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Research Paper

Human phenylalanine monooxygenase and thioether metabolism

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Abstract

Objectives The substrate specificity of wild-type human phenylalanine monooxygenase (wt-hPAH) has been investigated with respect to the mucoactive drug, S-carboxymethyl-L-cysteine and its thioether metabolites. The ability of wt-hPAH to metabolise other S-substituted cysteines was also examined.

Methods Direct assays of PAH activity were by HPLC with fluorescence detection; indirect assays involved following disappearance of the cofactor by UV spectroscopy.

Key findings wt-hPAH catalysed the S-oxygenation of S-carboxymethyl-L-cysteine, its decarboxylated metabolite, S-methyl-L-cysteine, and both their corresponding N-acetylated forms. However, thiodiglycolic acid was not a substrate. The enzyme profiles for both phenylalanine and S-carboxymethyl-L-cysteine showed allosteric kinetics at low substrate concentrations, with Hill constants of 2.0 and 1.9, respectively, for the substrate-activated wt-hPAH. At higher concentrations, both compounds followed Michaelis–Menten kinetics, with non-competitive substrate inhibition profiles. The thioether compounds, S-ethyl-L-cysteine, S-propyl-L-cysteine and S-butyl-L-cysteine were all found to be substrates for phenylalanine monooxygenase.

Conclusions Phenylalanine monooxygenase may play a wider role outside intermediary metabolism in the biotransformation of dietary-derived substituted cysteines and other exogenous thioether compounds.

Keywords metabolism; phenylalanine hydroxylase; phenylalanine monooxygenase; S-carboxymethyl-L-cysteine; thioethers

Introduction

The addition of an oxygen atom into a molecule is one of the major routes by which pharmaceutical agents are metabolised within mammalian organisms. Such biotransformation usually, but not always, reduces the pharmacological activity of a drug and increases its water solubility, thereby enhancing excretion. Although viewed as a necessary body 'defence' mechanism, this phenomenon may be grossly inconvenient to desired therapeutic intervention.

The cysteine amino acid derivatives, S-methyl-L-cysteine (SMC) and S-carboxymethyl-L-cysteine (SCMC), are both prescribed as mucoregulatory agents. In particular, the latter compound is widely used as a free radical scavenger in adjunctive treatment of chronic pulmonary disease and otitis media with effusions.[1,2] The underlying mechanism of protection provided by this drug requires the chemical oxidation of its thioether moiety by the pernicious reactive oxygen species, resulting in the formation of a stable S-oxide intermediate.[3] Hence, the administered parent sulfide is therapeutically active whereas the sulfoxide-containing species is not. Any additional metabolic enzyme-catalysed sulfur oxygenation, a known pathway of drug metabolism, thus decreases the required dose of active parent sulfide.

The enzyme systems responsible for drug oxidation are usually regarded as a separate and unrelated group, concerned only with 'foreign chemicals' or xenobiotics. The cytochrome P450 families CYP1, 2, 3 and 4, together with the flavin monooxygenases, are

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oft-cited examples. However, oxygenation of xenobiotics has been shown to be catalysed by other enzymes more commonly associated with intermediary metabolism. Included amongst these are aldehyde oxidase, dopamine-β-hydroxylase, lipoxygenase, monoamine oxidase, prostaglandin H-synthase and xanthine oxidase.[4] Appreciation of this situation is important because we may need to modify our viewpoint, as it appears that there is not an infinite capacity for oxidative drug metabolism in addition to the biochemistry of the body. The above intermediary enzymes already have a defined role to undertake without the extra burden of potential xenobiotic interference and overload. An awareness of which particular enzymes are at risk is vital to enhance our understanding of the biochemical interaction of pharmaceutical agents that underlie their therapeutic efficacy and biological safety.

Recently, another enzyme of intermediary metabolism, phenylalanine monooxygenase (PAH; phenylalanine hydroxylase; EC 1.14.16.1), has been implicated in the sulfur oxygenation of xenobiotic thioethers.[5–7] In this paper we confirm that such activity indeed exists and have demonstrated, for the first time, that pure recombinant human phenylalanine monooxygenase enzyme (wt-hPAH) can undertake the sulfur oxygenation of both SMC and SCMC.

Materials and Methods

Chemicals

Dithiothreitol, S-benzyl-L-cysteine, SCMC, S-ethyl-L-cysteine, SMC, phenylalanine, thiodiglycolic acid (TDA), tyrosine, NADH(H^+), bovine catalase and sheep dihydropteridine reductase were purchased from Sigma-Aldrich Co. Ltd (Poole, UK). Tetrahydrobiopterin (BH_4) was obtained from the Schricks Laboratories (Basel, Switzerland) and methanol (HPLC grade) from Rathburn Chemical Co. (Walkerburn, UK). PAH cDNA was acquired from LGC Promochem (Teddington, UK) and the pMAL-c2X expression vector was purchased from New England Biolabs (Massachusetts, USA). SCMC (*R/S*) S-oxides and SMC (*R/S*) S-oxides were synthesised and isolated by previously reported methods, as were N-acetyl-SMC, N-acetyl-SCMC and their S-oxides and TDA sulfoxide.[8,9] S-Pentyl-L-cysteine, S-hexyl-L-cysteine, S-heptyl-L-cysteine and S-octyl-L-cysteine were synthesised by various addition reactions involving halogeno-compounds, as previously detailed in the literature.[10–12]

Human recombinant phenylalanine monooxygenase

The subcloning and expression of wt-hPAH was based on a previously published method and data therein.[13] The wt-hPAH was amplified from PAH cDNA using the Expand High Fidelity^{PLUS} PCR system (Roche Diagnostics Ltd, Rotkreuz, Switzerland) using the following primers: CGGGGAGAATTCATGTCCACTGCGGTCCTGG and CTGTCCGTCGACTTACTTTATTTTCTGGAGGGC. The PCR product was digested with *EcoR*I and *Sal*I (restriction sites are underlined in the primer sequences above) and inserted into the pMAL-c2X expression vector. Expression of the wt-hPAH into *Escherichia coli* TB1 cells and the isolation of the maltose binding protein (MAP)–PAH fusion protein were based on the published method.[13] The cleavage of the MBP–PAH fusion protein by Factor Xa and separation of the MBP, Factor Xa and the wt-hPAH proteins by size exclusion chromatography were carried out as described previously.[13]

Enzyme assays

Direct PAH activity (measurement of product) was determined using a published method[14] with the following modifications. Each enzyme assay contained substrate (phenylalanine 0.0–5 mM; SCMC 0.8–50 mM; SMC 0.8–50 mM; N-acetyl-SCMC 0.8–40 mM; N-acetyl-SMC 0.8–40 mM or TDA 0.01–20 mM), potassium phosphate buffer (pH 6.8; 50 mM), catalase (7800 units/ml), tetrameric wt-hPAH (5 μg/ml), dithiothreitol (6 mM) and BH_4 (1–500 μM) in a total volume of 1.0 ml. Reactions were initiated by 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 10 min. Supernatants were prepared for analysis by centrifugation (3000*g* for 10 min). Control blanks were assayed as above but using heat-inactivated (100°C) wt-hPAH.

Indirect PAH activity (measurement of linked cofactor disappearance) was used for the metabolism of other thioether substrates. Each enzyme assay contained thioether substrate (1 mM), potassium phosphate buffer (pH 6.8; 50 mM), catalase (7800 units/ml), tetrameric wt-hPAH (5 μg/ml), NADH(H^+) (200 μM), dihydropteridine reductase (1.0 units/ml) and BH_4 (100 μM) in a total volume of 1 ml. The concentration of NADH(H^+) was monitored via spectrophotometry at 340 nm (using ε_{340nm} = 6220/per M cm) and the rate of disappearance calculated. All incubations were undertaken at 37°C for 10 min. Reactions were initiated by adding BH_4. Blanks were assayed as described above but using heat-inactivated wt-hPAH.

Both the direct and indirect assays were either substrate activated or lysophosphatidylcholine activated. This activation process involved pre-incubation of the fractions with either substrate or lysophosphatidylcholine (1 mM) for 5 min at 37°C before initiating the reaction.

HPLC determination of oxygenated products

Tyrosine (4-hydroxyphenylalanine) was measured by reverse-phase HPLC with fluorescence detection, as described previously.[14] The S-oxides of SCMC, SMC, N-acetyl-SCMC, N-acetyl-SMC and TDA were detected and quantified by reverse-phase HPLC with fluorescence detection following pre-column derivatisation with σ-phthalaldehyde/2-mercaptoethanol or 1-pyrenyldiazomethane, as reported previously.[5,6,15]

Enzyme kinetic analysis

Enzyme kinetic data were examined using WinNonLin 3.0 pharmacokinetic software (Pharsight Corporation, Mountain View, CA, USA) and Leonora kinetic analysis software (Oxford Sciences, Oxford, UK).

Statistical analysis

Each assay was carried out in duplicate, using heat-inactivated wt-hPAH as blanks. The results are the mean and SD of six assays. The data were analysed by Sigma Stat 3.5 (Statsys, San Jose, CA, USA) using two-way analysis of

variance (ANOVA) and Tukey's test. A significance level of $P < 0.05$ denoted significance in all cases.

Results

Enzyme preparation

The isolation and purification to homogeneity of the wt-hPAH resulted in the production of tetrameric (30.2 mg) and dimeric (4.3 mg) wt-hPAH proteins. Only the tetrameric wt-hPAH was used in the in-vitro enzyme studies in this work. For phenylalanine and SCMC as substrates, the velocity of reaction versus time (1–30 min) and wt-hPAH concentration (0.5–10 μg/ml) all displayed linearity (data not shown).

Substrate and lysophosphatidylcholine-activated wt-hPAH assays

The results of the kinetic investigations of substrate and lysophosphatidylcholine-activated wt-hPAH using phenylalanine as substrate are shown in Figure 1. Both showed classic Michaelis–Menten kinetics with non-competitive substrate inhibition profiles, as determined by both WinNonLin and Leonora software kinetic programs. However, the kinetics of the substrate-activated enzyme assay at lower phenylalanine concentrations (0.01–1 mM) displayed a sigmodal profile, with a calculated Hill constant (h) of 2.0. Table 1 shows the K_m and V_{max} values calculated by the Leonora program using the Michaelis–Menten equation with non-competitive substrate inhibition for phenylalanine in the substrate-activated and lysophosphatidylcholine-activated assays, together with the calculated clearances through the enzyme (CL_E). Pretreatment with lysophosphatidylcholine resulted in a 2.02-fold activation of wt-hPAH ($P < 0.01$, ANOVA; Figure 1). The K_m for phenylalanine was not significantly different between the substrate- and lysophosphatidylcholine-activated assays (Tukey's test). However, the V_{max} and CL_E values were significantly higher ($P < 0.01$, Tukey's test) for the lysophosphatidylcholine-activated assays than the substrate-activated assays (Table 1).

Similar to the above, substrate- and lysophosphatidylcholine-activated wt-hPAH using SCMC as substrate displayed classic Michaelis–Menten kinetics with non-competitive substrate inhibition profiles for both systems (Figure 2).

The kinetic profiles of the substrate-activated enzyme assays at lower SCMC concentrations (0.8–10 mM) were sigmodal,

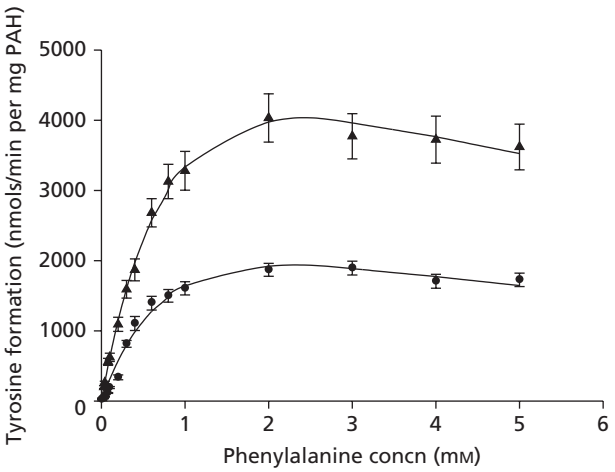

Figure 1 Michaelis–Menten kinetics with non-competitive substrate inhibition of the C-oxidation of phenylalanine by human phenylalanine hydroxylase (PAH) following activation by either the substrate (•) or lysophosphatidylcholine (▲). Data points represent means ± SD ($n = 6$ assays).

with a calculated Hill constant (h) of 1.9. Pre-treatment with lysophosphatidylcholine resulted in a 64-fold activation of wt-hPAH ($P < 0.001$, ANOVA, Figure 2). For comparison, the values for K_m and V_{max} calculated using the Michaelis–Menten equation with non-competitive substrate inhibition for SCMC in the substrate-activated and lysophosphatidylcholine-activated assays are given in Table 1, together with the calculated clearances through the enzyme. The K_m for SCMC was not significantly different between the substrate- and lysophosphatidylcholine-activated assays (Tukey's test). However, the V_{max} and CL_E values were significantly higher for the lysophosphatidylcholine-activated assays than the substrate-activated assays ($P < 0.001$, Tukey's test; Table 1).

S-Carboxymethyl-L-cysteine and metabolites

The kinetic parameters (K_m, V_{max} and CL_E) obtained for the substrate-activated assays using SCMC and its metabolites are given in Table 2. The decarboxylated metabolite, SMC, was cleared by wt-hPAH at 25% of the rate of the parent compound (SCMC) into its S-oxide metabolite. Values for the N-acetylated compounds (N-acetyl-SCMC; N-acetyl-SMC) were lower. TDA was found not to be a substrate.

Table 1 Kinetic data for phenylalanine (Phe) and S-carboxymethyl-L-cysteine (SCMC) in wild-type human phenylalanine hydroxylase (PAH) assays following activation by the substrate or 1 mM lysophosphatidylcholine (LPC)

Substrate	Activation	K_m	V_{max}	CL_E
Phe	substrate	1.1 ± 0.5	3896.0 ± 1081.0	3490.0 ± 510.0
Phe	LPC	1.2 ± 0.1	7875.0 ± 726.0*	6780.0 ± 700.0*
SCMC	substrate	8.3 ± 3.1	75.6 ± 20.5	9.0 ± 2.0
SCMC	LPC	8.1 ± 0.8	4803.0 ± 500.0**	580.0 ± 40.0**

K_m, Michaelis–Menten constant (in mM); V_{max}, maximum enzyme velocity (in nmols product formed/min per mg PAH); CL_E, clearance through enzyme (K_m/V_{max}, in μl/min per mg PAH).
Values are means ± SD ($n = 6$ assays).
*$P < 0.01$; **$P < 0.001$ substrate-activated vs LPC-activated assay for each substrate.

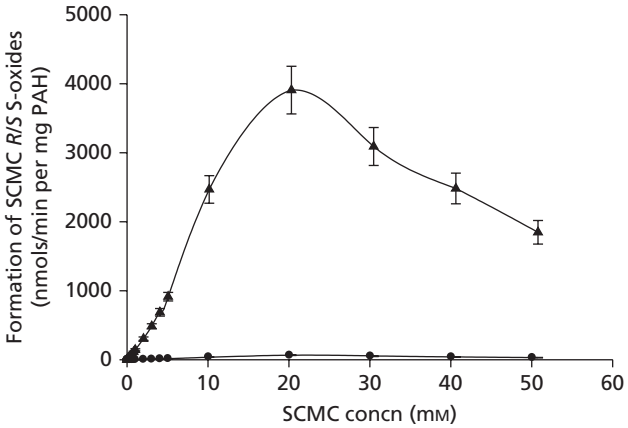

Figure 2 Michaelis–Menten kinetics with non-competitive substrate inhibition of the S-oxidation of S-carboxymethyl-L-cysteine by human phenylalanine hydroxylase (PAH) following activation with substrate (•) and lysophosphatidylcholine (▲). Data points represent means ± SD (n = 6 assays).

Table 3 Kinetic data for thioether substrates of wild-type human phenylalanine hydroxylase (PAH)

Substrate	Activity (nmols product formed/min per mg PAH)
L-Phenylalanine	1605.0 ± 158.7
S-Methyl-ergothionine	11.7 ± 1.3
L-Methionine	4.1 ± 0.6
S-Ethyl-L-cysteine	3.9 ± 0.8
S-Carboxymethyl-L-cysteine	2.4 ± 0.3
S-Propyl-L-cysteine	1.7 ± 0.4
S-Methyl-L-cysteine	1.5 ± 0.2
S-Butyl-L-cysteine	0.2 ± 0.1

S-Pentyl-L-cysteine, S-hexyl-L-cysteine, S-heptyl-L-cysteine, S-octyl-L-cysteine, S-cyclopropyl-L-cysteine, S-cyclohexyl-L-cysteine, S-cycloheptyl-L-cysteine, S-benzyl-L-cysteine, S-phenylethyl-L-cysteine, S-(2-chloro-6-nitrophenyl)-L-cysteine, S-(2-nitro-5-chlorophenyl)-L-cysteine, S-(2-nitro-4,5-dichlorophenyl)-L-cysteine, S-(4-bromophenyl)-L-cysteine and S-(4-chlorophenyl)-L-cysteine gave no measurable activity (< 0.01 nmols product formed/min per mg PAH). Values are means ± SD (n = 6 assays).

Other thioether substrates

Following incubation of the thioether substrates at 1 mM (Table 3), the indirect assay suggested that S-ethyl-L-cysteine, S-propyl-L-cysteine and S-butyl-L-cysteine were also substrates (3.9 ± 0.8, 1.7 ± 0.4 and 0.2 ± 0.1 nmols product formed/min per mg wt-hPAH, respectively), whereas S-pentyl-L-cysteine, S-hexyl-L-cysteine, S-heptyl-L-cysteine, S-octyl-L-cysteine and S-benzyl-L-cysteine were not (< 0.01 nmols product formed).

Discussion

The enzyme activity profiles of phenylalanine- and lysophosphatidylcholine-activated wt-hPAH both showed sigmodal kinetics at low substrate concentrations (0.01–1 mM phenylalanine) and Michaelis–Menten kinetics with non-competitive substrate inhibition at higher substrate concentrations (1–5 mM phenylalanine, Figure 1). This is in agreement with previously reported data.[16] The calculated Hill constant (h = 2.0) indicated that allosteric regulation of the enzyme was seen at these lower substrate levels.[17,18] Lysophosphatidylcholine activation of wt-hPAH resulted in the loss of allosteric kinetics (Figure 1) and a 2.02-fold increase in wt-hPAH activity. This agrees with previously published results in which a 2.0-fold increase was obtained under these conditions.[17] Thus, the recombinant wt-hPAH protein was found to express enzyme kinetic profiles (K_m and V_{max} values) and showed post-translational activation characteristics similar to previously published data (Table 1).[16–18]

When SCMC was used as a substrate, a similar pattern of results were observed (Table 1 and Figure 2). At low SCMC concentrations (0.8–10 mM) the enzyme kinetic profiles was sigmodal, indicating allosteric regulation.[17,18] However, at higher SCMC concentrations (10–50 mM) Michaelis–Menten kinetics with non-competitive substrate inhibition were observed. These findings are in agreement with data reported in previous studies.[5–7] Post-translational activation of wt-hPAH with lysophosphatidylcholine was greater when SCMC was used as a substrate rather than phenylalanine (Table 1). From the results it can be deduced that SCMC is a relatively poor substrate for wt-hPAH; clearance through the enzyme is an order of magnitude lower than that for the endogenous substrate, phenylalanine (0.3% in the substrate-activated assay; 8.5% in the lysophosphatidylcholine-activated assay, P < 0.001, Tukey's test, Table 1). Nevertheless, SCMC is metabolised by the enzyme. Moreover, previous studies[5–7] using hepatic cytosol indicated that only one enzyme is involved in the S-oxygenation of SCMC (as only one K_m and one V_{max} were found). Interestingly, lysophosphatidylcholine-activation of the wt-hPAH resulted in a 64-fold increase in SCMC clearance through the enzyme, compared with a 1.9-fold increase for phenylalanine. Could this or some other similar process be a physiological means by which the enzyme deals with xenobiotic substrates?

The results of the investigation into the S-oxidation of the other thioether metabolites of SCMC are reported in Table 2. With the exception of TDA, all the thioether metabolites (SMC, N-acetyl-SCMC and N-acetyl-SMC) were found to be substrates of wt-hPAH and all produced S-oxide metabolites in the in-vitro enzyme assay. The rank order for rate of clearance was SCMC > SMC > N-acetyl-SCMC > N-acetyl-SMC. These

Table 2 Kinetic data for thioether metabolites of S-carboxymethyl-L-cysteine (SCMC) in wild-type human phenylalanine hydroxylase (PAH) assays following activation by the substrate

Substrate	K_m	V_{max}	CL_E
SCMC	8.3 ± 3.1	75.6 ± 20.5	9.0 ± 2.0
SMC	20.4 ± 4.1	47.1 ± 9.4	2.3 ± 0.6
N-acetyl-SCMC	29.8 ± 6.0	18.9 ± 3.8	0.6 ± 0.1
N-acetyl-SMC	32.1 ± 6.3	11.3 ± 2.2	0.4 ± 0.1
Thiodiglycolic acid	Not a substrate for PAH		

K_m, Michaelis–Menten constant (in mM); V_{max}, maximum enzyme velocity (in nmols product formed/min per mg PAH); CL_E, clearance through enzyme (K_m/V_{max}, in μl/min per mg PAH).
Values are means ± SD (n = 6 assays).

results are similar to those observed *in vivo* in man with respect to urinary recovery of S-oxygenated metabolites following SCMC dosing (SCMC S-oxides > SMC S-oxides > N-acetyl-SCMC S-oxides > N-acetyl-SMC S-oxides).[19,20]

Since wt-hPAH undertakes the S-oxidation of a number of substituted cysteine-like compounds, the substrate specificity of the enzyme was further investigated. The appearance of S-oxygenated metabolites correlated with the disappearance of cofactor for four substrates examined by both wt-hPAH assay systems (coupling efficiency was 98–99%). As S-oxide derivatives were unavailable, cofactor disappearance was used as an indication of substrate metabolism. It was appreciated that this was not proven to involve S-oxygenation but it was deemed highly probable. From these investigations (Table 3), the thioethers S-ethyl-L-cysteine, S-propyl-L-cysteine and S-butyl-L-cysteine were also found to be substrates for wt-hPAH.

Conclusions

A previous report had indicated that the cell cytosol, and not the microsomal fraction, was the site of SCMC S-oxygenation in mammalian species, but no indication of the enzyme responsible was forthcoming.[21] The present investigation, together with recent studies cited in the literature,[5–7] and coupled with the lack of evidence to the contrary, establishes that phenylalanine monooxygenase is the cytosolic enzyme responsible for this reaction. Kinetic data (one K_m and one V_{max}) also suggests that there is only one enzyme, hence this enzyme, undertaking this reaction. In addition, phenylalanine monooxygenase is not restricted to this one substrate but is capable of sulfur-oxygenating other thioether compounds. The number of known dietary thioether compounds is large, and many are toxic to cells in high concentrations and thus have not been advocated as possible therapeutic agents.[22,23] The overall intake of such compounds within the diet, as health food supplements and as established or future therapeutic agents, will lead to an extra substrate load and subsequent burden upon this enzyme. The slowly acknowledged acceptance that enzymes that are more usually associated with intermediary metabolism may also play a role in drug or xenobiotic metabolism is of concern for the future, especially as new chemical entities, designed via information gleaned from the human genome project and systems biology, will, in all probability, closely resemble endobiotic compounds, and endobiotic metabolism enzyme systems will come to the fore, causing more potential problems for the pharmaceutical industry.

Conflict of interest

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

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