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Co-expression of different subunits of human phenylalanine hydroxylase: Evidence of negative interallelic complementation

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Abstract

To study the interaction between two different subunits of the heteromeric human phenylalanine hydroxylase (hPAH), present in hyperphenylalaninemic (HPA) compound heterozygous patients, heteroallelic hPAH enzymes were produced. A dual vector expression system was used (PRO™ Bacterial Expression System) in which each mutant subunit was expressed from a separate compatible vector, with different epitope tags, in a single bacterial host. Experimental conditions were selected in order that each plasmid produced equivalent levels of mutant subunits. In this study, we demonstrated that both subunits were expressed and that the purified heteroallelic enzymes, were catalytically active. As expected, the produced proteins displayed enzymatic activities levels lower than the predicted catalytic activity, calculated by averaging in vitro PAH activities from both alleles, and were strongly dependent on the proteins subunit composition. The obtained data suggest that interactions between the studied hPAH subunits, namely the I65T, R261Q, R270K and V388M, and the wild-type protein occurred. As postulated, this phenomenon could be a source of phenotypic variation in genetic diseases involving multimeric proteins.

Keywords: Heteroallelic human phenylalanine hydroxylase; Dual expression system; Interallelic complementation

1. Introduction

Human phenylalanine hydroxylase (hPAH; E.C. 1.14.16.1) is a homotetrameric non-heme iron dependent enzyme that hydroxylates phenylalanine (L-Phe) to tyrosine (L-Tyr). In man, hPAH dysfunction leads to phenylketonuria (PKU; OMIM 261600) and related forms of hyperphenylalaninemia (HPA) [1]. The enzymatic phenotype of this recessive metabolic disease results from the combined expression of the two mutant alleles. Most HPA patients are heteroallelic for PAH mutations being classified as compound heterozygous [2].

There is now considerable direct evidence indicating that it is possible to establish genotype/phenotype correlations [3,4] in the homoallelic state and in most cases of functional hemizygous patients (resulting from the combination with a "null" allele that completely abolishes PAH activity) [5]. However, in the heteroallelic state inconsistencies exist between the observed metabolic phenotype and the "predicted residual activity" (PRA) [6], as calculated from the mean of the monoallelic in vitro PAH enzyme activities for each mutation comprising the genotype [3]. In these patients, the presence of several mutations, namely the I65T, R261Q and V388M mutations, associated either among each other or with other mutations gives rise to more severe phenotypes than those anticipated by the PRA [6]. This phenomenon was already known since 1975 when Kaufman and Max [7] observed, in parents of PKU patients (obligate heterozygous), a deviation from proportionality in the determined PAH activity. These authors introduced the term negative interallelic complementation to illustrate the protein–protein interactions occurring between the subunits of the multimeric hPAH enzyme.

Recently, using a yeast two-hybrid approach [8,9], it was demonstrated that the wild-type hPAH subunits interact with different hPAH mutant subunits, thus, indicating the production of heteromeric PAH enzymes. However, a central question

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remains concerning the enzymatic activity of the postulated produced hybrid hPAH proteins. Till now, the use of conventional expression systems had never allowed to test for the hypothesis of a negative impact of a particular mutation on the activity of a heteromeric hPAH, since in these systems, a single mutant allele is expressed, thus, producing a homoallelic protein phenotype.

Using a dual vector expression strategy, for the co-production of two mutant hPAH subunits, we were able to mimic the natural heteroallelic state occurring in heterozygous individuals and compound heterozygous patients. Two different subunits were simultaneously produced in equivalent amounts in the same bacterial host, using two different plasmids (PRO™ Bacterial Expression System). The studied mutations included the I65T, R261Q, R270K and V388M mutant forms, which contribute mostly for the observed genotype/phenotype inconsistencies in the Portuguese PKU population [10].

The determined enzymatic activities showed that when co-expressed the produced mutant enzymes presented lower catalytic activities than the predicted by individual expression of the mutant subunits. The obtained results were in full accordance with the postulated phenomenon of negative interallelic complementation.

2. Material and methods

2.1. Materials

The PRO™ Bacterial Expression System was from Clontech (Clontech Laboratories, Palo Alto, USA). The synthetic cofactor 6-methyl-tetrahydropterin (6-MPH_4), L-Phe, HEPES and dithiothreitol (DTT) were from Sigma Chemical Co (St. Louis, USA). Catalase was purchased from Roche Diagnostics GmbH (Mannheim, Germany). All reagents were of analytical grade.

2.2. Construction of recombinant dual expression vectors

The PRO™ Bacterial Expression System comprises two expression vectors developed by Lutz and Bujard [11], namely the pPROLar and pPROTet. These vectors present different replication origins, a Myc epitope (–Glu–Gln–Lys–Leu–Ile–Ser–Glu–Glu–Asp–Leu–) and encode resistance to different antibiotics such that only cells expressing both vectors would be resistant to both kanamycin (pPROLar) and chloramphenicol (pPROTet). The pPROLar vector contains the $P_{lac/ara-1}$ promoter, induced by isopropylthio-β-D-galactoside (IPTG) and arabinose (Ara), and the pPROTet vector includes the $P_{LtetO-1}$ promoter, induced in response to anhydrotetracycline (aTc). A recombinant-deficient host (DH5αPRO; Clontech Laboratories, Palo Alto, USA) was used to minimize recombination between plasmids.

The original vectors were subjected to Site Directed Mutagenesis (Quikchange II; Stratagene, La Jolla, USA) in order to introduce a 6xHis purification tag (Table 1). Moreover, the pPROLar Myc-tag was removed and the Xpress epitope (–Asp–Leu–Tyr–Asp–Asp–Asp–Asp–Lys–) was introduced. The pPROLar–XPress–His and pPROTet–Myc–His expression vectors were obtained. The enterokinase (EK) recognition sequence was maintained just before the hPAH coding sequence in order to be possible to remove the 6xHis tag. However, the EK treatment it is not necessary, since we have demonstrated previously that these tags did not interfere with the activity of the recombinant protein [12].

For prokaryotic expression the coding sequence of hPAH [13] (GenBank accession U49897) was introduced in the BamHI site of pPROLar–XPress–His and pPROTet–Myc–His expression vectors, in frame with the start codon. Using the mutagenic oligonucleotides described in Table 1, the studied mutations were introduced by Site Directed Mutagenesis (Quikchange II). The obtained constructs were sequenced to confirm the introduction of the desired modifications and were used to transform competent DH5αPRO cells. Propagation of pPROTet–Myc–His constructs was performed in LB plates supplemented with spectinomycin (50 μg/ml, final concentration) and chloramphenicol (34 μg/ml, final concentration). The pPROLar–XPress–His constructs were propagated in LB plates supplemented with spectinomycin and kanamycin (both at 50 μg/ml, final concentration).

The strategy to achieve dual vector expression was to transform *E. coli* host cells simultaneously with two different hPAH subunits, each cloned in a different vector. To select the co-expressed constructs, LB plates supplemented with spectinomycin, kanamycin (both at 50 μg/ml final concentration) and chloramphenicol (34 μg/ml, final concentration) were used.

2.3. Co-expression of recombinant hPAH subunits

The protein expression was initially optimised, for each system individually, by testing the optimal inducers concentration and induction time. Final concentrations of aTc from 1 to 100 ng/ml were used for induction of pPROTet–Myc–His constructs. The pPROLar–XPress –His constructs were assayed for induction with a constant IPTG concentration (1 mM) and variable amounts of arabinose in the culture medium (0 to 0.2%). Kinetic induction studies were performed with an incubation period of 12 hours, with samples collected at 0, 1, 2, 3, 4, 5 and 12 hours after induction.

For the co-production of two different 6xHis–hPAH subunits, fresh overnight cultures were diluted 1:100 in LB medium containing 34 μg/ml of chloramphenicol, 50 μg/ml of spectinomycin and 50 μg/ml of kanamycin. Cells were grown at 37 °C with expression induced by addition of the optimised amounts of IPTG, arabinose and aTc when the A_{600} was about 0.6. Simultaneously 0.1 mM ferrous ammonium sulfate (Fe^{2+}) was added to the culture. Induction was performed for a period determined to maximize the prokaryotic expression of the heteroallelic hPAH enzyme forms under investigation.

Table 1
Oligonucleotides used for site directed mutagenesis

Primers	Position[a]	Sequence (5′–3′)
6xHis–tag F	pPROTet 96–131; pROLar 122–158	AGAAAGGTACCCATGGGTCATCATCATCATCATCATGAACAGAAACTGATCAGC
6xHis–tagR		GCTGATCAGTTTCTGTTCATGATGATGATGATGATGACCCATGGGTACCTTTCT
I65T-F	cDNA 183–203	CTAGATTCAGTGTGGGTCAGG
I65T-R		CCTGACCCACACTGAATCTAG
R261Q-F	cDNA 772–792	GTGGAAGACTTGGAAGGCCAG
R261-R		CTGGCCTTCCAAGTCTTCCAC
V388M-F	cDNA 1152–1172	CTCTCTGCCATGTAATACAGG
V388M-R		CCTGTATTACATGGCAGAGAG
R270K-F	cDNA 799–819	CAGTACATGAAACATGGATCC
R270K-R		GGATCCATGTTTGATGTACTG

[a] Nucleotide positions refer to Konecki-Kwok PAH cDNA (PAH Mutation Analysis Consortium Database; http://www.mcgill.ca/pahdb). Nucleotide mismatch positions are underlined.

Bacterial pellets were obtained by centrifugation at 4000×g for 10 min at 4 °C. The pellets (stored at −20 °C until used) were resuspended (40%, w/v) in lysis buffer (50 mM potassium phosphate; 300 mM NaCl, pH 7.8; 10% glycerol) containing 1 mM phenylmethanesulphonyl fluoride (PMSF) and 1 mg/ml lysozyme. After a 30 min incubation at 4 °C, cells were disrupted by passage through a Carver Press (Model C, from F.S. Carver Inc., Wabash, IN, USA) at 4000 psi. The crude extract was then centrifuged at 10,000×g at 4 °C for 20 min. The concentrations of imidazole and β-mercaptoethanol (β-ME) in the supernatants were adjusted to 10 mM and processed immediately.

The recombinant 6xHis–hPAH proteins expressed from the pPROLar–XPress–His and pPROTet–Myc–His vectors were designated 6xHis–hPAH$_{Lar}$ and 6xHis–hPAH$_{Tet}$, respectively. Expression of heteroallelic proteins was always carried out with both possible combinations (e.g. wt$_{Lar}$/V388M$_{Tet}$ and wt$_{Tet}$/V388M$_{Lar}$).

2.4. Purification and analysis of recombinant proteins

The presence of the 6xHis tag allowed the purification of the recombinant protein by IMAC using a Ni-chelating resin (Ni-NTA-Resin; Qiagen, Valencia, USA), as previously described [12]. Aliquots of the purified enzyme samples were analysed by SDS-PAGE (10% gel) after Coomassie brilliant blue R staining. Western immuno-analysis was performed according to standard methods [14] using the anti-Myc or the anti-Xpress as the primary antibodies (Invitrogen; Carlsbad, USA) and the anti-mouse IgG-HRP (Cell Signalling; Beverly, USA) as the secondary antibody. Protein concentrations were determined by the method of Bradford [15] using bovine serum albumin (BSA) as standard. Protein purity was assessed by densitometric scanning of destained gels or membrane blots followed by analysis using the Gel-Pro Analyzer (version 2.0) software (Media Cybernetics).

2.5. Assay of enzymatic activity

Enzyme assays were performed with the purified fusion proteins, essentially as described by Martinez et al [16] with some minor modifications [12]. The PAH activity was assayed using the synthetic cofactor 6-methyl-tetrahydropterin (6-MPH$_4$) and the substrate (L-Phe) at final concentrations of 500 μM and 1 mM, respectively. All stages of protein purification, analysis and enzyme assays were performed without intervening freeze–thaw cycles.

2.6. Cleavage of the fusion peptide

The leader fusion peptide 6xHis was excised from the recombinant fusion protein by cleavage with EK (Invitrogen, Carlsbad, USA). In this assay 50 μg of purified enzyme were incubated with 1 U of EK in 50 mM Tris–HCl pH 8.0, 1 mM $CaCl_2$ (ratio EK: fusion protein about 1:30) for 5 h at 4 °C.

3. Results

The wild-type and mutant hPAH cDNAs were cloned into the pPROLar–XPress–His and pPROTet–Myc–His expression vectors. Western Blot analysis of crude cell lysates revealed the presence of a single fusion protein band with a molecular mass of about 55 kDa. Assuming a molecular mass of 3 kDa for the fusion peptide [12] the 52-kDa protein corresponds to the expected full-length hPAH wild-type or mutant forms [17,18].

Expression conditions, including inducers concentration and induction time, were analysed in order to achieve equimolecular levels of each subunit comprising the hPAH heteroallelic state. Regulation of wild-type hPAH expression from the $P_{LtetO-1}$ and $P_{lac/ara-1}$ promoters showed that the highest and equivalent protein levels were reached using 100 ng/ml of aTc, for the pPROTet–Myc–His construct, and 0.2% arabinose/1 mM IPTG for the pPROLar–Xpress–His construct (Fig. 1). Using the above inducers concentration maximum yields of recombinant proteins were obtained between 4 and 5 h, for the wild-type form and at 4 h induction for the studied mutant forms (Fig. 2). After these time points, a decrease in the protein production was observed. Based on these observations, a 4-h induction time was chosen to produce the wild-type and the mutant recombinant 6xHis–hPAH proteins.

After IMAC purification the 6xHis hPAHwt protein presented, for both expression vectors, 95% purity. Similar to the results obtained with other expression systems [12,19] the recombinant mutant forms showed lower purity grades, namely 75–80% for the I65T, R261Q and V388M (pPROLar–XPress–His and pPROTet–Myc–His constructs). The R270K$_{Lar}$ protein was produced almost to homogeneity, whereas the R270K$_{Tet}$ mutant protein (Fig. 3) presented only 60% purity. In addition to a main band of full-length hPAH (55 kDa), a minor band of higher molecular mass was observed for the R270K$_{Tet}$ mutant form (Fig. 3). However, Western blot analysis showed that this band did not represent immunoreactive hPAH protein (results not shown).

The 6xHis fusion peptide was efficiently cleaved from the recombinant enzymes (EK digestion), yielding a protein with a MW of about 52 kDa (results not shown).

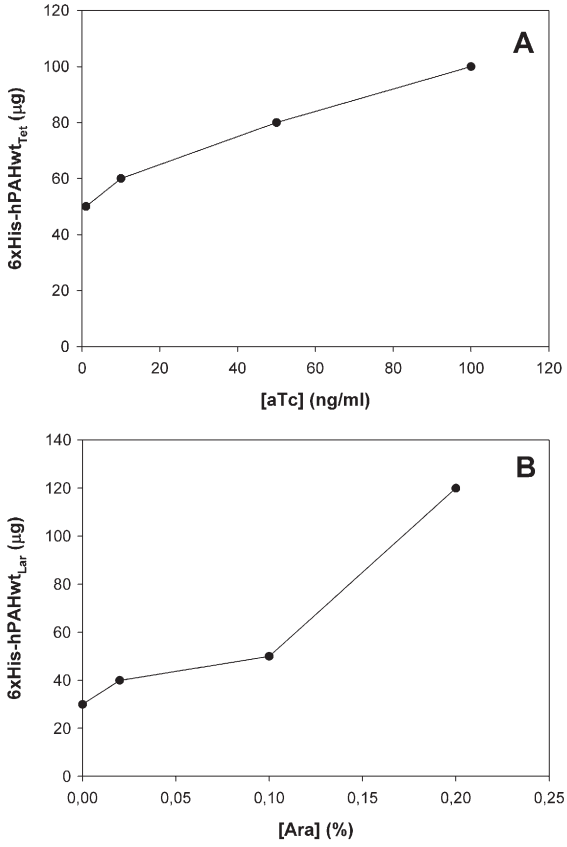

Fig. 1. Regulation of wild-type 6xHis–hPAH expression from the $P_{LtetO-1}$ (A) and $P_{Lac/ara-1}$ (B) promoters. Assays were performed with 500 ml of growth medium and 4-h incubation. Each value represents the mean of two independent analysis. Arabinose induction was performed with a constant 1 mM IPTG concentration.

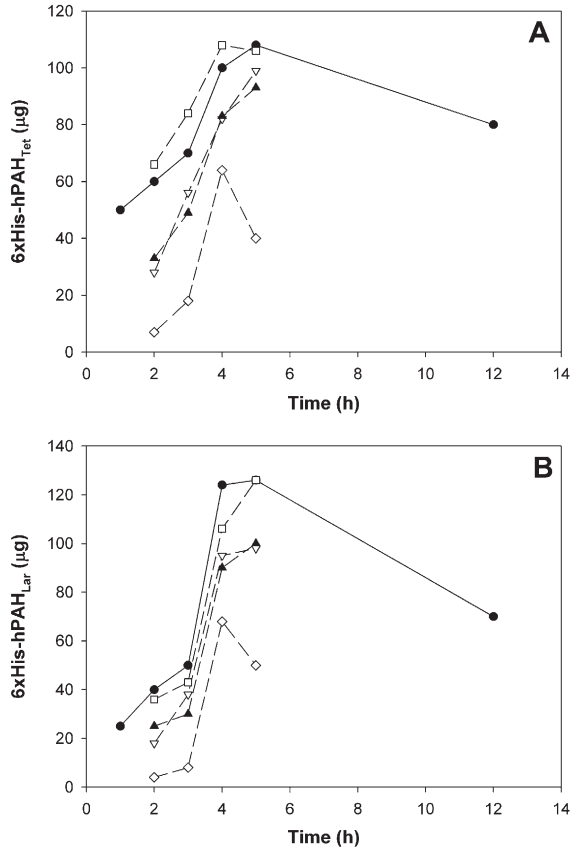

Fig. 2. Expression of wild-type and mutant 6xHis–hPAH enzymes encoded by the recombinant vectors pPROTet–Myc–His (A) and pPROLar–XPress–His (B), along time induction, using the optimized inducers concentrations. (●) hPAH wt; (□) R261Q mutant form; (▽) I65T mutant form; (▲) V388M mutant form; (◊) R270K mutant form. Each value represents the mean of two independent experiments.

Co-production of two different subunits was demonstrated by Western blot analysis of the purified recombinant proteins, using the anti-Myc and the anti-Xpress antibodies. The presence of the two subunits possessing both epitopes is illustrated in Fig. 4 for the co-produced 6xHis–hPAHwt$_{Tet}$/V388M$_{Lar}$.

In order to perform a comparative analysis, the enzymatic activities of the homomeric forms of 6xHis hPAH proteins were also determined (Table 2). When assayed at standard conditions (1 mM L-Phe and 500 µM 6-MPH$_4$), the wild-type form revealed a catalytic activity of 5818 nmol Tyr min^{-1} mg^{-1}. The

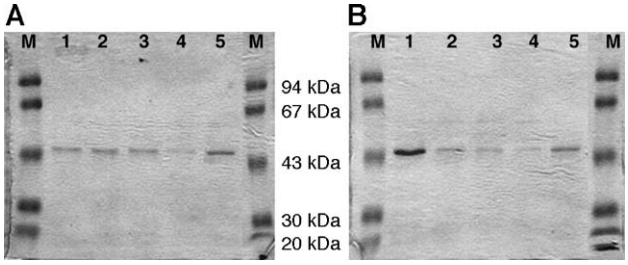

Fig. 3. SDS-PAGE analysis of hPAH mutant forms expressed from recombinant vectors pPROLar–XPress–His (A) and pPROTet–Myc–His (B). (M) Molecular weight marker; (1–4) 6xHis–hPAH recombinant forms of R261Q (1); I65T (2); V388M (3) R270K (4) and; wild-type (5).

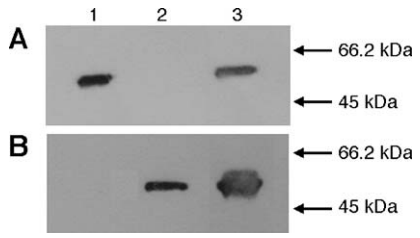

Fig. 4. Western blotting analysis of purified (1) 6xHis–hPAHwt$_{Tet}$ (Myc epitope); (2) 6xHis–hPAH V388M$_{Lar}$ (Xpress epitope) and (3) co-produced 6xHis–hPAHwt$_{Tet}$/6xHis–hPAH V388M$_{Lar}$ protein (Myc and Xpress epitope). Immunodetection using the anti-Myc (A) and anti-Xpress (B) antibodies (see text for details).

purified homomeric R270K protein exhibited only 2.1% (121 nmol Tyr min^{-1} mg^{-1}) of residual activity, whereas mutations I65T, R261Q and V388M presented 26.5% (1539 nmol Tyr min^{-1} mg^{-1}), 27.6% (1603 nmol Tyr min^{-1} mg^{-1}) and 27% (1569 nmol Tyr min^{-1} mg^{-1}) respectively, of the activity of the native enzyme.

The subunits of the co-produced 6xHis hPAH proteins were expressed alternatively by each of the vectors and their enzymatic activities were determined. The obtained values were compared (Student's *t* test) in order to determine if their difference were statistically significant (Table 2).

As shown in Table 2, the enzymatic activities of the purified co-produced mutant proteins were always lower than the PRA. The R270K/R261Q protein presented an experimental activity 21% lower than the PRA. For the V388M/I65T, V388M/R261Q and I65T/R261Q we could observe a similar effect in the decrease of the enzymatic activity (38, 35 and 28%, respectively). Proteins produced by co-expressing the R270K and V388M alleles showed the higher activity decrease (88%), while the R270K/I65T co-produced protein presented an experimental activity 52% lower than the PRA. The hPAHwt/V338M protein, mimicking a heterozygous condition, also presented a residual activity (38.5%) lower than the PRA (63.5%).

4. Discussion

Interallelic complementation is a phenomenon that occurs when a hybrid protein is expressed from two different mutated alleles of a gene. When compared to the predicted residual activity the produced heteromeric protein could present a higher (positive complementation) or lower (negative complementation) catalytic activity.

The observation that some PKU patients present a more severe phenotype than the predicted [6] suggests that the resulting heterotetrameric enzyme activity should be lower than the predicted value, determined from the independent enzyme activities of the respective homomeric mutant proteins. To give an explanation for this phenomenon the occurrence of a negative interallelic complementation mechanism has been evoked. While interactions of different subunits of a hybrid hPAH protein were already proven to occur [8,9], the enzymatic activities of such heteromeric proteins had never been determined.

In order to co-produce two different subunits of a heteroallelic hPAH phenotype, a prokaryotic dual vector co-

Table 2
Enzymatic activities of homoallelic and heteroallelic 6xHis hPAH recombinant proteins produced by simultaneous expression of two subunits

PAH sub-unit composition	Specific activity [a] (nmol Tyr min^{-1} mg^{-1})	Residual enzyme activity [b] (%)	PRA [c] (%)	Observed decrease (%)
$hPAHwt_{Lar}/hPAHwt_{Tet}$	5818±82.7 (100%)		–	–
$I65T_{Lar}/I65T_{Tet}$	1539±42.4 (26.5%)		–	–
$R261Q_{Lar}/R261Q_{Tet}$	1603±49.5 (27.6%)		–	–
$R270K_{Lar}/R270K_{Tet}$	121±18.4 (2.1%)		–	–
$V388M_{Lar}/V388M_{Tet}$	1569±41.0 (27.0%)		–	–
$V388M_{Lar}/hPAHwt_{Tet}$	2336 (40%)	38.5	63.5	39
$V388M_{Tet}/hPAHwt_{Lar}$	2200 (37%)			
	(*P* n.d.)			
$V388M_{Lar}/I65T_{Tet}$	918±49.0 (15.8%)	16.5	26.7	38
$V388M_{Tet}/I65T_{Lar}$	1003±20.8 (17.2%)			
	(*P*>0.05)			
$V388M_{Lar}/R261Q_{Tet}$	1062±34.9 (18.2%)	17.6	27.3	35
$V388M_{Tet}/R261Q_{Lar}$	987±35.5 (17.0%)			
	(*P*>0.05)			
$V388M_{Lar}/R270K_{Tet}$	118±7.4 (2.03%)	1.77	14.4	88
$V388M_{Tet}/R270K_{Lar}$	88±15.6 (1.51%)			
	(*P*>0.05)			
$R270K_{Lar}/I65T_{Tet}$	474±6.0 (8.15%)	6.91	14.3	52
$R270K_{Tet}/I65T_{Lar}$	330±10.5 (5.67%)			
	(*P*<0.001)			
$R270K_{Lar}/R261Q_{Tet}$	676±10.5 (11.6%)	11.8	14.8	21
$R270K_{Tet}/R261Q_{Lar}$	691±8.3 (11.9%)			
	(*P*>0.1)			
$I65T_{Lar}/R261Q_{Tet}$	1185±15.9 (20.4%)	19.4	27.0	28
$I65T_{Tet}/R261Q_{Lar}$	1075±20.5 (18.5%)			
	(*P*<0.01)			

[a] Values are means ± standard deviation determined from three independent experiments, except for the $V388M_{Lar}/hPAHwt_{Tet}$ and $V388M_{Tet}/hPAHwt_{Lar}$ constructs (two experiments); Statistical analysis compared subunits produced from different expression vectors; statistical significance (*P* value) was determined using the Student's *t* test; values in parenthesis refer to % of activity relative to the wild-type form (residual activity).

[b] Mean of residual enzyme activity determined for both constructs.

[c] (PRA) Predicted enzymatic activity, calculated by averaging the experimental enzymatic activities of the corresponding homomeric proteins. (n.d.) not determined.

expression system (PRO system) was used. The different modes of promoter regulation allowed us to control independently the expression of the two cloned alleles. Actually, the use of 100 ng/ml of aTc (pPROTet–Myc–His constructs) and 0.2% arabinose/1 mM IPTG (pPROLar–XPress–His constructs) produced similar quantities of each different protein (Fig. 1). This fact represents one of the greatest advantages of the dual vector system when compared to the bicistronic system [20].

We first studied each expression vector individually in order to confirm that they were suitable for the production of catalytically active PAH enzyme. The use of pPROLar–XPress–His and pPROTet–Myc–His expression vectors allowed the purification of the full-length recombinant 6xHis–hPAH proteins (55 kDa), with purity grades ranging from 60 to 95%. The resulting mutant homoallelic forms presented relative enzymatic activities (Table 2) similar to those obtained previously, using the pTrcHis prokaryotic expression system [12,19].

The presence of different epitopes in the constructs (Myc and Xpress) allowed us to confirm the presence of both subunits in the coexpressing assays. As shown by Western blot analysis (Fig. 4), cotransformation with the different pPROLar–XPress–His and pPROTet–Myc–His constructs into the *E. coli* strain DH5αPRO resulted in the production of similar levels of the two different subunits of the co-produced $hPAHwt_{Tet}/V388M_{Lar}$ protein.

In order to prove that the determined enzymatic activities of the co-produced proteins were independent of the vector used to express the studied allele, each hPAH subunit, comprising the heteroallelic state, was always synthesized in the two possible combinations (Table 2). The obtained enzymatic activities were not significantly different (*P*>0.05), except for the R270K/I65T (*P*<0.001) and R261Q/I65T (*P*<0.01) proteins. In these two co-produced proteins the determined *P* value (<0.05) could not be explained neither by the presence of a particular subunit, namely the I65T subunit as it was not observed for the co-produced V388M/I65T mutant protein, nor by an higher expression level of one of the vectors. Moreover, the same range of residual activity was calculated (Table 2) for the R270K/I65T combinations (8.1 and 5.67%) and for the R261Q/I65T combinations (18.5 and 20.4%). Therefore, any of the construct combinations could be used to study the interallelic complementation phenomenon.

We could demonstrate for the heteroallelic mutant proteins, a decrease in the enzymatic activity when compared to the predicted residual activity (PRA). These values, ranging from 88% (V388M/R270K) to 21% (R270K/R261Q), reflect a negative interaction between the studied mutant subunits.

Furthermore, we can conclude that the subunit interactions are strongly dependent on the nature of the mutant proteins present. Among these, the R270K subunit presented a higher negative effect, particularly over the V388M subunit. It has been postulated that reduced stability is likely the most important attribute for the association of the R270K mutation with PKU in vivo [19]. Therefore, the observed drastic effect of this mutant subunit could be related to an altered protein oligomerisation.

Each subunit of the tetrameric hPAH comprises a N-terminal regulatory domain (Ser2–Ser110), a catalytic domain (Ser110–Ser411) a dimerisation motif (Ser411–Thr427) and a C-terminal tetramerisation domain (Glu428–Lys452) [21]. The regulatory domain from one subunit establishes contacts with the catalytic domain of the other subunit in the dimer. The tetramers are considered to be a dimer of dimers in which the subunits in one dimer contact the subunits in the adjacent dimer only via the α-helical tetramerisation motif. As none of the studied mutations affect the hPAH tetramerisation domain (I65T and R261Q are dimer interface mutations) [22,23] probably they will affect the existing interactions between the subunit interfaces and not directly the tetrameric unit assembly. As a consequence, the enzymatic function could be directly affected as the studied mutations could interfere with the necessary conformational changes occurring at the dimer interface upon activation by L-Phe [24]. We could also admit that they result in quaternary structures relatively less stable. In this regard, the dual expression system described herein will allow the study of the oligomerisation pattern of the produced proteins as well as their enzymatic properties (studies in course). It must be noted that albeit there is now a high-resolution structure available for the hPAH enzyme, there is still no direct proof of subunit interaction or a clear insight into the possible structural basis of any such interaction.

In conclusion, from our observations, it seems clear that a phenomenon of negative interallelic complementation exists between the studied hPAH subunits, mimicking heterozygous and heterozygous compound patients. Furthermore, it appears likely that this phenomenon could be a general source of phenotypic variation in genetic diseases involving multimeric proteins. Such interactions must be considered in any attempt to establish genotype/phenotype correlations in patients affected by such disorders.

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