Acta Biochimica Polonica

Vol. 47 No. 2/2000

365–369

QUARTERLY

In vitro expression analysis of R68G and R68S mutations in phenylalanine hydroxylase gene

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Received: 18 April, 2000

Key words: hyperphenylalaninemia, mutation, expression analysis, phenylalanine hydroxylase

Phenylketonuria (PKU), an autosomal recessive disorder caused be a deficiency of hepatic phenylalanine hydroxylase (PAH), is clinically very heterogeneous. At the molecular level, more than 400 mutations in the *PAH* gene are known to date, which in different genotype combinations could account for biochemical and clinical variability of symptoms. *In vitro* expression studies on R68G and R68S mutations causing mild phenylketonuria are presented.

Mutations in the gene coding for the hepatic enzyme phenylalanine hydroxylase (PAH) result in various degrees of enzyme impairment, resulting in different metabolic and clinical outcome of hyperphenylalaninemia (HPA). The phenotypic classes form a gradient from classical phenylketonuria (PKU), requiring phenylalanine-free diet, to mild hyperphenylalaninemia, not requiring medical treatment. The degree of PAH enzyme impairment depends on the nature and position of mutations. Missense mutations located in exon 3, coding for part of the regulatory domain of PAH, cause either PKU, mild PKU or mild hyperphenylalaninemia [1] Most mutations studied so far reduce PAH activity *in vitro*, and the severity of metabolic phenotype correlates generally with the "predicted residual activity" (PRA) [2]. Mutations with PRA of about 0–15% are "severe" mutations, caus-

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Abbreviations: PAH, phenylalanine hydroxylase; HPA, hyperphenylalaninemia; PKU, phenylketonuria; PRA, predicted residual activity; MBP, maltose binding protein; BH_4, tetrahydrobiopterin; $6MPH_4$, 6-methyltetrahydrobiopterin; COS cells, a cell line derived from African green monkey and transformed with origin-defective SV40 genome.

ing classical PKU in homozygosity. Residual PAH activity sometimes varies from one expression systems to another.

The aim of this study was to elucidate the effect of R68G and R68S mutations on PAH activity in bacterial and mammalian expression systems. Both mutations when combined with "severe" mutations cause mild PKU, requiring partial phenylalanine free diet.

SUBJECTS AND METHODS

Three probands and one sibling were detected at the Center of Molecular Biology "Severo Ochoa" (Spain) and were presented in previous papers [3, 4]. Two probands and one sibling bearing two different mutations in exon 3 of the *PAH* gene were detected at the National Research Institute of Mother and Child (Poland) and have been presented in the previous paper [5]. The mendelian mode of inheritance was confirmed. In the case of R68G mutation all exons of the PAH gene have been sequenced. Brief description of genotypes and related biochemical and clinical phenotypes are listed in Table 1. All mild PKU patients were on partial phenylalanine free diet. The scale of psychometric evaluation by Terman and Merril was applied in patients aged 30 months to 5 years, and that of Wechsler to children above 5 years of age.

In vitro mutagenesis was performed using Quick Change kit (Stratagene). Expression analysis was performed in *Escherichia coli*, using pMAL-c2 expression system. The in-frame gene encoding a fusion protein with human phenylalanine hydroxylase and maltose binding protein (MBP) was obtained as described previously [6]. PAH activity was measured using conversion of [^{14}C]phenylalanine to [^{14}C]tyrosine, with total cell lysate protein and fusion protein purified using affinity chromatography (amylose resin) as described previously [7]. The assay was performed with artificial PAH cofactor: 6-methyltetrahydrobiopterin ($6MPH_4$). Thin-layer chromatograms were autoradiographed and the results were quantified using Gel Doc system software (BioRad). pMAL-c2 with the wild type *PAH* coding sequence was used as a positive control. Total cell lysate protein and purified fusion protein were analysed using SDS/PAGE. Both mutated and wild type fusion proteins were electrophoresed and electroblotted onto nylon membrane, and their immunoreactivity was measured. Purified mouse anti-tryptophan, anti-tyrosine

Table 1. Genotype-phenotype relations in patients with R68G and R68S mutations

Initials, gender and age	Genotype	PRA (%)	Pretreatment serum phenylalanine level (μmol/liter)	Phenylalanine serum levels range (μmol/liter)	Mental status		Phenylalanine tolerance		Clinical phenotype
					age (y)	IQ	age (y)	mg/kg per day	
MB, m, 7y	R68G/R243Q	100/10	1140	120–600	5	98	5	34	mild PKU
JB, f, 4y	R68G/R243Q	100/10	925	240–480	3	95	2	48	mild PKU
MM, f, 8y	R68S/IVS7nt3	100/0	1250	270–440	7	97	5	32	mild PKU
NL, f, 3y	R68S/S349P	100/0	600	500		nd	5	30	mild PKU
DS, m 20y	R68S/V388M	100/40	210	off diet		nd		-	mild HPA
ES, f, 19y	R68S/V388M	100/40	380	off diet		nd		-	mild HPA
PZ, m, 4y	R68S/R408W	100/0	360	120–480	2	112	3	41	mild PKU

PRA, predicted residual activity; f, female; m, male; nd, no data. Normal phenylalanine serum content is below 120 μmol/liter.

and anti-phenylalanine hydroxylases monoclonal antibody (Pharmingen) was used.

Expression analysis was performed also in COS cells using human expression vector pRc/CMV and the same *in vitro* mutagenesis kit. COS cells were transfected with lipofectin reagent (BRL). In COS cells β-galactosidase cDNA was cotransfected with normal and mutant construct and transfection efficiency was comparable in both cases, as monitored by β-galactosidase activity. Determination of enzymatic PAH activity was measured in crude cell lysate, Western blotting analysis and measurement of immunoreactivity were done essentially as described previously [8].

RESULTS AND DISCUSSION

The R68S mutation was identified previously in different populations and is connected with mild PKU. The R68G mutation was identified for the first time in a Polish patient with mild PKU. Both mutations located in codon 68 were connected with one of "severe" mutations: R243Q, IVS7nt3, S349P or R408W, producing mild PKU phenotype. In one patient R68S mutation was connected with V388M mutation, with a predicted residual activity of about 40%, thus the resulting phenotype was mild hyperphenylalaninemia. Despite great differences in pretreatment phenylalanine levels between mild PKU patients, there were no substantial differences in phenylalanine serum contents range and mental status of the patients. On this basis both mutations were assessed to be of intermediate severity. *In vivo* assessment of individual mutations in functionally hemizygous patients, was found very useful previously, and results are generally consistent with different *in vitro* expression analysis systems.

The residual catalytic activity of R68G and R68S mutations were analysed in bacterial and mammal expression system using pMAL-c2 and pRC/CMV expression vectors.

In the bacterial system PAH activity was measured using conversion of [^{14}C]Phe to [^{14}C]Tyr. There was no difference in residual activity between crude extract and purified fusion protein. In both cases the activity was the same as that of normal PAH fusion protein (98–104%, see Fig. 1). We observed no tendency to aggregation, as prolonged storage of fusion protein did not affect residual activity. The immunoreactivity of R68G and R68S fusion proteins was normal.

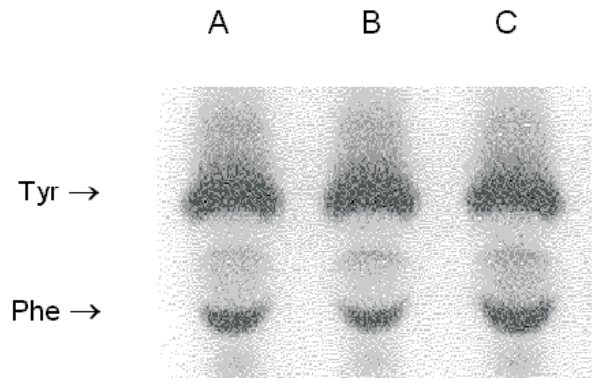

Figure 1. Relative PAH activity as a fusion protein (PAH-MBP) was assayed by measuring the conversion of [^{14}C]Phe to [^{14}C]Tyr using an artificial cofactor (6MPH$_4$).

Lane A is normal protein activity. Lanes B and C are R68G and R68S mutant proteins, respectively.

Three independent transfections were performed using COS cells, and activities of both mutant proteins were close to that of wild type enzyme (100% for R68G and 98% for R68S; see Fig. 2). Western blotting indicates normal amounts of PAH protein with normal immunoreactivity. We did not perform the analysis of mutant *PAH* mRNAs steady-state levels.

The data presented suggests that R68G and R68S mutations produce PAH with normal activity as a fusion protein in bacterial system and in mammalian *in vitro* expression system. Proteins in both systems are produced in normal amounts and display normal immunoreactivity.

A similar situation was observed with A403V mutation which appeared to be silent in the *E. coli in vitro* expression system [9]. This mutation was connected with mild hyperphenylalaninemia in different populations, including Spanish and Polish ones.

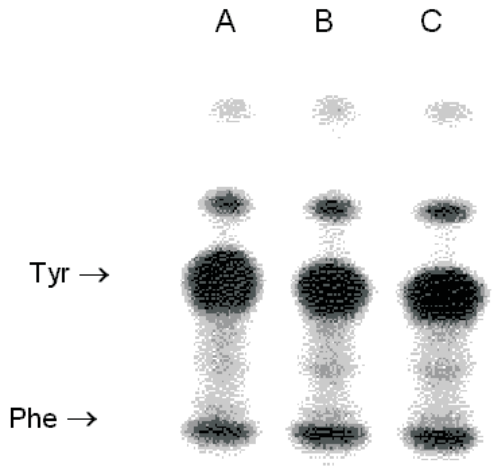

Figure 2. Relative PAH activity in COS cells was assayed by measuring the conversion of [^{14}C]Phe to [^{14}C]Tyr using a natural cofactor (BH_4).

Lane A is normal protein activity. Lanes B and C are R68S and R68G mutant proteins, respectively.

It should be stressed that no single expression system can provide definitive information concerning the effects of mutations in the *PAH* gene and their significance *in vivo*. Even when there are qualitative similarities between systems, there could be considerable quantitative differences between data from different systems. We did not measure e.g. positive cooperativity of PAH enzyme or different kinetic parameters of the enzymatic reaction [10]. Moreover, *in vitro* enzymatic activities are higher than those observed in liver biopsies or deduced from *in vivo* [^{13}C]phenylalanine oxidation studies. The results of *in vitro* assays at single phenylalanine and artificial cofactor concentrations could differ widely from those under physiological conditions.

In vivo assessment of mutations at the *PAH* gene, especially in functionally hemizygous patients, was found very useful [11]. The R68G and R68S mutations presented result in mild PKU phenotype when combined *in vivo* with a null mutation. It could be postulated that these mutations affect protein stability or some regulatory function of phenylalanine hydroxylase *in vivo* [12].

The authors (C.Ż. and W.W.) thank Dr. Maria Nowacka and Dr. Jolanta Ulewicz-Filipowicz for helpful clinical comments.

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