids, respectively. The hydroxy group of Tyr is also derivatized by the addition of a propyl formate group.

2.7. Instrumentation

For RP (reversed phase)-HPLC separation of amino acids, a 250×2 mm C18 column (Phenomenex EZ:faast™) was used. The derivatized amino acids were separated using the following program: (i) isocratic flow 75% solvent B for 6 min; (ii) linear gradient from 75% to 95% solvent B (v/v) in 9 min; (iii) linear gradient from 95% to 100% solvent B in 0.1 min; (iv) isocratic flow 100% solvent B for 3 min; (v) linear gradient from 100% to 75% solvent B in 0.1 min; (vi) isocratic flow 75% solvent B for 2 min. Solvents A and B were 10 mmol/L