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Research Paper

Recombinant heteromeric phenylalanine monooxygenase and the oxygenation of carbon and sulfur substrates

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Abstract

Objectives The aim of this investigation was to provide in-vitro enzyme kinetic data to support the hypothesis that the in-vivo heterozygous dominant phenotype for phenylalanine monooxygenase (hPAH) was responsible for the S-oxidation polymorphism in the metabolism of S-carboxymethyl-L-cysteine reported in humans. Using a dual-vector expression strategy for the co-production of wild-type and mutant human hPAH subunits we report for the first time the kinetic parameters (K_m, V_{max}, CL_E) for the C-oxidation of L-phenylalanine and the S-oxidation of S-carboxymethyl-L-cysteine in homomeric wild-type, heteromeric mutant and homomeric mutant hPAH proteins *in vitro*.

Methods A PRO™ dual-vector bacterial expression system was used to produce the required hPAH proteins. Enzyme activity was determined by HPLC with fluorescence detection.

Key findings The heteromeric hPAH proteins (I65T, R68S, R158Q, I174T, R261Q, V338M, R408W and Y414C) all showed significantly decreased V_{max} and CL_E values when compared to the homomeric wild-type hPAH enzyme. For both substrates, all calculated K_m values were significantly higher than homomeric wild-type hPAH enzyme, with the exception of I65T, R68S and Y414C heteromeric hPAH proteins employing L-phenylalanine as substrate.

Conclusions The net outcome for the heteromeric mutant hPAH proteins was a decrease significantly more dramatic for S-carboxymethyl-L-cysteine S-oxidation (1.0–18.8% of homomeric wild-type hPAH activity) when compared to L-phenylalanine C-oxidation (25.9–52.9% of homomeric wild-type hPAH activity) as a substrate. Heteromeric hPAH enzyme may be related to the variation in S-carboxymethyl-L-cysteine S-oxidation capacity observed in humans.

Keywords enzyme activity; phenylalanine monooxygenase; S-carboxymethyl-L-cysteine; S-oxidation

Introduction

It has been customary in the field of drug metabolism to regard the enzymes involved in xenobiotic metabolism as a separate group, being distinct and different from those concerned with the endobiotic biochemistry of the body. Xenobiotic metabolism enzymes are usually less demanding in terms of substrate specificity, often foregoing a high turnover rate in exchange for the capacity to metabolise a wider range of substrates. However, several enzymes noted for their highly specific reactions and a defined role within endobiotic (intermediary) metabolism have been shown to be able to metabolise foreign compounds.[1] Phenylalanine monooxygenase (PAH) is one enzyme that recently has been added to this latter category, following its implication in the oxygenation of several thioether molecules, especially the mucoactive drugs S-methyl-L-cysteine (SMC) and S-carboxymethyl-L-cysteine (SCMC).[2–6] The biotransformation of these two drugs is interlinked, with SCMC being decarboxylated to SMC; both compounds may then be N-acetylated and all four may undergo S-oxygenation.[7,8]

Human phenylalanine monooxygenase (hPAH; E.C. 1.14.16.1; phenylalanine hydroxylase) is a homotetrameric non-heme iron-dependent protein that catalyses the C-oxidation

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of L-phenylalanine (Phe) to L-tyrosine (Tyr; 4-hydroxyphenylalanine) in the presence of (6*R*)-L-*erythro*-5,6,7,8-tetrahydrobiopterin (BH_4), utilising molecular oxygen as an additional substrate.[9] In humans, severe PAH dysfunction, arising from the combined expression of two mutant recessive alleles, results in the inborn error of metabolism known as phenylketonuria (PKU; OMIM 261600). Individuals who are heteroallelic for PAH mutations and classified as compound heterozygotes usually present with the milder, but nevertheless serious, condition of hyperphenylalaninaemia (HPA).[10] The establishment of genotype–phenotype correlations is now possible in the homoallelic state[11,12] and in the vast majority of functional hemizygous cases,[13] but there remain considerable inconsistencies between the observed metabolic phenotype and the predicted residual activity arising from a heteroallelic state.[14]

This is clinically relevant in the field of drug metabolism as it is now thought that SCMC acts as a free-radical scavenger, its thioether moiety combining with harmful reactive oxygen species to form stable S-oxide metabolites. Thus the administered parent sulfide is therapeutically active whereas the S-oxide metabolites are not.[15] Unfortunately, any additional metabolic enzyme-catalysed sulfur oxygenation, a recognised pathway of drug metabolism, thus decreases the amount of active sulfide available. Moreover, individuals within a population show differing abilities to undertake this S-oxidation reaction, resulting in some ('non-S-oxidisers', 'poor S-oxidisers') receiving the maximum therapeutic effect whilst others ('good S-oxidisers') benefit less. In a first attempt to correlate the molecular genetics of the PAH gene with the poor SCMC S-oxidation phenotype, it has been suggested that a subject who possesses one or two mutant alleles for the PAH gene would be a 'non-S-oxidiser' or 'poor S-oxidiser' and also that individuals carrying only one PAH mutant allele would show no clinical symptoms (of PKU or HPA) but would still be incapable of undertaking the S-oxidation reaction.[6]

Recent reports in the literature that utilised a yeast two-hybrid approach[16,17] demonstrated that wild-type (wt) hPAH subunits can interact with different PAH mutant subunits, resulting in functional heteromeric mutant PAH proteins. Using the dual vector expression strategy for the co-production of wt and mutant hPAH subunits as described previously,[18] we are able to report on the C-oxidation of Phe and the S-oxidation of SCMC in heteromeric mutant hPAH proteins in an attempt to gain insight into the interactions between hPAH wild-type and mutant monomers in an in-vitro system.

Materials and Methods

Chemicals

The chemicals in this investigation have been reported in detail in previous publications.[3,18,19] The synthesis of S-carboxymethyl-L-cysteine (*R*/*S*) S-oxides diasterioisomers was performed by hydrogen peroxide oxidation of SCMC. The isolation of the (*R*) and (*S*) SCMC S-oxides was carried out by fractional recrystallisation of the racemic mixture. The experimentally determined melting points for S-carboxymethyl-L-cysteine (*R*/*S*) S-oxides, S-carboxymethyl-L-cysteine (*R*) S-oxides and S-carboxymethyl-L-cysteine (*S*) S-oxides are in agreement with those reported in the literature.[20] The ^{1}H-NMR of the racemic mixture and the (*R*) and (*S*) SCMC S-oxide were converted to their methyl esters and their spectrum recorded in the presence of the chiral shift reagent Eu(hfc)$_3$.[21] The S-carboxymethyl-L-cysteine (*R*/*S*) S-oxides mixture appeared as colourless crystals; ^{1}H-NMR: δ (ppm) = 3.31–3.52 (m, 3H), 3.83–3.99 (m, 1H), 4.24–4.29 (m, 1H); ^{13}C-NMR: δ (ppm) = 18.47, 48.99 (CH), 49.70, 50.03 (CH_2-CH), 55.45, 56.01 (CH_2-COOH), 168.46, 169.46, 169.57 (C); ESI-MS: m/z = 196 ($(M \pm H)^+$). These results confirm the identity of the compound as SCMC (*R*/*S*) S-oxide.[21]

Expression and purification of recombinant PAH enzymes

The PRO™ bacterial expression system comprising the pPROLar and pPROTet expression vectors together with a recombinant-deficient host (DH5αPRO) to minimise recombination between the plasmids was used to express the recombinant human hPAH enzymes.[18] The mutations, R158Q, I174T, R408W, I65T, R68S, R261Q, V388M and Y414C, were introduced into the hPAH coding sequence by site-directed mutagenesis.[18] The growth of *Escherichia coli* transformed with the 6xHis purification tag vectors for the co-expression of wt, R158Q, I174T, R408W, I65T, R68S, R261Q, V388M and Y414C PAH subunits were also performed using a previously established method.[18] The tetrameric co-expressed wt, R158Q, I174T, R408W, I65T, R68S, R261Q, V388M and Y414C PAH fusion proteins were isolated using the IMAC Ni-chelating resin[18] and were subsequently cleaved by incubation with enterokinase. The resulting tetrameric proteins were isolated by size exclusion chromatography and concentrated using Centricon 30 microconcentrators. The cleavage of the 6xHis-PAH fusion proteins by enterokinase and separation of the resulting 6xHis, enterokinase and the PAH proteins by size exclusion chromatography were carried out using the method of Martinez *et al*.[22] The PAH proteins were stored in liquid nitrogen. Protein concentration was measured spectrophotometrically using ε_{280nm} (1 mg/ml) = 1.63.[22]

Tyr and SCMC S-oxides quantification by HPLC with fluorimetric detection

Tyr was measured by reverse-phase HPLC with fluorescence detection as previously described in detail.[19] The reversed-phase column used was a Spherisorb C_{18} 10 µm column (250 × 4.6 mm id). The mobile phase consisted of 100 mM ammonium acetate containing 300 µM tetrabutylammonium hydrogen sulphate (pH 4.6) and was delivered at a flow rate of 1.5 ml/min. The eluant was monitored at an excitation wavelength of 274 and an emission wavelength of 304 nm. The retention times were Tyr 3.5 min, 3-hydroxyphenylalanine (IS) 5.5 min and Phe 7.5 min.

The (*R*)- and (*S*)-S-oxides of SCMC were measured by reverse-phase HPLC with fluorescence detection following pre-column derivatisation with σ-phthalaldehyde/2-mercaptoethanol.[3] The reverse-phase column used was a Hypersil-OPA amino acid C_{18} 5 µm column (30 × 2.1 mm id).

The mobile phase consisted of solvent A (90% v/v 50 mM sodium acetate, pH 5.5 and 10% v/v methanol) and solvent B (100% v/v methanol). The column was eluted with the following gradient: isocratic with 100% A (0.0–5.0 min, flow rate 0.2 ml/min); linear gradient to 5% A and 95% B (5.0–6.5 min, flow rate 0.5 ml/min); isocratic 5% A and 95% B (6.5–11.5 min, flow rate 0.5 ml/min); linear gradient to 100% A (11.5–14.0 min, flow rate 0.2 ml/min); isocratic 100% A (14.0–16.0 min, flow rate 0.2 ml/min.). The eluant was monitored at an excitation wavelength of 344 and an emission wavelength of 433 nm. The retentions times were L-cysteic acid (IS) 7.5 min, SCMC (*S*)-S-oxide 18.5 min, SCMC (*R*)-S-oxide 23.7 min and SCMC 35 min.

PAH in-vitro enzyme assays

Enzyme activity was determined by a previously published method[19] with the following modifications. Each enzyme assay contained substrate (phenylalanine 0.0–5.0 mM or SCMC 0.8–50.0 mM), 50.0 mM potassium phosphate buffer (pH 6.8), catalase (7800 units/ml), tetrameric PAH (5.0 μg/ml), dithiothreitol (6.0 mM) and BH_4 (1.0–1000.0 μM) in a total volume of 1.0 ml. Reactions were initiated by the addition of BH_4 in dithiothreitol and terminated by the addition of trichloroacetic acid (TCA; 100 μl; 10% w/v). All incubations were undertaken at 37°C for 1.0 min. Supernatants were prepared for analysis by centrifugation (3000*g* for 10 min). Controls were assayed as above but using heat-inactivated (100°C) PAH.

Data analysis

Statistical data analyses were performed with Sigma Stat 3.5. Differences in enzyme kinetic parameters were examined using the ANOVA analysis of variance and Tukey's test and were regarded as statistically significant when *P* values were less than 0.05. Enzyme kinetic data was analysed by non-linear regression analysis and the curve-fitting programme Sigma Plot Enzyme Kinetic module v1.3. Experimental data was modelled against the Hill, Michaelis–Menten and Michaelis–Menten with non-competitive substrate inhibition equations.

Results

The purified 6xHis-PAH fusion proteins were cleaved with enterokinase since, although the 6xHis tag does not affect the C-oxidation of Phe,[18] it dramatically reduced the S-oxidation of SCMC when compared to the enterokinase-cleaved and purified homomeric and heteromeric PAH proteins (2–5% of the cleaved and purified forms of the hPAHs). Hence the data presented in this study is for the enterokinase-cleaved and purified homomeric hPAH and the purified heteromeric hPAH proteins.

To assess if the experimentally determined enzyme activities of the co-expressed systems were independent of the vectors employed, both the homomeric hPAH proteins and heteromeric hPAH proteins were always synthesised in two possible combinations. The measured enzyme activities (specific activity and/or V_{max}) for all of these respective homomeric and heteromeric pairs (PAH_{Lar}/PAH_{Tet} or PAH_{Tetr}/PAH_{Lar}) showed no significant differences ($P > 0.05$, Tukey's test) (Tables 1 and 2). (V_{max} is the maximal velocity of the enzyme reaction.)

Homomeric hPAHs with L-phenylalanine and S-carboxymethyl-L-cysteine as substrates

Obvious differences were observed between the homomeric wt hPAH proteins and the homomeric mutant hPAH proteins. Measured activities for tyrosine production from the allelic combinations that result in the classical PKU phenotype were significantly and dramatically decreased by 98% or more when compared to that of the wt (Table 1). Despite being quantifiable after incubation with homomeric wt hPAH, any SCMC (*R*/*S*) S-oxides that may have been produced by these particular homomeric mutant hPAH assemblies were below the level of quantification. The level of quantification for the in-vitro enzyme assay using SCMC as substrate was 1 nmole SCMC (*R*/*S*) S-oxides formed per minute per milligram and thus were assigned nominal values of ≤1(Table 1). The allelic combinations that give rise to a BH_4-responsive PKU phenotype also resulted in significantly decreased specific activities. With L-phenylalanine as substrate, the activities were reduced by between 65 and 75% and those measured in incubations employing SCMC as substrate were reduced by between 96 and 98%, when compared to activities seen with the homomeric wt hPAH (Table 1).

The predicted specific activities for the C-oxidation of Phe and the S-oxidation of SCMC were calculated as the sum of 50% of the mean experimentally determined specific activities of the homomeric wt hPAH plus 50% of the mean experimentally determined specific activities of the homomeric mutant hPAH. As expected, in all cases these simple estimates predicted that the heteromeric hPAH proteins would have decreased activities for both substrates, approximating half (Phe, 50.1–66.3%; SCMC, 50.5–51.3%) of that observed for the homomeric wt hPAH protein (Table 2).

Heteromeric hPAHs with L-phenylalanine and S-carboxymethyl-L-cysteine as substrates

Since specific activity values were determined using a single concentration of substrate (albeit chosen as it had resulted in maximal velocities in the enzyme assays) a direct comparison with V_{max} values is not strictly possible. Nevertheless, it can be seen that, in all instances, for both substrates, the experimentally determined V_{max} values were always lower than the predicted specific activities, this being particularly evident for SCMC S-oxidation (Table 2).

Results for the calculated V_{max} and CL_E (clearance through enzyme (V_{max}/K_m)) values, using Phe as substrate, in the heteromeric hPAH proteins all showed significantly decreased V_{max} values (R158Q, I174T, R408W, I65T, R68S, R261Q, V338M and Y414C, $P < 0.05$ ANOVA test) compared to the wt hPAH enzyme. A similar picture was seen with the CL_E values (R158Q, I174T, R408W, I65T, R68S, R261Q, V338M and Y414C, $P < 0.05$ ANOVA test). However, the calculated K_m values showed a more varied pattern of results with significantly increased values for R408W, I174T, R158Q, V338M and R261Q ($P < 0.05$, Tukey's test). (K_m is the Michaelis–Menten constant, the substrate concentration that results in half maximal velocity of enzyme activity.) The I65T,

Table 1 Specific activity of homomeric hPAH using L-phenylalanine and S-carboxymethyl-L-cysteine as substrates

Allele	Phenotype	L-Phe (nmoles Tyr formed per min/mg)	SCMC (nmoles SCMC (*R*/*S*) S-oxides formed per min/mg)
$PAHwt_{Lar}/PAHwt_{Tet}$	Wild-type	2175 ± 203	86 ± 9
$PAHwt_{Tetr}/PAHwt_{Lar}$	Wild-type	2362 ± 189	106 ± 12
$R158Q_{Lar}/R158Q_{Tet}$	Classical PKU	39 ± 4*	<1*
$R158Q_{Tet}/R158Q_{Lar}$	Classical PKU	44 ± 4*	<1*
$I174T_{Lar}/I174T_{Tet}$	Classical PKU	24 ± 2*	<1*
$I174T_{Tet}/I174T_{Lar}$	Classical PKU	27 ± 3*	<1*
$R408W_{Lar}/R408W_{Tet}$	Classical PKU	5 ± 1*	<1*
$R408W_{Tet}/R408W_{Lar}$	Classical PKU	4 ± 1*	<1*
$I65T_{Lar}/I65T_{Tet}$	BH_4-responsive	584 ± 55*	3 ± 0.4*
$I65T_{Tet}/I65T_{Lar}$	BH_4-responsive	615 ± 61*	2 ± 0.3*
$R68S_{Lar}/R68S_{Tet}$	BH_4-responsive	599 ± 54*	2 ± 0.2*
$R68S_{Tet}/R68S_{Lar}$	BH_4-responsive	604 ± 57*	2 ± 0.3*
$R261_{Lar}/R261Q_{Tet}$	BH_4-responsive	732 ± 69*	2 ± 0.2*
$R261_{Tet}/R261Q_{Lar}$	BH_4-responsive	699 ± 72*	3 ± 0.3*
$V388M_{Lar}/V388M_{Tet}$	BH_4-responsive	631 ± 61*	2 ± 0.2*
$V388M_{Tet}/V388M_{Lar}$	BH_4-responsive	586 ± 61*	2 ± 0.2*
$Y414C_{Lar}/Y414C_{Tet}$	BH_4-responsive	735 ± 69*	2 ± 0.3*
$Y414C_{Tet}/Y414C_{Lar}$	BH_4-responsive	752 ± 75*	2 ± 0.3*

Each enzyme assay contained substrate (Phe 1.0 or SCMC 40.0 mM), 50.0 mM potassium phosphate buffer (pH 6.8), catalase (7800 units/ml), tetrameric PAH (5.0 μg/ml), dithiothreitol (6.0 mM) and BH_4 (50.0 μM) in a total volume of 1.0 ml. Reactions were initiated by the addition of BH_4 in dithiothreitol and terminated by the addition of TCA (100 μl; 10% w/v). All incubations were undertaken at 37°C for 1.0 min. Supernatants were prepared for analysis by centrifugation (3000*g* for 10 min). Controls were assayed as above but using heat (100°C) inactivated PAH. Each assay was carried out in duplicate with blanks and the results reported are the mean ± SEM of $n = 4$ experiments. *All values were significantly different from their respective wild type control ($PAHwt_{Lar}/PAHwt_{Tet;}$ $PAHwt_{Tetr}/PAHwt_{Lar}$) ($P < 0.05$ ANOVA test).

R68S and Y414C heteromeric hPAH proteins showed no significant difference in the calculated K_m values for Phe with the wt hPAH protein ($P > 0.05$, Tukey's test).

The calculated kinetic parameters (K_m, V_{max} and CL_E) arising from investigation using SCMC as substrate showed that for all the heteromeric hPAH proteins (R158Q, I174T, R408W, I65T, R68S, R261Q, V338M and Y414C), the V_{max} and CL_E values were significantly reduced compared to the wt hPAH protein ($P < 0.05$ for both V_{max} and CL_E, ANOVA test). The calculated K_m values for the R158Q, I174T, R408W, R261Q, V338M and Y414C heteromeric hPAH proteins were all significantly higher than for the wt hPAH enzyme ($P < 0.05$, Tukey's test), whereas the calculated K_m values for SCMC in the I65T and R68S heteromeric hPAH proteins were not significantly different from the wt hPAH enzyme ($P > 0.05$, Tukey's test for both).

Discussion

The fully functional PAH enzyme requires a complex arrangement of four independent but intimately interacting monomers, and any factors that are able to interfere with this delicate assembly may alter function, usually decreasing the enzyme's efficiency to process substrate. In this context, and before any conclusions could be drawn, it was shown that the use of the two different expression vectors employed in this study to produce the homomeric hPAH and heteromeric hPAH proteins did not significantly alter the experimentally determined enzyme activities, hence having little influence on overall catalytic function.

Earlier reports in the literature have suggested that negative intrallelic complementation was seen both *in vitro*[18] and *in vivo*[23] with regard to the heterozygous dominant phenotype for hPAH, which resulted in a lower than predicted hPAH activity with respect to the C-oxidation of Phe. The V_{max} data for the heteromeric hPAH proteins expressing wt in combination with classical PKU alleles (R158Q, I174T, R408W) or BH_4-responsive PKU alleles (I65T, R68S, R261Q, V388M, Y414C) showed experimentally determined values that were dramatically reduced compared to the predicted values for both the C-oxidation of Phe and the S-oxidation of SCMC (Tables 1 and 2). Similarly, other workers, using Phe as a substrate with 6xHis hPAH fusion proteins for the $PAHwt_{Lar}/V388M_{Tet}$ and the $PAHwt_{Tet}/V388M_{Lar}$ heteromeric hPAH proteins, found only 38.5% of the wt hPAH activity, which was a 60.5% decrease on their predicted activity.[18] In the present study, experimental data for the wt/V338M proteins (not 6xHis-PAH fusion proteins) showed only 63.2% of the expected predicted value (a decrease of 36.8%) for the C-oxidation of Phe and a 73.5% decrease for the experimental versus predicted S-oxidation of SCMC. Overall, experimentally determined activities were always less than predicted values, ranging from 20.0% (wt/R68S) to 43.0% (wt/I174T) lower with Phe as substrate, and from 57.0% (wt/Y414C) to 96.5% (wt/I174T) lower with SCMC as substrate. In all cases, the S-oxidation of SCMC appeared to be most affected. Thus, evidence of negative interallelic complementation appears to be present, as suggested previously.[18]

When the kinetic parameters were examined in more detail, it was seen that the substrate-activated homomeric and

Table 2 Enzyme kinetic parameters of heteromeric hPAH proteins using L-phenylalanine and S-carboxymethyl-L-cysteine as substrates

Allelic combination	Phenylalanine				S-Carboxymethyl-L-cysteine			
	K_m (mM)	CL_E (ml/min/mg)	V_{max} (μmoles Tyr per min/mg)	(Sp.Act.†)	K_m (mM)	CL_E (μl/min/mg)	V_{max} (nmoles SCMC S-oxides per min/mg)	(Sp.Act.†)
$PAHwt_{Lar}/PAHwt_{Tet}$	0.1 ± 0.01	18 ± 2	3 ± 0.1	(2)	8 ± 3	14 ± 2	114 ± 10	(86)
$PAHwt_{Tet}/PAHwt_{Lar}$	0.1 ± 0.01	18 ± 2	2 ± 0.1	(2)	8 ± 3	13 ± 1	107 ± 10	(106)
$PAHwt_{Lar}/R158Q_{Tet}$	1 ± 0.1**	0.8 ± 0.1*	0.9 ± 0.1*	(1)	20 ± 2*	0.1 ± 0.02*	3 ± 1*	(43)
$PAHwt_{Tet}/R158Q_{Lar}$	1 ± 0.1**	0.8 ± 0.1*	0.7 ± 0.1*	(1)	19 ± 2*	0.1 ± 0.01*	2 ± 1*	(53)
$PAHwt_{Lar}/I174T_{Tet}$	2 ± 0.2**	0.3 ± 0.1*	0.7 ± 0.1*	(1)	19 ± 2*	0.1 ± 0.01*	2 ± 0.3*	(43)
$PAHwt_{Tet}/I174T_{Lar}$	2 ± 0.2**	0.3 ± 0.1*	0.6 ± 0.1*	(1)	20 ± 2*	0.1 ± 0.01*	2 ± 0.4*	(53)
$PAHwt_{Lar}/R408W_{Tet}$	3 ± 0.3**	0.2 ± 0.1*	0.7 ± 0.1*	(1)	21 ± 2*	0.1 ± 0.01*	2 ± 0.4*	(43)
$PAHwt_{Tet}/R408W_{Lar}$	3 ± 0.3**	0.2 ± 0.1*	0.7 ± 0.1*	(1)	20 ± 2.*	0.1 ± 0.01*	2 ± 1*	(53)
$PAHwt_{Lar}/I65T_{Tet}$	0.1 ± 0.02	8 ± 1*	1 ± 0.1*	(1)	15 ± 2	1 ± 0.2*	18 ± 4*	(44)
$PAHwt_{Tet}/I65T_{Lar}$	0.1 ± 0.02	8 ± 1*	1 ± 0.01*	(2)	17 ± 4	1 ± 0.1*	21 ± 2*	(54)
$PAHwt_{Lar}/R68S_{Tet}$	0.1 ± 0.01	10 ± 1*	1 ± 0.1*	(1)	18 ± 3	1 ± 0.1*	17 ± 4*	(44)
$PAHwt_{Tet}/R68S_{Lar}$	0.1 ± 0.02	10 ± 1*	1 ± 0.1*	(1)	18 ± 2	1 ± 0.1*	20 ± 2*	(54)
$PAHwt_{Lar}/R261Q_{Tet}$	1 ± 0.1**	2 ± 0.2*	0.9 ± 0.1*	(1)	23 ± 2.*	0.6 ± 0.03*	15 ± 3*	(44)
$PAHwt_{Tet}/R261Q_{Lar}$	1 ± 0.1**	2 ± 0.2*	0.9 ± 0.1*	(2)	21 ± 3*	0.8 ± 0.1*	17 ± 3*	(54)
$PAHwt_{Lar}/V388M_{Tet}$	0.9 ± 0.1**	1 ± 0.1*	0.9 ± 0.1*	(1)	25 ± 3*	1 ± 0.04*	12 ± 3*	(44)
$PAHwt_{Tet}/V388M_{Lar}$	1 ± 0.1**	1 ± 0.1*	0.9 ± 0.1*	(2)	27 ± 3*	1 ± 0.04*	14 ± 3*	(54)
$PAHwt_{Lar}/Y414C_{Tet}$	0.2 ± 0.01	7 ± 0.8*	1 ± 0.1*	(2)	24 ± 3*	0.9 ± 0.1*	22 ± 4*	(44)
$PAHwt_{Tet}/Y414C_{Lar}$	0.1 ± 0.01	8 ± 0.8*	1 ± 0.1*	(2)	22 ± 2*	0.9 ± 0.1*	20 ± 4*	(54)

Each enzyme assay contained substrate (phenylalanine 0.0–5.0 mM or S-carboxymethyl-L-cysteine 0.8–50.0 mM), 50.0 mM potassium phosphate buffer (pH 6.8), catalase (7800 units/ml), tetrameric PAH (5.0 μg/ml), dithiothreitol (6.0 mM) and BH_4 (50.0 μM) in a total volume of 1.0 ml. Reactions were initiated by the addition of BH_4 in dithiothreitol and terminated by the addition of TCA (100 μl; 10% w/v). All incubations were undertaken at 37°C for 1.0 min. Supernatants were prepared for analysis by centrifugation (3000*g* for 10 min). Control samples were assayed as above but using heat (100°C) inactivated PAH. Each assay was carried out in duplicate with blanks and the results reported are the mean ± SEM of $n = 4$ experiments.
†The values in parentheses indicate the predicted specific activities of heteromeric hPAH proteins. The concentration of substrates, L-phenylalanine and S-carboxymethyl-L-cysteine, employed to determine specific activities were those that had resulted in maximal velocity in the enzyme assay (Table 1). These predicted activities were calculated as (50% mean specific activity of homomeric wt PAH protein) plus (50% mean specific activity of homomeric mutant hPAH protein) (Table 1). *$P < 0.05$ ANOVA test: these values were statistically significantly different from their respective wild type control values ($PAHwt_{Lar}/PAHwt_{Tet;}$ $PAHwt_{Tetr}/PAHwt_{Lar}$) **$P < 0.05$ Tukey's test. **$P < 0.05$ Tukey's test: these values were statistically significantly different from their respective wild-type control values ($PAHwt_{Lar}/PAHwt_{Tet;}$ $PAHwt_{Tetr}/PAHwt_{Lar}$) **$P < 0.05$ Tukey's test. Sp.Act.: specific activity. Rate at one specific concentration of substrate (that which resulted in maximal velocity in previous enzyme assays).

heteromeric hPAH proteins showed the classical Michaelis–Menten with non-competitive substrate inhibition V against S profiles (data not shown). In all instances, the calculated V_{max} were significantly reduced for all the heteromeric hPAH proteins with regard to the C-oxidation of Phe. The calculated K_m values for the wt/R158Q, wt/I174T, wt/R408W, wt/R261Q and wt/V338M hPAH proteins were significantly increased. However, the K_m values for the wt/I65T, wt/R68S and wt/Y414C hPAH proteins were not significantly different from the wt hPAH protein. The calculated CL_E of Phe was found to be significantly reduced for all the heteromeric hPAH proteins. When the kinetic constants (K_m, V_{max} and CL_E) for SCMC as substrate for the wt hPAH and heteromeric hPAH proteins were determined, a similar pattern of results were obtained. Again, the classical Michaelis–Menten with non-competitive substrate inhibition V against S profiles were seen (data not shown).

A previous publication concerning the possible role of hPAH in the S-oxidation of SCMC suggested that the heterozygous dominant (hPAH+/–) and heterozygous recessive (hPAH–/–) phenotypes underpin the reported polymorphic variation in SCMC S-oxidation.[6] This may have medical significance in terms of both disease susceptibility and clinical therapeutics. The 'non-metaboliser' phenotype for the S-oxidation of SCMC has been implicated as a biomarker of disease susceptibility in Parkinson's disease and motor neurone disease,[24] although the mechanism(s) by which PAH may be involved in the aetiology of these two neurological conditions is unknown. From a therapeutic standpoint, the muco-regulatory drug SCMC appears to act as a free-radical scavenger; its thioether moiety combining with harmful reactive oxygen species to form stable S-oxide metabolites.[15] Varying degrees of metabolic S-oxidation therefore deactivate varying amounts of the drug, leading to erratic and unpredictable responses in patients. Intriguingly, at the far end of the observed spread of SCMC S-oxidation capacities, the incidence of 'non-S-oxidisers' (i.e. the extreme 'poor S-oxidisers') in a white European population was found to be 5 per 200 or 2.5%,[25] whilst (perhaps co-incidentally) the incidence of the two combined PAH (+/– and –/–) phenotypes was 4 per 200 or 2.0%.[6]

The present investigation develops this idea and supplies initial in-vitro experimental evidence that heteromeric hPAH enzymes could be the cause of the pharmacogenetic polymorphism reported for the S-oxidation of SCMC.[25] The combination of an hPAH subunit from a PKU-causing allele was found to significantly decrease the calculated V_{max} and CL_E values for both Phe and SCMC as substrates, but the effects on

SCMC S-oxidation were more profound, on occasion preventing or decreasing this metabolic reaction to levels below detection. Conclusive proof will, of course, only be obtained from an in-vivo correlation study of the S-oxidation of SCMC in a population of individuals with the PAH (+/–) phenotype. Nevertheless, the results reported in this investigation provide the first experimental evidence and permit an insight into the possible molecular genetic defects that may underlie the spread of SCMC S-oxidation capacities previously observed in humans. Additionally, this may serve to emphasise the emerging belief in the overlapping role of classical intermediary enzymes in the metabolism of alternative substrates and foreign compounds.

Conclusions

Although a previous investigation into the C-oxidation of Phe by heteromeric mutant hPAH proteins has been described, only specific activities for the conversion of Phe to Tyr were reported.[18] The current study provides details of a full enzyme kinetic investigation into the C-oxidation of Phe and the S-oxidation of SCMC and presents experimentally determined K_m, V_{max} and Cl_E data from the following heteromeric hPAH proteins: wt/R158Q, wt/I174T, wt/R408W, wt/I65T, wt/R68S, wt/R261Q, wt/V338M and wt/Y414C. These results are of substantial interest and need to be investigated further. Initially the use of mouse models for PKU and HPA[24] and then volunteer studies in humans that are heterozygous dominant with the PAH (+/–) phenotype, will determine if the present in-vitro findings are important and are transmitted into the complex in-vivo situation. Confirming the role of PAH in polymorphic SCMC S-oxidation will engender opportunity for a deeper understanding of this SCMC S-oxidation capacity as a reported biomarker of disease susceptibility in several neurological conditions.[26]

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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