Heterotetrameric forms of human phenylalanine hydroxylase: Co-expression of wild-type and mutant forms in a bicistronic system

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A B S T R A C T

Hybrid forms of human phenylalanine hydroxylase (hPAH) mutants have been found to present catalytic activities lower than predicted from the individual recombinant forms, indicating that interallelic complementation could be a major determinant of the metabolic phenotype of compound heterozygous phenylketonuric (PKU) patients. To provide a molecular explanation for interallelic complementation we have here developed a bicistronic expression system and a purification strategy to obtain isolated hPAH heterotetrameric forms. On co-expression of WT-hPAH (~50% tetramer; ~10% dimer) and the N- and C-terminally truncated form ΔN102/ΔC24-hPAH (~80% dimer) no heterodimers were recovered. Moreover, by co-expression of WT-hPAH and the N-terminally truncated form ΔN102-hPAH (~95% tetramer), heterotetramers as a result of an assembly of two different homodimers, were isolated. The recovered (WT)/ΔN102)-hPAH heterotetramers revealed a catalytic activity deviating significantly from that calculated by averaging the respective recombinant heterotetrameric forms. The heterotetramer assembly also results in conformational changes in the WT-hPAH protomer, as detected by trypsin limited proteolysis. The finding that the presence of two homodimers with different kinetic parameters influences the properties of the resulting heterotetrameric protein indicates that the dimers exhibit interactions which are transmitted across the assembled tetramer. The bicistronic expression system developed here allowed the isolation of hybrid forms that exhibit negative interallelic complementation, and may represent a model system for studying the molecular pathogenic mechanisms of PAH gene mutations in compound heterozygous PKU patients, providing the rationale to understand the observed inconsistencies both in genotype/phenotype correlations and in the response to BH₄ supplementation.

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1. Introduction

Human phenylalanine hydroxylase (hPAH, human phenylalanine 4-monooxygenase, EC 1.14.16.1), is a non-heme iron enzyme that catalyzes the hydroxylation of L-phenylalanine (L-Phe) to L-tyrosine (L-Tyr) in the presence of the cofactor (6R)-6,7,8-tetrahydrobiopterin (BH₄) and dioxygen, the rate-limiting step in L-Phe catabolism [1]. hPAH is a homotetrameric/homodimeric protein with catalytic core domain (residues 118–411); and a C-terminal region (residues 412–452) including the dimerization and tetramerization motifs [1,2]. The tetramer is a dimer of two conformationally different dimers, related by a 2-fold noncrystallographic symmetry axis [3]. It is activated 5- to 6-fold on preincubation with L-Phe, reveals a positive kinetic cooperativity with respect to L-Phe and Michaelis–Menten kinetics with BH₄ for the non-preactivated enzyme [4] and BH₄-dependent positive cooperativity for the preactivated form [5].

Mutations in hPAH causing lost or impaired activity of the enzyme are associated with the autosomal recessively inherited disease phenylketonuria (PKU, OMIM 261600), the most frequent disorder of amino acid metabolism [6], with a high genotypic heterogeneity (more than 500 different PAH gene mutations identified to date; http://www.pahdb.mcgill.ca [7]). If untreated, PKU patients present neurological damages due to the increased levels of L-Phe in body fluids [8], and life-long dietary restriction of L-Phe remains the most effective treatment for PKU [9]. Some PKU patients respond to oral ingestion of the pterin cofactor (BH₄) by a reduction in blood L-Phe concentration (BH₄-responsive) and therefore, presently BH₄ is accepted as a novel therapeutic approach. However, it has been
shown that the full genotype determines the BH₄-responsive phenotype [10–12].

A unique property of homomeric enzymes associated with human genetic disorders is their potential ability to exhibit interallelic complementation (IC), a phenomenon that occurs in heteroallelic states when particular combinations of two different alleles, at a given locus, produce a less (positive IC) or a more (negative IC) severe phenotype than their homoallelic counterparts. IC is observed in homomeric enzymes where the protomers exhibit interactions resulting in hybrid proteins with functional or stability properties significantly different from the average of the parental enzymes. The phenomenon is proposed to contribute to the phenotypic diversity of several human diseases including metabolic disorders such as PKU [13,14], argininosuccinic aciduria [15–17], erythropoietic protoporphyria [18] and galactosemia [19,20], neurodegenerative disorders such as familial amyloid polyneuropathy [21,22] and cancer [23,24]. Interallelic complementation is assumed to be of particular importance in PKU since ~75% of PKU patients are compound heterozygous, encoding two different mutant hPAH protomers [25]. Experimental evidence lending support to a negative IC in some compound heterozygous PKU patients has been obtained by the observations that the patients present: (i) a more severe metabolic phenotype than that anticipated by the predicted residual activity (PRA) based on in vitro assays of recombinant proteins [26–28], and (ii) an absence or only a partial response to BH₄, although carrying two BH₄-responsive alleles [29]. Interactions between different hPAH protomers have been demonstrated by three different experimental strategies, i.e. in vitro formation of heterotetramers from different dimers [30], the yeast two-hybrid system [31] and a dual vector prokaryotic expression system producing two different hPAH subunits mimicking the natural heteroallelic state in heterozygous or in compound heterozygous patients [14]. However, the molecular mechanism of negative IC and its role in PKU pathogenesis is far from being solved since heterotetrameric forms of hPAH have only been demonstrated in vitro [30], but never isolated and characterized biochemically.

In order to provide some insight into the assembly process of heteroallelic hPAH proteins and their non-additive gene dosage effect, truncated forms of the protein (ΔN102-hPAH and ΔN102/ΔC24-hPAH) were co-expressed with WT-hPAH, and the hetero-meric forms characterized biochemically. To isolate the recombinant heteromorphic proteins in high yield and purity a bicistronic expression system was developed, presenting the different protomers as fusion proteins with the chaperone maltose binding protein (MBP) [30,32] and different affinity purification tags (calmodulin binding peptide (CBP), hexahistidyl peptide (6His) and StrepII peptide). The affinity isolated heteromic species were characterized by their steady-state kinetic properties and susceptibility to limited proteolysis.

2. Materials and methods

2.1. Materials

The DNA primers used for mutagenesis were provided by Eurogentec (Seraing, Belgium) and the restriction endonucleases were from New England Biolabs (USA). Escherichia coli BL21(DE3) and the prokaryotic expression vector pETDuet-1 were obtained from Novagen (Merck KGaA, Darmstadt, Germany). The restriction protease factor Xa was obtained from Protein Engineering Technology ApS (Aarhus, Denmark). SDS molecular mass standard was delivered by Bio-Rad and the pterin cofactor (GR)-α-erythro-5,6,7,8-tetrahydrobipterin (BH₄) was obtained from Schircks Laboratories (Jona, Switzerland). Trypsin and trypsin inhibitor were delivered by Sigma-Aldrich (St. Louis, MO, USA).

2.2. Construction of the bicistronic expression vectors

The calmodulin binding peptide (later replaced by the Strepl tag) and the hexahistidyl peptide (6His; HHHHHH) were firstly selected to produce different N-terminal tagged recombinant proteins using the bicistronic expression vector pETDuet-1. This vector contains two multiple cloning sites (MCS1 and MCS2), each preceded by a T7 promoter/lac operator and a ribosome binding site. A T7 terminator sequence is located at 3′ of the MCS2.

The strategy to construct the bicistronic expression vector is depicted in Supplementary Fig. S1. The sequence coding for the calmodulin binding peptide (CBP; KRRWKFIAYSAANRFKKISSSS-GAL) together with a Pmel restriction sequence were introduced 5′ to the MBP coding sequence by three steps of site-directed mutagenesis using the pMAL-hPAH vector as a template [33] and primers CBP1st-F/CBP1st-R, CBP2nd-F/CBP2nd-R and CBP3rd-F/CBP3rd-R (Table 1). The resulting vector pMAL-CBL-MBP-pep(Xₐ)₆WT-hPAH was double digested with Pmel/SfiI. The isolated expression cassette was blunted with T4 DNA polymerase, according to standard procedures, and cloned into the MCS1 of pETDuet-1 vector which had been previously digested with Ncol, blunted and dephosphorylated with calf intestinal alkaline phosphatase (CIP), thus generating the expression vector pETDuet-1-CBL-MBP-pep(Xₐ)₆WT-hPAH.

In order to replace the CBP tag by the Strepl tag (WSHPQFEK), two steps of site-directed mutagenesis were performed using the pETDuet-1-CBL-MBP-pep(Xₐ)₆WT-hPAH as template and primers STRII-F/STRII-R and CBPDel-F/CBPDel-R (Table 1). In the obtained construct (pETDuet-1-StrepII-MBP-pep(Xₐ)₆WT-hPAH) a new T7 terminator was introduced 3′ to MCS1, by site-directed mutagenesis, using primers CRE7T-F/CRE7T-R (Table 1).

To create the bicistronic expression construct sequence the coding for the 6His tag and the Pmel restriction sequence were previously introduced 5′ to the MBP coding sequence by site-directed mutagenesis, using pMAL-MBP-pep(Xₐ)₆AN102-hPAH or pMAL-MBP-pep(Xₐ)₆AN102/ΔC24-hPAH constructs as templates [34] and primers HisF/HisR (Table 1). The expression cassette was isolated, after Pmel/SfiI double digestion, blunt-ended and cloned into the MCS2 of pETDuet-1-Strepl-MBP-pep(Xₐ)₆WT-hPAH vector which had been previously digested with EcoRV (MCS2), blunt-ended and dephosphorylated. The pETDuet-1-Strepl-MBP-pep(Xₐ)₆WT-hPAH/6His-MBP-pep(Xₐ)₆AN102-hPAH and pETDuet-1-Strepl-MBP-pep(Xₐ)₆WT-hPAH/6His-MBP-pep(Xₐ)₆AN102/ΔC24-hPAH bicistronic constructs were obtained (Fig. 1). Site-directed mutagenesis reactions were always performed using the Quikchange® II XL system (Stratagene, La Jolla, CA, USA) and the authenticity of mutagenesis reactions and cloning was always verified by DNA sequencing using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) in an ABI 3730xl DNA Analyzer (Applied Biosystems). All the expression constructs present a cleavage site for factor Xa (IEGR) between the N-terminal fusion protein and the hPAH sequence.

2.3. Expression and purification of recombinant hPAH

E. coli BL21(DE3) competent cells were transformed with the pETDuet-1-[StrepII-MBP-pep(Xₐ)₆6His-MBP-pep(Xₐ)₆hPAH₄] (hPAH₄; WT-hPAH; hPAH₄; ΔN102-hPAH or ΔN102/ΔC24-hPAH) expression vectors and grown in LB medium, supplemented with 50 μg mL⁻¹ ampicillin and 0.2% glucose, at 37 °C. Protein expression was induced with 1 mM isopropyl β-D-galactoside (IPTG), when the A₆₀₀ was about 0.8. Ferrous ammonium sulfate, at 0.2 mM, was added simultaneously with IPTG and 3 h after induction. After 8 h incubation, at 28 °C, bacteria were harvested and the pellets stored at −20 °C until use. To rule out homologous recombination, plasmid integrity was checked. To purify the recombinant enzymes, cells were resuspended in lysis buffer
Table 1
Oligonucleotides used for site-directed mutagenesis.

<table>
<thead>
<tr>
<th>Template</th>
<th>Primers&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sequence (5′→3′)&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td>pMAL-hPAH&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>CBP1st F</td>
<td>CCACAGAAGGACCATAGATTATGTTAAACTTTAAACGAGATGGAAAAAGAAAATCTGAAGGCTAAACTGG</td>
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<tr>
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</tr>
<tr>
<td>pMAL-CBP2-hPAH&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>CBP2nd F</td>
<td>CCGGATTAACCCATGCGATTTTCATTTTTTTCCCACGTCGTTAGGGACACAATACG</td>
</tr>
<tr>
<td>pMAL-CBP2-hPAH&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>CBP2nd R</td>
<td>CCGGATTAACCCATGCGATTTTCATTTTTTTCCCACGTCGTTAGGGACACAATACG</td>
</tr>
<tr>
<td>pETDuet-1-CBP-hPAH&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>STRI F</td>
<td>GAAAATCTCATCCTCCGGGGCACTT</td>
</tr>
<tr>
<td>pETDuet-1-CBP-hPAH&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>STRI R</td>
<td>GAAAATCTCATCCTCCGGGGCACTT</td>
</tr>
<tr>
<td>pETDuet-1-CBP-MBP-hPAH&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>CBPDef F</td>
<td>CAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAAAATGCTGCCTTTTTCGAACTG</td>
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<tr>
<td>pETDuet-1-CBP-MBP-hPAH&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>CBPDef R</td>
<td>TATTTCATAGCCGTCTCAGCAGCCAACCGC</td>
</tr>
<tr>
<td>pETDuet-1-CBP-MBP-hPAH&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>HISP F</td>
<td>CAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAAAATGCTGCCTTTTTCGAACTG</td>
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<tr>
<td>pETDuet-1-CBP-MBP-hPAH&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>HISP R</td>
<td>TATTTCATAGCCGTCTCAGCAGCCAACCGC</td>
</tr>
</tbody>
</table>

<sup>a</sup> F—forward, R—reverse.
<sup>b</sup> Mismatch nucleotides are shown in boldface; nucleotides used to create new restriction sites are underlined.

(10 mM Tris–HCl, 200 mM NaCl, 0.5 μg·mL⁻¹ pepstatin, pH 7.25) supplemented with 1× Complete Protease Inhibitor Cocktail (Roche Applied Science) and disrupted by passage through a French Press. The presence of the MBP tag, in both hPAH subunits, allowed the purification of the fusion proteins using an amylose resin (New England Biolabs), previously equilibrated with 15 mM NaCl, and 1 mM EDTA, pH 7.4. Recombinant proteins were eluted with 10 mM maltose [33].

The tetramers and dimers were isolated by size-exclusion chromatography (SEC), using a HiLoad Superdex 200 HR column (1.6 cm × 80 cm, Amershams Biosciences) and a mobile phase containing 20 mM Na–Hepes, 200 mM NaCl, pH 7.0 (SEC buffer) pumped at a flow rate of 0.38 mL min⁻¹. The relative molecular mass of the different oligomeric forms was estimated from a calibration curve obtained with the following standard proteins: aprotinin (6.5 kDa), ribonuclease A (13.7 kDa), carbonic anhydrase (29 kDa), ovalbumin (43 kDa), BSA (66 and 132 kDa), conalbumin (75 kDa), aldolase (158 kDa), ferritin (440 kDa) and thyroglobulin (669 kDa). Blue Dextran 2000 and AMP were used to determine the void volume (V<sub>0</sub> = 45.2 mL) and the total exclusion volume (V<sub>T</sub> = 115.5 mL) of the column, respectively. All purification steps were carried out at 4 °C. The relative amounts of the different oligomeric states were calculated by deconvolution of the obtained chromatograms using the PeakFit software (Systat Software Inc). Protein concentration was estimated by the Bradford method [35] using bovine serum albumin (BSA) as the standard.

2.4. Isolation of hybrid proteins

The tetrameric or dimeric fractions obtained from SEC were applied to a Ni-chelating resin (Ni–NTA, Qiagen, Valencia, CA), pre-equilibrated in SEC buffer containing 10 mM imidazole, and stirred for 1 h at 4 °C. The flow-through was collected (Fraction 1: Strepll-MBP-hPAH<sub>1</sub> homomeric proteins) and the resin was washed with SEC buffer containing 20 mM imidazole, followed by a SEC buffer washing step containing 50 mM imidazole. The fusion proteins were eluted with SEC buffer containing 150 mM imidazole (Fraction 2: 6His-MBP-hPAH<sub>2</sub> homomeric and Strepll-MBP-hPAH<sub>1</sub>/6His-MBP-hPAH<sub>2</sub> heteromeric proteins).

Fraction 2 was then applied to a Strepl-Tactin resin (Strepl-Tactin Superflow high capacity, IBA, Göttingen, Germany) and stirred for 1 h at 4 °C. The flow-through was collected (Fraction 3: 6His-MBP-hPAH<sub>2</sub> homomeric proteins) and the resin was washed with SEC buffer. The hybrid proteins were eluted with SEC buffer containing 2.5 mM desthiobiotin (Fraction 4: Strepl-MBP-hPAH<sub>1</sub>/6His-MBP-hPAH<sub>2</sub> heteromeric proteins).

The fusion proteins obtained in Fractions 1, 3 and 4 were cleaved for 4 h at 4 °C by factor Xa (Protein Engineering Technology ApS), using a protease to substrate ratio of 1:30 (by mass), and applied to a second column of amylase resin, to remove MBP, followed by removal of factor Xa protease using the Xa Removal Resin (QIAGEN). The homomeric hPAH<sub>1</sub> and hPAH<sub>2</sub> and the heteromeric hPAH<sub>1</sub>/hPAH<sub>2</sub> forms were then obtained.
Protein purity was analyzed by SDS-PAGE on 10% (w/v) polyacrylamide gels. The gels were stained by 0.1% (w/v) Coomassie Brilliant Blue R250 (Bio-Rad) in 50% (v/v) methanol and 10% (v/v) acetic acid. Stained gels were scanned using VersaDoc 4000 (Bio-Rad) and the protein bands were quantified using the Quantity One 1-D Analysis Software (Bio-Rad).

2.5. Assay of enzymatic activity

The hPAH enzymatic activity was assayed essentially as described [33], using 0.1 mg mL⁻¹ of catalase and an enzyme activation step by prior incubation (5 min) with L-Phe. BSA, at 0.5% (w/v), was included in the reaction mixture to stabilize the diluted purified hPAH preparations. The amount of L-Tyr formed after 1 min was measured by HPLC and fluorimetric detection [36]. The steady-state kinetic data were analyzed by nonlinear regression analysis using the SigmaPlot® Technical Graphing Software and the modified Hill equation of LiCata and Allewell [37] for cooperative substrate binding as well as substrate inhibition [38]. In order to study the effect of preincubation with L-Phe on the specific activity, 1 mM L-Phe was added together with 75 μM BH₄ at the initiation of the hydroxylation reaction. In this case a 3 min time course was then followed.

2.6. Limited proteolysis with trypsin

Limited proteolysis of WT, ΔN102 and (WT)/(ΔN102)-hPAH tetrameric forms was performed at 25 °C in 20 mM Na–Hepes, 200 mM NaCl, pH 7.0 and a protease to hPAH ratio of 1:200 (by mass) in the absence or presence of 1 mM L-Phe. The final protein concentration was 0.3 mg mL⁻¹. At timed intervals (0–60 min) aliquots of the reaction were mixed with soybean trypsin inhibitor (at 1:1.5 (by mass) protease to inhibitor ratio) and subjected to SDS-PAGE analysis. Gels were stained by Coomassie brilliant blue and the band corresponding to the full-length protein was quantified by densitometry.

3. Results

3.1. Co-expression of hybrid hPAH proteins using a bicistronic expression system

Using a dual vector expression system encoding two different epitopes (Myc and Xpress) we have previously shown the presence of assembled hybrid hPAH proteins presenting a catalytic activity lower than the PRA [14]. In theory five different tetrameric species would be formed, from the combination of two different co-expressed hPAH subunits (hPAH₁:hPAH₂), in the following ratios: 0:4, 1:3, 2:2, 3:1 and 4:0. However, by performing a cross-linking reaction of the co-expressed Myc-WT-hPAH and Xpress-WT-hPAH prior to the co-immunoprecipitation step, heterotetramers but not heterodimers were detected in the immunoblots (R. Almeida, J. Leandro et al., personal communication). The absence of heterodimers indicates that the heterotetrameric species 1:3 and 3:1 (hPAH₁:hPAH₂) would not be formed and only the heterotetrameric 2hPAH₁:2hPAH₂ will be expected, as represented in Fig. 2. Nevertheless, using the described dual expression system [14], the low yields of obtained hybrid

Fig. 2. Flow-chart of the purification procedure of recombinantly co-expressed hPAH polypeptide chains (hPAH₁ and hPAH₂). The supernatant of lysed bacteria co-expressing two different hPAH proteins was applied to an amylose resin in the first purification step. The MBP fusion proteins were eluted with maltose and applied to a size-exclusion column (SEC). The tetrameric (or dimeric) fraction recovered was applied to a Ni–NTA resin. The StrepII tagged homotetramers (or homodimers) were collected in the flow-through (FT) and the 6His tagged homotetramers (or homodimers) and StrepII/6His tagged heterotetramers (or heterodimers) bound to the resin were eluted with imidazole. In the last step, the eluted fraction was applied to a Strep-Tactin resin. The 6His tagged homotetramers (or homodimers) were obtained in the FT and the Strepl/6His tagged heterotetramers (or heterodimers) were eluted with desthiobiotin. This procedure resulted in the separation of three different hPAH protein species: homomeric Strepl tagged, homomeric 6His tagged and heteromeric Strepl/6His tagged.
proteins precluded the isolation of heteromeric recombinant enzymes for further biochemical and structural characterization. To overcome this problem, we developed here a bicistronic expression strategy, where in addition to two different purification tags (CBP or Strepl, and 6His), the putative molecular chaperone maltose binding protein was used as fusion partner, N-terminally to the hPAH protein, as outlined in Fig. 1A. The presence of two different purification tags offers the possibility to obtain pure hetero-oligomeric fractions by performing two affinity-chromatographic steps (Fig. 2). MBP was chosen as fusion partner (common to both polypeptides), as MBP has been shown to have a chaperone like effect [32,39], improving the yield of recombinant hPAH [30,33]. Using this strategy the WT-hPAH (~50 kDa subunit) was co-expressed with truncated forms of hPAH (Fig. 1B), namely the ΔN102-hPAH (~38.8 kDa subunit) which lacks the first 102 residues (corresponding to the N- regulatory domain) and is mainly expressed as a tetramer (96%) (Table 2); and the double truncated form ΔN102/ΔC24-hPAH (~36.2 kDa subunit) which also lacks the 24 C-terminal residues (corresponding to the tetramerization motif) and is mainly expressed as a dimer (81%) (Table 2). The rationale for co-expressing the WT-hPAH with the truncated forms was fourfold. First, co-expressing the WT-hPAH with the double truncated form (which is isolated mainly as tetramer) will favor the assembly of a heterotetramer. Thirdly, due to the different catalytic properties of the wild-type and truncated forms [2,34] the impact of protomer interactions on the kinetic properties of the assembled heteromeric forms will be profusely assessed. Fourthly, using SDS-PAGE analysis a clear distinction and quantification of the ratio of the different subunits will be possible since the truncated forms present molecular masses lower than the wild-type.

Small tags characterized by a high specificity and mild elution conditions (Supplementary Table S1) were selected. The polyhistidine tag was our first choice since we had already used it to express and purify recombinant hPAHs [40]. The choice of the second affinity tag was more challenging given the large number of alternatives [41,42].

As a first candidate we tested the CBP peptide. However, during the purification process the hPAH polypeptide, preceded by the CBP tag (CBP-MBP-pep(Xa)-hPAH), suffered extensive aggregation as the result of the effect of a highly hydrophobic patch, present in the middle of the CBP tag (Supplementary Fig. S2). This aggregation was not overcome by the use of glycerol (10%), or by the mild non-ionic detergent n-octylglucoside (15 mM) nor by the use of the improved E. coli C41(DE3) strain [43,44] (data not shown). Hence, the CBP tag was replaced by the Strepl tag which allowed the recovery of soluble hPAH in high yields. Since the original pETDueT-1 vector presents only one T7 terminator after MCS2, a new T7 terminator was introduced after MCS1 (Fig. 1A), in order to guarantee the existence of just two mRNAs, each one corresponding to the transcribed sequence of the two different hPAH cDNAs.

The expression conditions (28 °C, 1 mM IPTG, 8 h) were chosen in a compromise between the maximum total recombinant protein produced, its amount in the soluble fraction and the minimization of hPAH deamination that occurs with long induction times [45]. The expression of the recombinant hPAHs as MBP-fusion proteins resulted in high yields (~12 mg/L culture) when compared with the values obtained with the dual expression system used in our previous studies (~0.3 mg of fusion protein/L culture [14]). After the introduction of the new T7 terminator similar levels of the translated proteins were produced, as shown by SDS-PAGE analysis of the co-expressed proteins after amylose affinity purification (Supplementary Fig. S3). The double truncated dimer ΔN102/ΔC24-hPAH was an exception since it was recovered in the soluble (Supplementary Fig. S3, lane 3) and dimeric fractions at higher levels due to its higher solubility. As expected, on size-exclusion chromatography the separately expressed Strepl-MBP-WT-hPAH and 6His-MBP-ΔN102-hPAH were recovered mainly in the tetrameric form whereas the double truncated 6His-MBP-ΔN102/ΔC24-hPAH was recovered as a dimer (Table 2). Accordingly, for the co-expressed Strepl-MBP-WT-hPAH and 6His-MBP-ΔN102/ΔC24-hPAH the dimer represented the major oligomeric form (Fig. 3A). SDS-PAGE of this fraction (peak 3) revealed one distinct band at ~96 kDa (Strepl-MBP-WT-hPAH) and another at ~82 kDa (6His-MBP-ΔN102/ΔC24-hPAH) of different intensities, as the double truncated form assembles as dimers with a higher solubility (Fig. 3A, inset, lane 3). SDS-PAGE of the tetrameric fraction (peak 2) gave a single band of ~96 kDa (Fig. 3A, inset, lane 2) corresponding to the Strepl-MBP-WT-hPAH protein. For the co-expressed Strepl-MBP-WT-hPAH and 6His-MBP-ΔN102-hPAH, the tetramers were the predominant species (Fig. 4A) with 96 and 84 kDa bands on SDS-PAGE (Fig. 4A, inset, lane 2), corresponding to the Strepl-MBP-WT-hPAH and 6His-MBP-ΔN102-hPAH, respectively. The dimeric fraction corresponding to the WT-hPAH (Fig. 4A, inset, lane 3), revealed a band corresponding to the truncated form, since the dimeric fraction (peak 3) was not completely resolved from the ΔN102-hPAH tetramer.

3.2. Study of the heterodimer and heterotetramer formation using WT and truncated forms of hPAH

3.2.1. (WT)/(ΔN102/ΔC24)-hPAH heterodimer is not formed

When the dimeric fraction of the co-expressed Strepl-MBP-WT-hPAH and 6His-MBP-ΔN102/ΔC24-hPAH was applied to the Ni–NTA resin, a protein with an apparent molecular mass of ~96 kDa, corresponding to the Strepl-MBP-WT-hPAH subunit, was recovered in the flow-through (Fig. 3B, lane 2). Only a trace amount of ~82 kDa protein was observed, corresponding to the 6His-MBP-ΔN102/ΔC24-hPAH, indicating that homodimeric Strepl-MBP-WT-hPAH was mainly obtained in the flow-through. Elution of bound proteins with imidazole gave essentially one band at 82 kDa (Fig. 3B, lane 3), suggesting that only the 6His-MBP-ΔN102/ΔC24-hPAH species was recovered. In fact, when this fraction was applied to the Strep-Tactin resin (Fig. 3C) a band corresponding to the 6His-MBP-ΔN102/ΔC24-

<table>
<thead>
<tr>
<th>Expressor or co-expressed fusion proteins</th>
<th>Subunit molecular mass (fusion protein)(^b) (kDa)</th>
<th>Oligomeric state</th>
<th>Apparent molecular mass(^b)</th>
<th>Relative amounts(^b,c)</th>
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<td>WT-hPAH</td>
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<td></td>
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<td>Tetramer</td>
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<td></td>
<td></td>
<td>Tetramer</td>
<td>310 kDa</td>
<td>(96%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dimer</td>
<td>156 kDa</td>
<td>(81%)</td>
</tr>
<tr>
<td>(WT)/(ΔN102)-hPAH</td>
<td>95.6/84.4</td>
<td>Aggregated</td>
<td>—</td>
<td>(9%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tetramers</td>
<td>—</td>
<td>(62%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dimer</td>
<td>219 kDa</td>
<td>(12%)</td>
</tr>
<tr>
<td>(WT)/(ΔN102/ΔC24)-hPAH</td>
<td>95.6/81.8</td>
<td>Aggregated</td>
<td>—</td>
<td>(20%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tetramer</td>
<td>412 kDa</td>
<td>(14%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dimer</td>
<td>—</td>
<td>(55%)</td>
</tr>
</tbody>
</table>

\(a\) Subunit molecular mass calculated from the amino acid sequence.

\(b\) The apparent molecular mass of the oligomeric forms and their relative amounts were calculated by deconvolution analysis of the size-exclusion chromatograms using the Peakfit software (Systat Software Inc.) of the MBP-fusions proteins before Ni-NTA and Strep-Tactin purification.

\(c\) Degradation products not included.

\(d\) Data from [34].

\(e\) Apparent molecular mass not determined, as the broad peak correspondent to 2 or more different species.
hPAH (82 kDa), which did not bind to the resin, was obtained in the flow-through (Fig. 3C, lane 1), and only negligible amounts of recombinant 82 kDa and 96 kDa fusion proteins were eluted with desthiobiotin (Fig. 3B, lane 2). This fraction was acetone-precipitated, and the recovered proteins, representing less than 1% of the homodimeric proteins, may correspond to proteins that remained bound to the column from the previous steps of purification. The results indicate that only StrepII-MBP-WT-hPAH and 6His-MBP-ΔN102/ΔC24-hPAH homodimers, but no StrepII-MBP-WT-hPAH/6His-MBP-ΔN102/ΔC24-hPAH heterodimers, were formed.

3.2.2. The (WT)/(ΔN102)-hPAH heterotetramer

The His-tag affinity purification of the co-expressed StrepII-MBP-WT-hPAH and 6His-MBP-ΔN102/ΔC24-hPAH proteins resulted in the recovery of the StrepII-MBP-WT-hPAH homotetramer (~96 kDa subunit) in the flow-through when applied to the Ni-chelating resin (Fig. 4B, lane 2), and the imidazole elution recovered the StrepII-MBP-WT-hPAH (~96 kDa) and 6His-MBP-ΔN102/ΔC24-hPAH homodimers (~82 kDa, and the potential StrepII-MBP-WT-hPAH/6His-MBP-ΔN102/ΔC24-hPAH heterodimers (subunits Mₙ of ~96 and ~82 kDa, respectively). (C) SDS-PAGE analysis of the Strep-Tactin purification. Lane 1, flow-through containing 6His-MBP-ΔN102/ΔC24-hPAH homodimers; lane 2, desthiobiotin eluted fraction after acetone-precipitation (see main text for further explanation — Results section). Lane M, low-molecular-mass marker (104.4, 83.2, 49.3, 36.9 and 28.9 kDa). At the bottom of each lane is indicated the expected hPAH forms as depicted in Fig. 2, with the StrepII-MBP-WT-hPAH polypeptide chain (□) and the 6His-MBP-ΔN102/ΔC24-hPAH polypeptide chain (○).
The WT/(ΔN102)-hPAH heterotetramer revealed a lower [S]0.5 (L-Phe) value (76±17 μM) than the WT-hPAH homotetramer ([S]0.5 =151±6 μM), but only slightly higher than the truncated homotetramer ΔN102-hPAH ([S]0.5=55±3 μM) (Table 3). The heterotetramer is also characterized by a marked decrease in the kcat (0.34±0.03×10^3 nmol Tyr−1⋅nmol tetramer−1), a value lower than that predicted by averaging the kcat of the homotetrameric counterparts (kcat,PRA=1.12×10^3 nmol Tyr−1⋅nmol tetramer−1) (Table 3). Moreover, the (WT)/(ΔN102)-hPAH heterotetramer revealed no kinetic cooperativity (nH =1.0±0.1) with respect to L-Phe and is not activated by L-Phe preincubation (1.1-fold), properties also observed for the truncated homotetramer ΔN102-hPAH (nH =1.2±0.1 and 1.0-fold, respectively). By contrast the WT-hPAH homotetramer revealed a kinetic positive cooperativity (nH =2.1±0.1) and a 3.6-fold activation.

**Table 3**

Steady-state kinetic constants for the substrate (L-Phe) of WT-hPAH homotetramer, ΔN102-hPAH homotetramer and (WT)/(ΔN102)-hPAH heterotetramer.

<table>
<thead>
<tr>
<th>hPAH</th>
<th>Vmax</th>
<th>kcat</th>
<th>kcat (PRA)</th>
<th>Vl</th>
<th>[S]0.5</th>
<th>K</th>
<th>kcat/[S]0.5</th>
<th>nE</th>
<th>Fold activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>10.4±0.27</td>
<td>0.56±0.01</td>
<td>—</td>
<td>1.55±0.14</td>
<td>151±6</td>
<td>2.20±0.44</td>
<td>4</td>
<td>2.1±0.1</td>
<td>3.6</td>
</tr>
<tr>
<td>ΔN102</td>
<td>1.85±0.18</td>
<td>0.34±0.03</td>
<td>1.12</td>
<td>4.53±0.27</td>
<td>55±3</td>
<td>1.56±0.17</td>
<td>30</td>
<td>1.2±0.1</td>
<td>1.0</td>
</tr>
<tr>
<td>(WT)/(ΔN102)</td>
<td>1.85±0.18</td>
<td>0.34±0.03</td>
<td>1.12</td>
<td>1.03±0.05</td>
<td>76±17</td>
<td>0.86±0.23</td>
<td>4</td>
<td>1.0±0.1</td>
<td>1.1</td>
</tr>
</tbody>
</table>

The catalytic activity was measured as described in the Materials and methods section at 25 °C, 75 μM BH4 (L-Phe variable). [S]0.5 represents the L-Phe concentration at half-maximal activity and kcat/[S]0.5, the catalytic efficiency which was calculated on the basis of the tetramer molecular mass of 200, 161.2 and 180.6 kDa for WT-hPAH, ΔN102-hPAH and (WT)/(ΔN102)-hPAH, respectively. PRA, predicted residual activity of the heterotetramer, is the mean of the kcat of the corresponding homotetramers.
on preincubation by l-Phe (Table 3). The catalytic efficiency \( k_{cat}/[S]_{0.5} \) was 4 \( \mu \text{M}^{-1} \cdot \text{min}^{-1} \) for both the heterotetrameric form and WT-hPAH.

### 3.4. Limited proteolysis of \( (WT)/(\Delta N102)\)-hPAH heterotetramer with trypsin

On limited proteolysis by trypsin, the WT-hPAH homotetramer (Fig. 6A) was found to be more resistant than the homotetrameric N-terminal truncated form both in the absence and presence of l-Phe (Fig. 6B). A small proteolytic fragment is rapidly cleaved off in the \( \Delta N102\)-hPAH homotetramer, during the first 10 min of incubation, generating a core fragment of ~35 kDa resistant to further trypsin proteolysis. A core fragment of similar molecular mass was also obtained from the WT-hPAH trypsin proteolysis (data not shown). Whereas the WT-hPAH became markedly more susceptible to limited proteolysis in the presence of l-Phe, only a slight increase in the rate of proteolysis was observed for the truncated form (Fig. 6A and B). While the heterotetrameric \( (WT)/(\Delta N102)\)-hPAH revealed no significant difference in the rate of proteolysis for the \( \Delta N102\)-hPAH protomer (Fig. 6D), the WT protomer is more rapidly degraded (Fig. 6C).

### 4. Discussion

#### 4.1. The developed bicistronic expression system is a valuable tool to produce and isolate heteromeric forms of hPAH

Co-expression of different proteins in E. coli can be achieved using three different strategies: (i) a multi-vector expression system (e.g. dual vector system with plasmids with different replication origins and selection markers and with identical or different promoters), (ii) a single polycistronic plasmid with a single promoter, encoding a long polycistronic mRNA, and (iii) a single polycistronic plasmid with individual promoters [46]. In our previous work [14] we followed the first strategy but the low yield preclude the isolation of the hybrid species. Since the use of the second strategy (one polycistronic vector with individual promoters) has been reported to lead to a lower expression of the more downstream encoded protein [47], we decided to follow the third strategy. Using a bicistronic vector with individual but identical promoters (T7 promoter) and two terminators, we obtained similar and high expression levels of both proteins. By using a single plasmid we also assured that the two proteins should localize within the same region of the cell, in contrast to replication from different plasmids which could have a different subcellular localization within the bacterium [48]. Additionally, due to expression from two similar sequences, verification of plasmid integrity is necessary to rule out homologous recombination, as E. coli BL21(DE3) (recA) is one of the most commonly used expression strains. Alternatively, a recA derivative of BL21 should be used (e.g. BLR (DE3) [49]).

In the present work the hPAH variants (WT and truncated forms) were recognized by their different molecular mass. In studies on missense mutations, the detection and quantification can readily be achieved by immuno-detection with monoclonal antibodies specific for the epitope tags (hexahistidyl peptide and StrepII peptide) and MS peptide analyses. Moreover, the depicted vector can be easily adapted to an in vitro transcription-translation system (the large tags can be removed and a consensus Kozak sequence introduced at the MCS2), as a potential tool to study the observed inconsistencies in BH4 supplementation (see below).

#### 4.2. The formation of hPAH heteromers occurs by a non-random assembly process

The C-terminal oligomerization domain of WT-hPAH (residues 412–452) consists of two short \( \beta \)-strands (Ser411-Tyr414 and Ile421-Leu424), connected by a loop (residues 415–420), and a 40 Å long \( \alpha \)-helix (Gln428-Lys452). Dimerization is mediated by several van der
Waals and hydrogen bond interactions of the two symmetry-related loops (residues 415–420), one of each protomer [1,50], and through a relatively polar interface contributed by the regulatory domain in one protomer and the catalytic domain in the other protomer [51].

In the present study, the co-expression of WT-hPAH and truncated forms of the protein allowed the isolation of heterotetramers, resulting from the assembly of different homodimers (2hPAH 1/2hPAH2), but no heterodimers were recovered. Thus, the absence of a random assortment of hPAH protomers suggests that hPAH dimerization, via the Asp415-Arg420 loop of the dimerization motif, is a co-translational process and that tetramer formation (dimer of dimers) occurs post-translationally. Tetramerization occurs from preformed dimers through domain swapping of the C-terminal α-helices (Gln428-Lys452) [1], and our data indicate that these interactions will not be affected by the presence of two different stable dimers with a WT like C-terminal α-helix structure. A co-translational assembly of dimers could explain why hPAH monomers were never found for WT-hPAH [34] or in mutant forms, except for Y417H mapped to the dimerization motif preventing the formation of dimers [52].

### 4.3. The WT protomer within the heterotetramer reveal conformational changes

Binding of L-Phe at the active site triggers reversible conformational changes in the catalytic domain [53], which are transmitted globally (dimer and tetramer) through several hinge-bending regions [53–56] resulting in a more open structure [54]. This conformational change results in an increased susceptibility to limited proteolysis by trypsin (Fig. 6A). The WT protomer revealed a higher susceptibility to limited proteolysis when present in the heterotetrameric (WT)/(ΔN102)-hPAH than in the homomeric form, both in the absence and presence of L-Phe, indicating that the WT protomer adopts a more open conformation. These results suggest that the assembly of the two different dimers (WT and ΔN102) induces a conformational change in the WT protomers mediated by the C-terminal 40-Å long α-helix (Gln428-Lys452), as the only contact between the two dimers in the assembled tetramer occurs by domain swapping of the four C-terminal helices (two from each dimer) [1].

### 4.4. Functional implications of the assembly of hPAH heterotetramers

A comparison of the steady-state kinetic parameters of the (WT)/(ΔN102)-hPAH heterotetramer with the homotetrameric counterparts (Table 3) revealed that the heterotetramer has acquired a catalytic efficiency similar to that of WT-hPAH. However, it has lost the cooperativity with respect to L-Phe and the ability to be activated by the substrate, which are characteristic properties of the ΔN102-hPAH tetramer. Moreover, the $V_{max}$-value was only ~30% of the predicted residual activity (PRA), thus demonstrating a negative interallelic complementation. However, it should be noted that the isolated WT-hPAH dimeric form revealed no kinetic cooperativity and only a minor fold activation by L-Phe when compared to the WT-hPAH tetramer [4], and the kinetic properties of the isolated ΔN102-hPAH...
dimer are unknown as the truncated form is recovered mainly as tetramers (Table 2). If the two dimers in the heterotetramer were acting isolated, the lack of cooperativity and substrate activation in the hybrid could be explained. However, the l-Phe concentration at half-maximal activity (S0.5) of the WT-hPAH dimeric form is much higher than the WT-hPAH tetramer [4], whereas the hybrid tetramer displays a S0.5 (l-Phe) lower than the WT-hPAH tetramer. Taking these results into consideration and the ones obtained from the trypsin limited proteolysis, indicating that the WT dimer in the heterotetramer underwent a conformational change, it was not unexpected to find that the catalytic properties of the heterotetramer were consistently different from those obtained theoretically by averaging the homomeric counterparts. The fact that the presence of two homodimers with different kinetic parameters can alter the properties of resulting hybrid protein indicates that interactions are transmitted across the overall assembled protein and that the dimers are not acting isolated.

4.5. Implications of heterotetrameric assembly in PKU

Although the mutant partner used in the present study is a truncated protein and not a PKU-mutant enzyme, the results obtained in this work can give some insight into the mechanisms involved in PKU, as we report here the first isolation of a heterotetrameric form of hPAH with properties deviating significantly from the average of those of the parental enzymes. The developed expression/purification protocol provides the necessary experimental tools to study on disease-associated hPAH mutations, notably those demonstrating the observed 2hPAH1:2hPAH2 association of hPAH dimers and if they system. It will be important to understand how the mutations alter hPAH with properties deviating significantly from the average of those of the parental enzymes. The developed expression/purification strategy provides the necessary experimental tools for studies on disease-associated hPAH mutations, notably those demonstrating inconsistencies both in genotype/phenotype correlations and in the response to BH4 supplementation. The mechanisms underlying BH4-responsiveness are multifactorial, including correction of catalytic defect (decreased affinity for BH4) and stabilization of the mutant protein against degradation/inactivation in vivo and in vitro [57–59]. Until now, in vitro studies to unravel the mechanism of BH4-responsiveness (e.g. steady-state kinetic analysis, isothermal titration calorimetry and thermostability followed by circular dichroism spectroscopy) have been limited to the study of homotetrameric proteins. However, they do not represent the hPAH protein population of a compound heterozygous patient. The isolation of the heterotetramers will allow the in vitro characterization of these species in terms of the cofactor effect. However, one limitation of the prokaryotic expression system is that it is not amenable to test the effect of precise BH4 concentrations when the protein assembles in vivo. One alternative is to use the described system (with minor modifications) in an in vitro transcription–translation expression system. It will be important to understand how the mutations alter the observed 2hPAH1:2hPAH2 association of hPAH dimers and if they also exert a dominant negative impact on the function of the heteromeric species. As a large fraction of PKU mutations affects stability and/or folding efficiency, this system will also be a valuable tool to understand how an aggregation-prone mutant (e.g. G46S [30,60]) affects the other mutant partner within the heteromeric protein.

The characterization of the interactions between wild-type and mutant dimers and between different mutant dimers (compound heterozygous) is clinically relevant, as they will contribute to a better understanding of the metabolic phenotype in the HPA/PKU patients and BH4-responsiveness. It will also be essential in the development of new emerging therapies (e.g. pharmacological chaperones), as the hybrid proteins could respond differently from the parental enzymes.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbadis.2011.02.001.

References


