PHENYLALANINE 4-MONOOXYGENASE AND THE *S*-OXIDATION OF *S*-CARBOXYMETHYL-L-CYSTEINE

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SUMMARY

The identity of the enzyme responsible for the *S*-oxidation of the mucolytic *S*-substituted L-cysteine drug, *S*-carboxymethyl-L-cysteine (SCMC), has been actively investigated for the last 10 years. A genetic polymorphism exists in the oxidation of the thioether moiety that has been identified as a disease susceptibility factor in a number of degenerative diseases. This polymorphism has also been implicated in the wide variation in clinical response to SCMC therapy in man. To date little is known about the molecular enzymology of this reaction but a previous investigation revealed that rat activated phenylalanine 4-monooxygenase (PAH) could *S*-oxidise both Met- and *S*-methyl-L-cysteine (SMC) to their *S*-oxide metabolites. We have investigated the hypothesis that SCMC was also a substrate for activated PAH in the cytosolic faction of the Wistar rat. 1. Substrate and inhibitor investigation revealed that SCMC was a substrate for activated PAH activity *in vitro*. 2. The large aromatic amino acid hydroxylase monoclonal

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antibody and the Fe^{3+} chelator, deferoxamine, completely inhibited both Phe and SCMC oxidation to their respective metabolites. 3. Analysis of the Dixon plots revealed that both Phe and SCMC competitively inhibited each other's oxidation. 4. Correlation studies showed that the rate of production of Tyr was positively correlated to the production of both SCMC and SMC *S*-oxides in 20 female Wistar rat hepatic cytosolic fractions. These results strongly support the hypothesis that PAH is the enzyme responsible for SCMC *S*-oxidation in the rat.

KEY WORDS

phenylalanine 4-monooxygenase, *S*-carboxymethyl-L-cysteine, *S*-oxidation, rat hepatic cytosolic fractions, pharmacogenetics

INTRODUCTION

The biotransformation of the mucolytic drug, *S*-carboxymethyl-L-cysteine (SCMC), has been explored since the early 1960s. Initial studies were undertaken in a number of diverse mammalian species, but since the mid-1970s the majority of this work has focussed on man /1/. The main conclusion that can be drawn from these *in vivo* investigations is that, although SCMC undergoes several metabolic alterations including decarboxylation to *S*-methyl-L-cysteine (SMC), the major urinary metabolites are *S*-oxidation products /2/. It has also been shown that the ability to produce these *S*-oxides, and presumably the enzyme protein(s) involved in this activity, are polymorphic in nature and under genetic control /3/. In addition, this *S*-oxidation reaction shows a diurnal variation in activity, suggesting that hormonal influences may be another important controlling factor /4/.

There is a paucity of data concerning *in vitro* investigation of the *S*-oxidation of SCMC, but the enzyme activity has been localised to the crude hepatic cytosolic fraction of rat, guinea-pig and man (also hamster, rabbit and sheep) /5/. The addition of a microsomal fraction (contains the mixed function oxidase systems), SKF525A (cytochrome P450 inhibitor), iproniazid (monoamine oxidase inhibitor), hydroxylamine (inhibitor of pyridoxal phosphate dependent enzymes), Cu^{+}, Mg^{2+} and Fe^{3+} had no effect on this reaction. Heat treatment of the

hepatic cytosolic fraction, the addition of EDTA (divalent cation chelator), superoxide dismutase (an enzyme that converts O_2^{2-} to H_2O_2) and $HgNO_3$ (a compound that interacts with sulphydryl dependent enzymes) all abolished the SCMC *S*-oxidation activity. The divalent cation Cu^{2+} resulted in 1.5-fold activation in the guinea-pig, whilst Fe^{2+} caused a 3.3 fold activation of SCMC *S*-oxidation /5/. The authors of this paper concluded that the enzyme 'SCMC *S*-oxygenase' was located in the cytosolic fraction of the liver, required molecular oxygen and a divalent metal cation for activity, that the oxidising species may be superoxide, or derived from superoxide, and that the protein was sulphydryl dependent /5/.

The present investigation now focuses on a number of important observations. The first was a report that activated phenylalanine 4-monooxygenase (PAH), an enzyme that converts phenylalanine (Phe) into tyrosine (Tyr), was responsible for the conversion of L-methionine (Met) to L-methionine *S*-oxide and SMC to SMC *S*-oxide /6/. PAH is an allosteric cytosolic protein found in the cytosol of the liver and kidney and is a tetramer composed of four identical monomers; the protein itself is a 'dimer of dimers' with each monomer in the dimer lying in a 'head-to-tail' arrangement /7/. Each monomer has an N-terminal regulatory domain, a catalytic domain and a C-terminal polymerisation domain for dimer formation /8/. This N-regulatory region is not only in contact with the catalytic domain of its own monomer but is also in contact with the catalytic domain of the adjacent monomer that makes up the dimer /9,10/. No monomer has catalytic activity but both dimers and tetramers possess catalytic activity. The enzyme requires molecular oxygen and Fe^{2+} for the oxidation reaction, being inactivated by H_2O_2, and utilises tetrahydro-biopterin (BH_4) as a cofactor. Since PAH is an allosteric enzyme it can be activated by post-translational modification via a number of different mechanisms. These include the binding of Phe to the catalytic domain, modification of the Cys^{237} residue in the catalytic domain, phosphorylation of Ser^{16} residue in the N-terminal regulatory domain, binding of lysophosphatidylcholine or limited proteolysis /7/.

These observations suggest that PAH may be involved in the *S*-oxidation of SCMC. A more detailed investigation of PAH as the protein responsible for 'SCMC *S*-oxygenase' activity was therefore undertaken to evaluate this possibility and is reported herein.

MATERIALS AND METHODS

Materials

Deferoxamine, lysophospatidylcholine, L-methionine, L-phenylalanine, *S*-methyl-L-cysteine, *S*-carboxymethyl-L-cysteine, tetrahydrobiopterin and L-tyrosine were purchased from Sigma-Aldrich Co. Ltd. (Dorset, UK). The large aromatic amino acid hydroxylase monoclonal antibody was obtained from Biodesign International (Saco, ME, USA). High-pressure liquid chromatography grade methanol was from Rathburn Chemical Company (Walkerburn, Scotland). The two *S*-carboxymethyl-L-cysteine *S*-oxides (*R*- and *S*-), and those of its decarboxylated metabolite, *S*-methyl-L-cysteine (*R*- and *S*- *S*-oxides), were synthesised and isolated by the methods described in detail by Meese and coworkers /11,12/.

Preparation of crude rat cytosolic fractions

Female Wistar rats (200-250 g, BSU King's College London) were reduced by cervical dislocation and the liver was removed immediately and covered with 100 mM NaH_2PO_4/Na_2HPO_4 (pH 7.4) at 4°C. A 20% (w/v) whole liver homogenate was prepared using 100 mM NaH_2PO_4/Na_2HPO_4 (pH 7.4) at 4°C and was separated into its subcellular fractions by high-speed centrifugation. The cell debris, nuclei and mitochondria were removed by centrifugation at 12,500 *g* for 15 min at 4°C. The pellet was discarded, the supernatant was made up to a $CaCl_2$ concentration of 8 mM and then stirred at 4°C for 5 min before centrifugation at 27,000 *g* for 15 min at 4°C to remove microsomes. The remaining soluble fraction was decanted and assayed for protein content by the method of Bradford /13/ and for marker enzyme activity, lactate dehydrogenase (cytosol) /14/, isocitrate dehydrogenase NAD^+-specific form (mitochondria) /15/ and glucose-6-phosphatase (endoplasmic reticulum/microsomes) /16/, to assess potential organelle contamination.

Activated phenylalanine 4-monooxygenase enzyme assay

Rat hepatic cytosolic fractions were assayed for PAH activity, employing L-phenylalanine, SCMC and SMC as substrates, utilising methods previously reported in detail /17/. Briefly, this entailed the

following. To 500 µl of 100 mM potassium phosphate buffer (pH 6.8) containing 1.0 mM lysophosphatidylcholine (activator) and 2,500 units/ml bovine catalase the following solutions were added in the following sequence: 100 µl of substrate (Phe, SCMC, SMC, see results section for concentrations) in 100 mM potassium phosphate buffer (pH 6.8); 200 µl of cytosol fraction which was then incubated at 37°C for 3 min; 100 µl of 6 mM dithiotreitol (DTT) solution in H_2O; 100 µl of BH_4 solution in 5 mM HCl (see results section for concentrations). The enzyme assay was stirred and the mixture incubated for 30 min at 37°C. The reaction was terminated by the addition of 100 µl of 20% (w/v) trichloroacetic acid (TCA) solution. Samples were centrifuged at 5,000 rpm for 5 min and the supernatant removed and neutralised with 10 µl of 6 M NaOH. All samples were frozen at -20°C until analysed for product formation /17,18/.

Effect of various compounds on activated phenylalanine 4-monoxygenase activity

The above assay system was employed to investigate the effects of varying the BH_4 (cofactor) concentration and also co-incubation with potential enzyme inhibitors on the production of L-tyrosine and SCMC S-oxides. The inhibitors included deferoxamine (Fe^{3+} chelator), L-methionine, L-phenylalanine, *S*-methyl-L-cysteine, *S*-carboxymethyl-L-cysteine and large aromatic amino acid hydroxylase monoclonal antibody (inhibits phenylalanine hydroxylase [PAH], tyrosine hydroxylase [TYH], tryptophan hydroxylase [TPH]).

Non-enzymatic production of SCMC *S*-oxides

The enzyme incubation system employed above for the PAH activity assay was utilised to investigate potential non-enzymatic oxidation of SCMC. Rat hepatic cytosol was excluded from the incubation mixtures but oxidising agents, H_2O_2 (0.0, 0.1, 1.0 or 10 mM) and ascorbic acid (0.0, 0.1, 1.0, 10 mM), from the Fenton/ Udenfriend systems, were added /19-21/. Any potential reaction was initiated by the addition of $FeSO_4$ (0.0, 0.1, 1.0, 10 mM), with aliquots being removed at known time intervals thereafter (0, 5, 10, 20, 30 min) and added to EDTA (100 mM) to stop any reaction via cation chelation. The SCMC substrate concentration used in these experiments was 66.6 mM.

Reaction product quantification

The detection and quantification of SCMC and SMC *S*-oxides were carried out by high pressure liquid chromatography (hplc) with fluorescence detection as previously described /18/. *o*-Phthaldehyde/ 2-mercaptoethanol derivatizing reagent was prepared as follows: 22.0 ml of 100 mM sodium borate buffer (pH 9.5) was added to 18.0 ml of *o*-phthaldehyde/2-mercaptoethanol (2ME) solution (144 mg of *o*-phthaldehyde in 18.0 ml of methanol containing 90 µl of 2ME). This is prepared daily and protected from the light in a brown glass reagent bottle. 90 µl of the derivatization mixture was then added to 20 µl of standard/sample by the TSP AS3500 pre-column derivatizing auto-sampler. Both preparation and sample syringe were washed out before each injection with water:methanol mixture (50:50 v/v). This procedure took a total of 2 min. 5 µl of the standard/sample-derivatization solution was injected onto a Hypersil Amino Acid 5µ C_{18} (30 x 2.1 mm i.d.) analytical column protected by a Hypersil Amino Acid guard column. SCMC, L-cysteic acid and SCMC *R* and *S* *S*-oxides were separated by a gradient elution. Solvent A was 90% v/v (50 mM sodium acetate, pH 5.5) and methanol (10% v/v) and solvent B was 100% (v/v) methanol. The analytical column was eluted isocratically with 100% A (0-5 min) at a flow rate of 0.2 $ml \cdot min^{-1}$ followed by a linear gradient to 5% A:95% B (5.0-6.5 min) at a flow rate of 0.5 $ml \cdot min^{-1}$. This was held isocratically at 5% A:95% B (6.5-11.5 min) at a flow rate of 0.5 $ml \cdot min^{-1}$ before a second linear gradient to 100% A was initiated (14.0-16.0 min) at a flow rate of 0.2 $ml \cdot min^{-1}$. The column temperature was 25°C and the excitation and emission wavelengths were 344 and 443 nm.

The PAH activity in rat liver cytosol was investigated using a modification of the method of Kaufman /17/. 0.5 ml of the acid supernatant was added to 0.25 ml of a 'nitrosonaphthol solution' (0.1% w/v 1-nitroso-2-naphthol in 95% v/v ethanol). To this mixture was added 0.25 ml of a nitric acid-sodium nitrite solution (1 ml 2.5% w/v sodium nitrite plus 10 ml of concentrated nitric acid diluted to 50 ml with water). The mixture was heated at 55°C for 30 min. To this mixture was added 2.5 ml of ethylene dichloride and the resulting solution was well shaken. 1.0 ml of clear supernatant was then removed and the absorbance determined at 450 nm.

Data analysis

Statistical data analyses were performed with SPSS 10.0 /22/. Enzyme kinetic data was analysed by the Leonora enzyme kinetic programme 1.0 /23/ using the Michaelis-Menten equation, $V = V \cdot [S]/K_m + [S]$, where V = velocity, S = substrate, K_m = Michaelis-Menten constant.

RESULTS

The cytosolic preparations employed for these studies displayed the expected lactate dehydrogenase activity but no measurable isocitrate dehydrogenase or glucose 6-phosphatase activities and thus were deemed to be free of mitochondrial and microsomal (endoplasmic reticulum) contamination. Investigation of potential non-enzymatic oxidation showed that measurable amounts of SCMC *S*-oxides were only detectable at the highest concentrations (10 mM) of additives, with Fe^{2+} producing a maximum of 5 μM (0.008% conversion), H_2O_2 producing 3 μM (0.005% conversion) and ascorbate giving 8 μM (0.012% conversion) after 30 min incubation. At levels of Fe^{2+} normally present within the enzyme incubations used no non-enzymatic production would be detectable. In addition, control (background) incubations were also employed for all experiments.

The activated PAH assays were found to show linear production of metabolites for all substrates investigated over the range of 0-60 min (results not shown) and hepatic cytosolic protein concentrations of 0-2 mg/ml (results not shown). The V against S plots for the activated PAH assay using varying concentrations of substrates with a constant cofactor concentration (20 μM BH_4 as the physiological concentration in rat liver is 10 μM) can be seen in Figure 1. These results showed that saturation kinetics for production of Tyr from Phe in the PAH assay was reached at a substrate concentration of 600 μM. The two sulphide substrates do not reach saturation kinetics until a concentration of 20 mM for SCMC and 40 mM for SMC. The Leonora calculated apparent K_m and V_{max} values for the data in Figure 1 can be seen in Table 1. The K_m values ranged from 260.0 ± 80.0 μM for Phe ω 14.73 ± 8.91 and 43.25 ± 7.76 mM for SCMC and SMC, respectively. Similar differences were also seen in the Leonora calculated apparent V_{max} results with values ranging from 13.72 ± 4.74

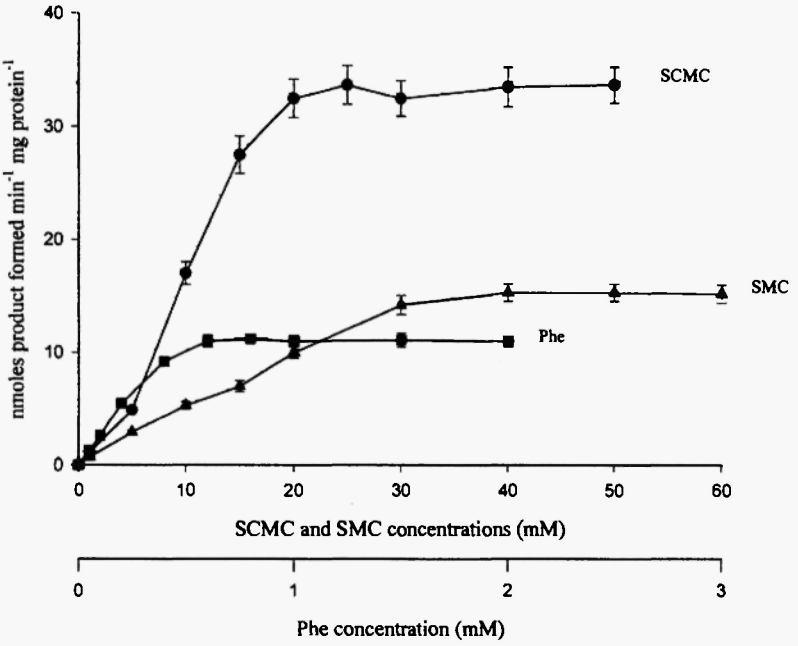

Fig. 1: The V against S plots of varying Phe, SMC and SCMC substrate concentrations with BH_4 (cofactor) concentration set at 20 μM for activated PAH activity in female Wistar rat cytosolic fractions *in vitro*. Each value is the mean ± SD of 6 experiments.

nmoles product formed min^{-1} mg cytosolic $protein^{-1}$ for Phe to 29.73 ± 3.79 and 48.11 ± 10.69 for SMC and SCMC. In all subsequent assays the substrate concentrations were set at 1 mM for Phe, 40 mM for SCMC and 50 mM for SMC.

The V against S plots for the activated PAH assays using varying BH_4 concentrations and constant substrate concentrations can be seen in Figure 2. All three substrates showed saturation kinetics at a BH_4 concentration of 80 μM. However, Phe reached saturation kinetics at a BH_4 concentration of 40 μM while SCMC and SMC reached saturation kinetics at a BH_4 concentrations of 60 and 80 μM, respectively. The Leonora calculated apparent K_m and V_{max} values for the data in Figure 2 can be seen in Table 1. The K_m values for BH_4 ranged from 9.36 ± 0.60 μM for Phe to 48.96 ± 3.22 and 114.13 ± 16.89 μM

TABLE 1

Leonora 1.0 /23/ calculated K_m and V_{max} values for activated phenylalanine 4-monooxygenase (PAH) activity using **A:** varying Phe, SMC and SCMC substrate concentrations with BH_4 (cofactor) concentration set at 20 μM, and **B:** varying BH_4 concentrations with 1 mM Phe, 50 mM SMC and 40 mM SCMC as substrates

A. Varying substrate concentrations, [BH_4] = 20 μM

Substrate	n	K_m (mM)	V_{max}*
Phe	6	0.26 ± 0.08	13.72 ± 4.74
SMC	6	43.25 ± 7.76	29.73 ± 3.79
SCMC	6	14.73 ± 8.91	48.11 ± 10.69

B. Varying cofactor concentration, [Phe] = 1 mM, [SMC] = 50 mM, [SCMC] = 40 mM

Substrate	n	K_m (μM)	V_{max}
Phe	6	9.36 ± 0.60	637.2 ± 13.3
SMC	6	114.13 ± 16.89	1,438.0 ± 153.0
SCMC	6	48.96 ± 3.22	962.6 ± 40.4

Each value is the mean ± SD of 6 experiments.
Each experiment was undertaken in duplicate with associated controls.
n = number of experiments.
* V_{max} as nmoles product formed min^{-1} mg cytosolic $protein^{-1}$
Phe = phenylalanine; SMC = *S*-methyl-L-cysteine; SCMC = *S*-carboxymethyl-L-cysteine.
PAH was activated by pre-incubation with 1.0 mM lysophosphatidylcholine for 3.0 min at 37°C.

for SCMC and SMC and apparent V_{max} values ranged from 637.2 ± 13.3 nmoles product formed min^{-1} mg cytosolic $protein^{-1}$ for Phe to 962.6 ± 40.4 and 1,438.0 ± 153.0 for SCMC and SMC. In all subsequent assays the BH_4 concentrations were set at 80 μM.

The results of the inhibitor studies on activated PAH in relation to Phe and SCMC metabolism can be seen in Table 2. These results

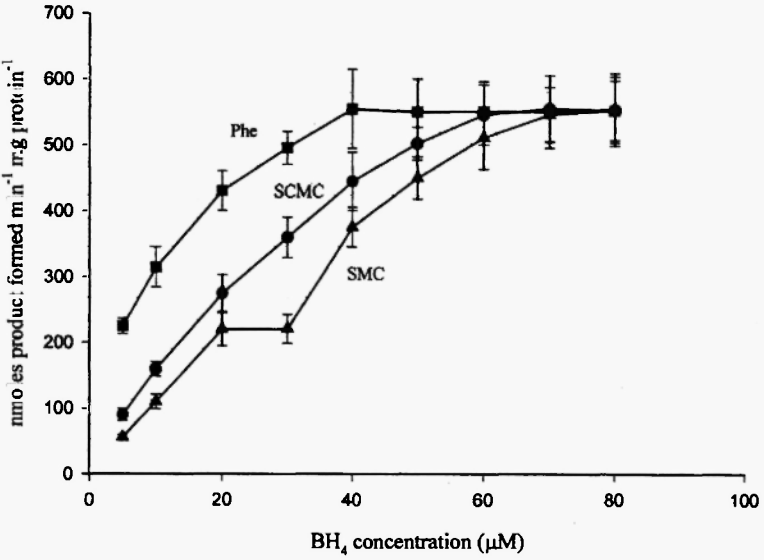

Fig. 2: The V against S plots of varying BH_4 (cofactor) concentrations with 1 mM Phe, 50 mM SMC and 40 mM SCMC as substrates for activated PAH activity in female Wistar rat cytosolic fractions *in vitro*. Each value is the mean ± SD of 6 experiments.

indicate that an activated PAH assay using either Phe (1 mM) or SCMC (40.0 mM) as substrate was inhibited by methionine (Met) and SMC and was almost completely prevented by the inclusion of deferoxamine (Fe^{3+} chelator) or the large aromatic amino acid hydroxylase monoclonal antibody (inhibits PAH, TYH and TPH). The results obtained were very similar for both substrates. The Dixon plots for both Phe and SCMC as inhibitors of activated PAH can be seen in Figures 3 and 4. The Leonora calculated K_i for SCMC as an inhibitor of activated PAH using Phe as substrate was 13.14 ± 20.9 mM and the K_i for Phe as an inhibitor of activated PAH using SCMC as substrate was 0.38 ± 0.04 mM. From the plot in Figure 3 it can be seen that SCMC was a competitive inhibitor of Phe C-oxidation. A similar pattern was seen in Figure 4, in which Phe was a competitive inhibitor of SCMC *S*-oxidation.

The correlation of Tyr, SMC *S*-oxide and SCMC *S*-oxide formation in 20 different Wistar strain rat hepatic cytosolic fractions

TABLE 2

The effects of various inhibitors on activated phenylalanine 4-monooxygenase (PAH) activity using Phe and SCMC as substrates

Compound	(concentration)	n	% of control activity	
			Phe substrate	SCMC substrate
Met	(5 mM)	6	55.0 ± 7.0	75.3 ± 6.2
SMC	(5 mM)	6	89.0 ± 6.0	92.1 ± 7.4
SCMC	(5 mM)	6	74.2 ± 6.5	ND
Phe	(5 mM)	6	ND	0.5 ± 0.5
MCA	(0.5 mg/ml)	6	<0.1	<0.1
Deferoxamine	(1 mM)	6	1.2 ± 0.7	0.5 ± 0.4

Each value is the mean ± SD of 6 experiments.
Each experiment was undertaken in duplicate with associated blanks.
n = number of experiments.
Met = methionine; SMC = *S*-methyl-L-cysteine; SCMC = *S*-carboxymethyl-L-cysteine; Phe = phenylalanine; MCA = large aromatic amino acid hydroxylase monoclonal antibody.
PAH was activated by pre-incubation with 1.0 mM lysophosphatidylcholine for 3.0 min at 37°C.
ND = not determined.

using the activated PAH enzyme assay can be seen in Figures 5 and 6. There was a significant linear correlation between the production of Tyr and SMC *S*-oxide (Spearman's rank correlation coefficient, $r_s = 0.75$, $p < 0.01$) and Tyr and SCMC *S*-oxide ($r_s = 0.85$, $p < 0.001$), indicating a close association between these oxidative reactions.

DISCUSSION

It has been demonstrated that hepatic cytosol derived from female Wistar strain rats from King's College London BSU possess both PAH and 'SCMC S-oxygenase' activity. The kinetic characteristics of

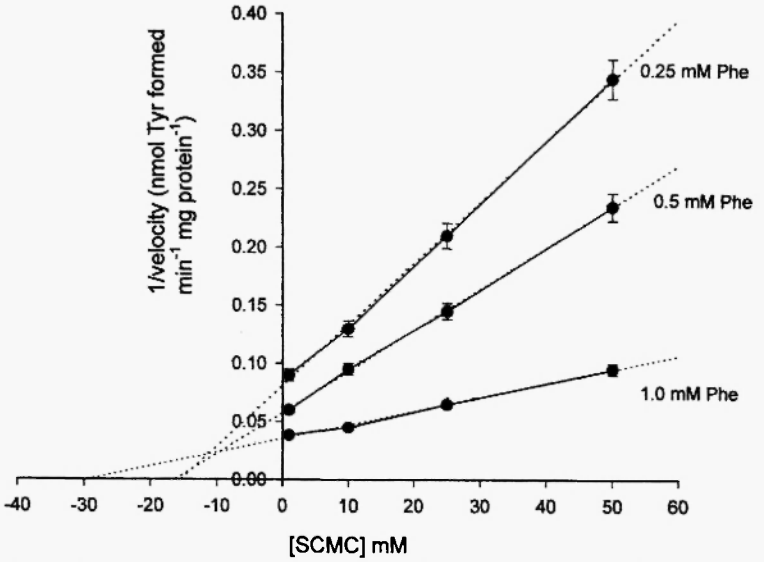

Fig. 3: The Dixon plot of SCMC as an inhibitor of activated PAH activity at 0.25 mM, 0.5 mM and 1.0 mM Phe substrate concentrations in female Wistar rat cytosolic fractions *in vitro*. Each value is the mean ± SD of 6 experiments.

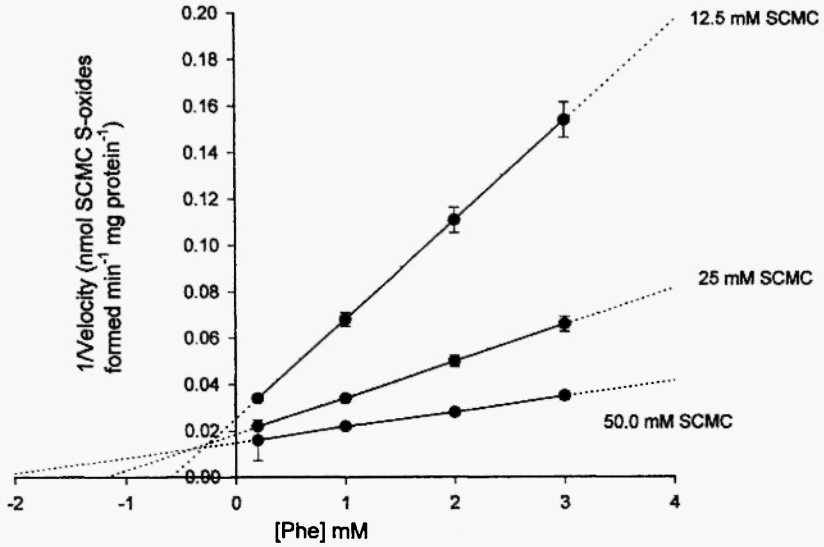

Fig. 4: The Dixon plot of Phe as an inhibitor of activated PAH activity at 12.5 mM, 25.0 mM and 50.0 mM SCMC substrate concentrations in female Wistar rat cytosolic fractions *in vitro*. Each value is the mean ± SD of 6 experiments.

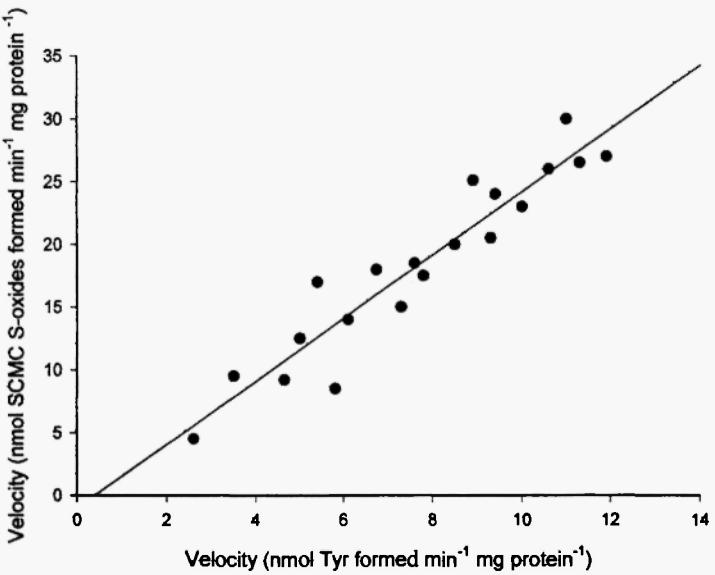

Fig. 5: The correlation of activated PAH activity using 1.0 mM Phe and 40.0 mM SCMC as substrates in 20 different female Wistar rat cytosolic fractions *in vitro*.

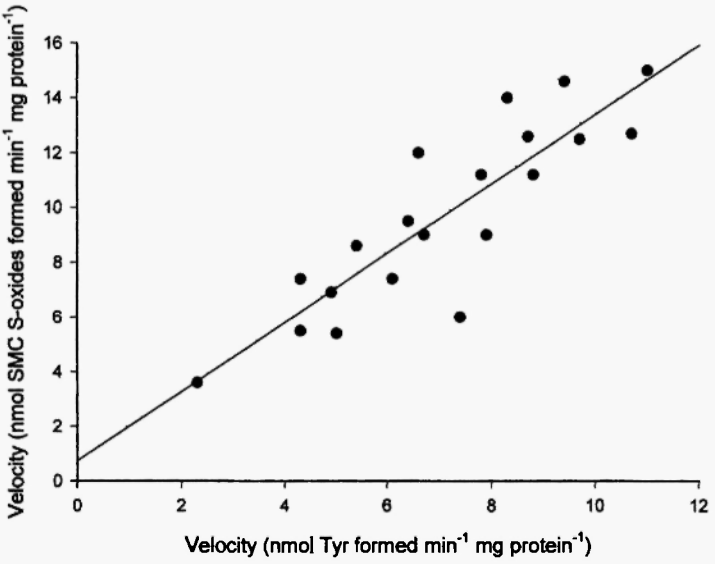

Fig. 6: The correlation of activated PAH activity using 1.0 mM Phe and 50.0 mM SMC as substrates in 20 different female Wistar rat cytosolic fractions *in vitro*.

the present PAH activities were similar to values previously reported (apparent K_m present 0.26 mM, reported 0.19 mM /23/; apparent V_{max} present 13.72 nmoles/min/mg, reported 11.0 nmoles/min/mg /23/). Using the different assay condition described in a previous study /5/, 'SCMC *S*-oxygenase' activity gave an *S*-oxide production rate of 37.43 ± 15.11 nmoles of *S*-oxides formed/min/mg cytosol protein (n = 20) (results not shown), comparing favourably with the literature (39.7 ± 12.1 nmoles/min/mg /5/). However, when compared to Phe, SCMC was not as efficient a substrate but had similar kinetic values to its decarboxylated metabolite, SMC, itself confirmed as a substrate in the present studies. The K_m and V_{max} values for SMC as a substrate are 166.3- and 2.1-fold higher than Phe as a substrate. When SCMC was used as a substrate the K_m and V_{max} values were 56.6- and 3.5-fold higher than those for Phe.

Varying and increasing the cofactor (BH_4) concentration markedly altered the kinetic parameters and increased both Tyr and SMC *S*-oxide and SCMC *S*-oxide production, with a 12.1- and 5.2-fold increase in K_m values for BH_4 when SMC and SCMC were used as substrates compared to Phe. Similar increases in V_{max} values for BH_4 were also seen for SMC (2.25-fold increase) and SCMC (1.51-fold) compared to Phe. In addition, both activities behaved in a very similar manner following the inclusion of various potential reaction inhibitors (Met, SMC, SCMC, Phe, deferoxamine, large aromatic amino acid hydroxylase monoclonal antibody). The large aromatic amino acid hydroxylase monoclonal antibody was designed to react with a penta-peptide sequence common to the active sites of TYH, TPH and PAH. However, since the liver does not possess either TYH or TPH /7/, the monoclonal antibody was specific for PAH activity. In fact, both Phe and SCMC were found to be competitive inhibitors of the other compound's oxidation. Together with the results of the correlation studies showing a close linear association between Tyr production and SCMC *S*-oxide formation, the correlation coefficient (r_s = 0.85, $p < 0.001$) is certainly noteworthy for a biological system.

All the presently reported data provides strong experimental evidence that the same enzyme undertakes both reactions and, by implication, that activated PAH was the enzyme responsible for the *S*-oxidation of the mucolytic compound, SCMC, and also of its metabolite, SMC. It is appreciated that absolute proof will only be available from studies employing authentic purified enzyme but, until

such investigations are forthcoming, it appears reasonable to conclude that the identity of an 'SCMC *S*-oxygenase' has been found. This is of profound importance in two major research areas of disease susceptibility factors and clinical therapeutics. This defect in the *S*-oxidation of SCMC has been associated with a number of chronic degenerative disease states as a disease susceptibility factor. These include Parkinson's disease, motor neuron disease and rheumatoid arthritis /4/. To date the gene and protein responsible for this biotransformation has been unknown. Thus a detailed investigation into the mechanism(s) of the regulation of substrate specificity of PAH at either the genomic and or the proteomic level is now urgently required. The second area that may be improved from the results of this investigation is the chemotherapy of both chronic obstructive pulmonary disease (COPD) and otitis media with effusions (OME). Both COPD and OME are diseases that result in an increased accumulation of viscous mucus in the lungs or inner ear, and treatment of patients with SCMC has produced a wide spectrum of clinical endpoints from successful treatment to no improvement. Thus being able to identify which patients may respond to SCMC treatment will enable clinicians to successful target patient therapy to a successful outcome. Thus the roles of PAH in disease aetiology (with the exception of phenylketonurea [PKU]) and clinical drug therapy need to be urgently investigated.

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