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Lack of congruence between cysteine dioxygenase activity and *S*-carboxymethyl-L-cysteine *S*-oxidation activity in rat cytosol

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Abstract

The identity of the enzyme(s) responsible for the *S*-oxidation of the mucoactive drug *S*-carboxymethyl-L-cysteine (SCMC) is unknown but the protein(s) are a susceptibility factor for a number of chronic degenerative diseases. The structural similarities between the amino acid L-cysteine and SCMC have raised the possibility that cysteine dioxygenase (CDO) may be responsible for this biotransformation reaction. Both CDO and SCMC *S*-oxygenase were found to require Fe^{2+} for enzymatic activity, and both enzyme activities were inhibited by Fe^{2+} and Fe^{3+} chelators. However, sulphydryl group modification of the enzymes resulted in the activation of the *S*-oxidation of SCMC but inhibition of the *S*-oxidation of L-cysteine. When the two enzyme activities were quantified in 20 female hepatic cytosolic fractions no linear correlation in the production of their respective metabolites was seen. The results of this investigation indicate that CDO is not responsible for the *S*-oxidation of SCMC in the rat.

Introduction

The biotransformation of the mucoactive drug *S*-carboxymethyl-L-cysteine (SCMC) has been the subject of intense investigation during the 1980–1990 period (Steventon 1999), following the suggestion that the *S*-oxidation of SCMC in a UK Caucasian population displayed a genetic polymorphism (Waring et al 1982; Mitchell et al 1984). Subsequent investigations reported that, although they were present, only small amounts of the *S*-oxide of SCMC could be found (Brockmoller et al 1991). It was stated that thiodiglycolic acid *S*-oxide was the major *S*-oxide metabolite of SCMC (Hofmann et al 1991) and that the SCMC *S*-oxide metabolite had been mistakenly identified and was in fact *S*-(carboxymethylthio)-L-cysteine (Meese et al 1991). These latter reports could be explained by the diurnal variation in SCMC metabolism that causes both quantitative and qualitative differences in the metabolite profile (Steventon 1998, 1999). The *S*-oxidation of SCMC to SCMC *S*-oxide is now accepted as the major biotransformation pathway of the mucoactive compound following its early morning administration (Steventon 1998, 1999).

The enzyme responsible for the *S*-oxidation of SCMC was initially thought to be the cytochrome P450 isozyme 2D6, owing to the co-segregation of the "poor sulphoxidizer" phenotype for SCMC metabolism with the "poor metabolizer" phenotype for debrisoquine (CYP2D6) (Waring et al 1981). However, this was later found to be incorrect (Haley et al 1985). To date, little is known about the enzymology of "SCMC *S*-oxygenase" (Waring et al 1986) and the assumption that the enzyme cysteine dioxygenase (CDO), the enzyme responsible for the conversion of L-cysteine to L-cysteine sulphinic acid and L-cysteic acid, was responsible for this *S*-oxidation reaction. This was based simply on the structural similarity of SCMC to the amino acid L-cysteine (Mitchell & Waring 1989). To date, only one investigation has reported on the cofactor/inhibitor specificities of SCMC *S*-oxygenase, indicating that the enzyme contained a sulphydryl group and also required molecular oxygen and a cation for its activity (Waring et al 1986). The "S-oxidation" genetic polymorphism has been associated with a number of chronic degenerative diseases as a potential susceptibility

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factor (Steventon 1999; Steventon et al 2001) and identifying the enzyme(s) responsible for the *S*-oxidation of SCMC would be a major step forward in investigating the aetiology of these diseases. The purpose of the present investigation was to compare the enzyme kinetics, cofactor, activator and inhibitor specificities of the cytosolic enzyme-mediated *S*-oxidation of both L-cysteine and SCMC, and to determine the role of rat hepatic CDO in SCMC *S*-oxidation.

Materials and Methods

Chemicals

Bathocuproine disulfonate, bovine liver catalase, bovine liver superoxide dismutase, SCMC, D-cysteine, L-cysteine, L-cysteic acid, L-cysteine *S*-sulphate, L-cysteine sulphinic acid, deferoxamine, 2,2′ dipyridyl, disodium hydrogen phosphate, 1,4-dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), ethyleneglycoltetraacetic acid (EGTA), *N*-ethylmaleimide, glacial acetic acid, glutathione, hydrochloric acid (35% w/v), hydrogen peroxide (30% w/v), hydroxylamine, 2-mercaptoethanol, methanol (analar grade), nicotinamide-adenine dinucleotides (NAD^+, $NADH(H^+)$ $NADP^+$, $NADPH(H^+)$), 2-(*N*-morphilino) ethanesulfonic acid (Mes), σ-phthaldehyde, sodium acetate, sodium borate, sodium dihydrogen phosphate, sodium hydrogen carbonate, $MgCl_2.6H_2O$, $CaCl_2$, $FeCl_2$, $FeCl_3$, CuCl, $CuCl_2$ and trichloroacetic acid were all purchased from Sigma-Aldrich Chemical Company (Poole, Dorset, UK). High-pressure liquid chromatography (HPLC) grade methanol was from Rathburn Chemical Company (Walkerburn, Scotland). β-Diethylaminoethyl diphenylpropylacetate (SKF525A, a cytochrome P450 inhibitor) was a kind gift from Glaxo-SmithKline (Welwyn, Herts, UK). SCMC *R*- and *S*-*S*-oxides were synthesized and isolated by the method of Meese (1987). All other chemicals were readily available in pure form in our laboratory.

Biological material

All animals used were killed using a Schedule 1 method as stated in the Animals (Scientific) Procedures Act 1986.

Female Wistar rats (200–250 g; BSU King's College London) were killed by cervical dislocation and the liver was immediately removed and covered with 50 mM Mes (pH 6.0) buffer at 4°C. A 40% (w/v) whole liver homogenate was prepared using a 50 mM Mes (pH 6.0) buffer at 4°C. The homogenate 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 and the supernatant was made to a $CaCl_2$ concentration of 8 mM. The supernatant was then stirred at 4°C for 5 min before centrifugation at 27 000 *g* for 15 min at 4°C. The supernatant was removed and assayed for protein content (Bradford 1976), lactate dehydrogenase, a marker for the cytosolic fraction (Doroshenko & Doroshenko 2003), isocitrate dehydrogenase, a marker for the mitochondrial fraction (Schon et al 1994), and glucose-6-phosphatase activity, a marker for the microsomal fraction (Pears et al 1989).

Biochemical assays

CDO activity and the various activator/inhibitor investigations were carried out as described by Bagley et al (1995) and Waring et al (1986). A total of 0.3 mL of crude hepatic cytosol fraction in 50 mM Mes (pH 6.0) buffer was kept on ice in 3.5-mL microcentrifuge tubes. Next, 0.3 mL of the 50 mM Mes (pH 6.0) buffer was added to the crude homogenate. This was followed by the addition of 0.5 mL of a 200-mM Mes (pH 6.1) buffer and 0.4 mL of the cofactor solution (2.5 mM ferrous ammonium sulphate, 25 mM hydroxylamine, 10 mM NAD^+). The reaction was started by the addition of 0.5 mL of a freshly prepared 20 mM L-cysteine solution containing 2 mM bathocuproine disulfonate. The microcentrifuge tubes were immediately incubated at 37°C for 24 min. The assays were terminated by the addition of 1 mL of 1 M trichloroacetic acid. The microcentrifuge tubes were then centrifuged at 5000 rev min^{-1} for 10 min and 1 mL of supernatant was removed. Following the addition of 5 μL of L-cysteine *S*-sulphate, the internal standard (IS), all samples were frozen at −20°C until analysed for L-cysteine sulphinic acid and L-cysteic acid. The final concentration of IS in samples was 250 μM. All concentrations are final concentrations. When the effects of Cu^+ and Cu^{2+} were investigated the copper chelator bathocuproine disulfonate was not included in either the control or Cu^+ and Cu^{2+} experiments.

SCMC *S*-oxygenase activity and the various activator/inhibitor investigations were carried out as described by Waring et al (1986) and Bagley et al (1995). An aliquot (0.5 mL) of crude hepatic cytosol fraction in 50 mM Mes (pH 6.0) buffer was pre-incubated for 10 min at 37°C in 1.5-mL microcentrifuge tubes. The reaction was initiated by the addition of 0.5 mL of SCMC (25 mM) in 100 mM NaH_2PO_4/Na_2HPO_4 (pH 7.4) at 37°C and allowed to proceed for 30 min at 37°C. The reaction was terminated by the addition of 0.1 mL of 10% (w/v) trichloroacetic acid. The microcentrifuge tubes were then centrifuged at 5000 rev min^{-1} for 10 min and 1 mL of supernatant was removed. Following the addition of 5 μL of L-cysteic acid (IS), all samples were frozen at −20°C until analysed for SCMC *R*- and *S*-*S*-oxide formation. The final concentration of IS in samples was 250 μM. All concentrations are final concentrations.

HPLC assays

L-Cysteine sulphinic acid and L-cysteic acid were assayed by pre-column derivatization with σ-phthaldehyde/2-mercaptoethanol and separation by reverse-phase chromatography with fluorescence detection as reported by Stipanuk et al (1987). SCMC *R*- and *S*-*S*-oxide was analysed by pre-column derivatization with σ-phthaldehyde/2-mercaptoethanol and separation by reverse-phase chromatography with fluorescence detection as reported by Bednar et al (2004).

Data analysis

Statistical data analyses were performed with SPSS 10.0. Enzyme kinetic data were analysed by the Leonora enzyme kinetic program 1.0 using the following two equations.

Michaelis–Menten equation

$$V = V[S]/K_m + [S]$$

Michaelis–Menten with substrate inhibition equation

$$V = V[S]/(K_s + [S](1 + [S]/K_{si}))$$

V, velocity; S, substrate; K_m, Michaelis–Menten constant; K_s, Michaelis–Menten constant for substrate when substrate inhibition was present; K_{si}, inhibitor constant for the substrate.

Results

Marker enzymes for cell fractions

The results from the marker enzyme assays for the cytosolic fractions showed high levels of lactate dehydrogenase activity and no isocitrate dehydrogenase or glucose-6-phosphatase activity (results not shown). Thus, there appeared to be no contamination of the cytosolic fraction with microsomes or mitochondria.

V_{max} and K_m determinations

CDO was assayed as described by Bagely et al (1995) and the V versus S plot is shown in Figure 1A. Two curves were fitted to the data by the Leonora program V1. The Leonora-engineered best fit for the Michaelis–Menten equation resulted in a calculated apparent K_m of 1.35 ± 0.13 mM and a calculated apparent V_{max} of 1.29 ± 0.07 nmol L-cysteine sulphinic acid formed min^{-1} (mg protein)$^{-1}$. However, the best curve that "fitted" the experimental data was that for the Michaelis–Menten equation with substrate inhibition. The Leonora calculated an apparent V_{max} of 2.87 ± 0.18 nmol L-cysteine sulphinic acid formed min^{-1} (mg protein)$^{-1}$, an apparent K_m of 3.48 ± 0.25 mM, and an apparent K_{si} of 2.11 ± 0.21 mM for the substrate L-cysteine. A similar pattern of results can be seen in Figure 1B for the SCMC *S*-oxygenase assay, carried out as described by Waring et al (1986). Again, the Leonora-engineered best fit for the Michaelis–Menten equation resulted in a calculated apparent K_m of 10.99 ± 1.74 mM and a calculated apparent V_{max} of 72.60 ± 7.30 nmol SCMC *S*-oxides formed min^{-1} (mg protein)$^{-1}$. Similar to the CDO results, the best curve that fitted the experimental data was again in accordance with the Michaelis–Menten equation with substrate inhibition. The Leonora calculated an apparent V_{max} was 541.07 ± 261.31 nmol SCMC *S*-oxides formed min^{-1} (mg protein)$^{-1}$, an apparent K_m of 99.18 ± 50.06 mM, and an apparent K_{si} of 2.57 ± 1.38 mM for SCMC as a substrate. Boiling the cytosol for 10 min before assay completely abolished activity, as did precipitation with trichloroacetic acid (0.5 mL, 33% w/v).

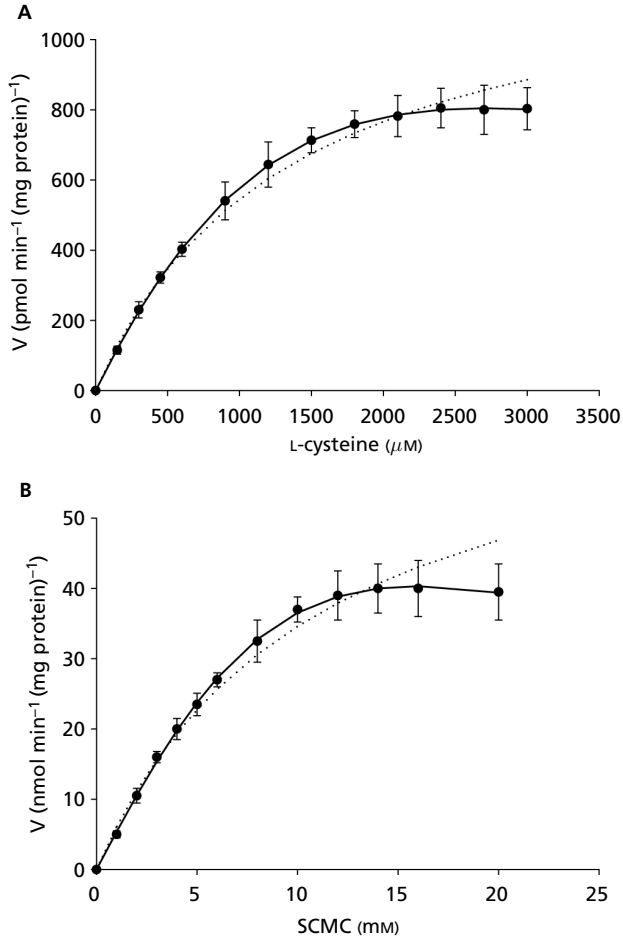

Figure 1 A. Effects of varying L-cysteine concentration on rat hepatic cysteine dioxygenase activity in-vitro. The solid line represents the best fit to the experimental data using the Michaelis–Menten equation with substrate inhibition. The dashed line represents the best fit to the experimental data using the Michaelis–Menten equation. Each data point is the mean ± s.d. of six experiments. One experiment is an enzyme assay carried out in duplicate with blanks. A total of four rats were used in this experiment. B. Effects of varying *S*-carboxymethyl-L-cysteine (SCMC) concentration on rat hepatic SCMC *S*-oxygenase activity in-vitro. The solid line represents the best fit to the experimental data using the Michaelis–Menten equation with substrate inhibition. The dashed line represents the best fit to the experimental data using the Michaelis–Menten equation. Each data point is the mean ± s.d. of six experiments. One experiment is an enzyme assay carried out in duplicate with blanks. A total of four rats were used in this experiment.

Previously known cofactors for CDO activity

The known cofactor requirements of rat CDO enzyme and their effects on SCMC *S*-oxygenase are shown in Figure 2. The effect of varying NAD^+ concentration on both enzyme activities showed that this cofactor was not required by SCMC *S*-oxygenase but that NAD^+ caused a linear increase in CDO activity over the range 0.1–2.0 mM before showing a slow linear decrease over the range 2.0–10.0 mM (Figure 2A). Both CDO and SCMC *S*-oxygenase showed

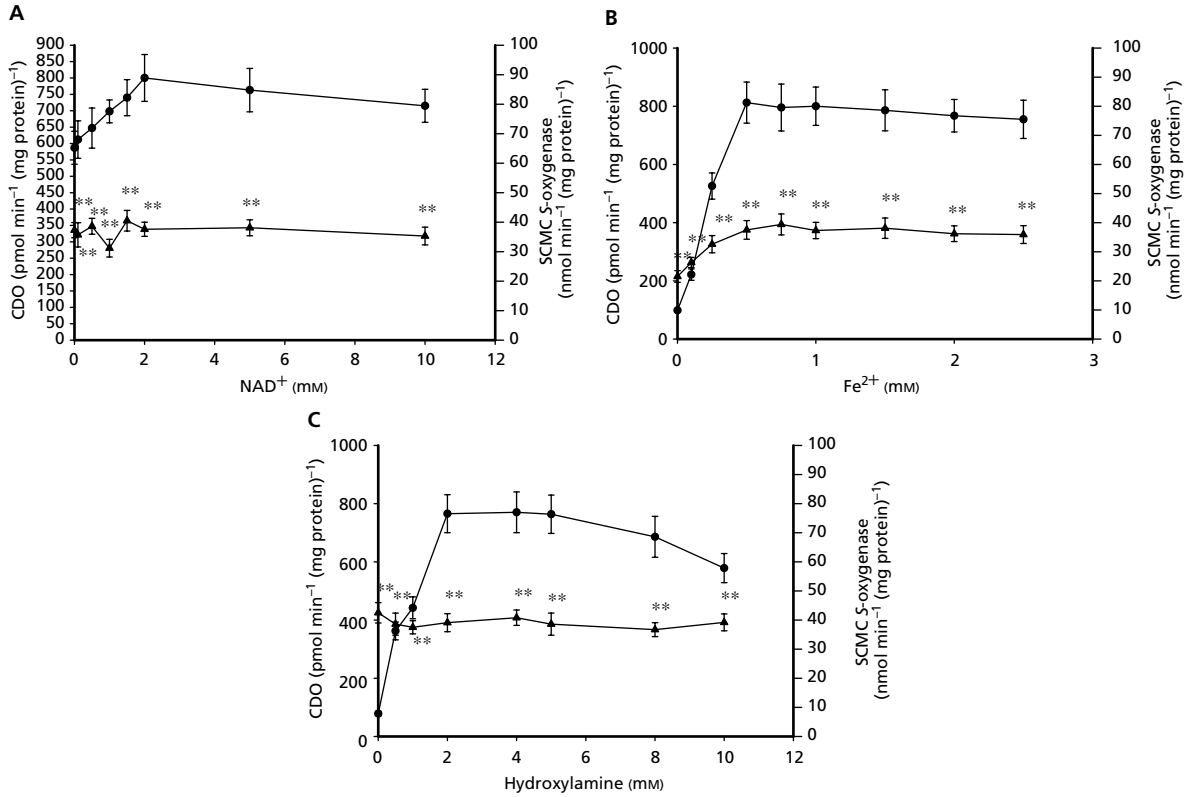

Figure 2 A. Effects of varying NAD^+ concentration on rat hepatic cysteine dioxygenase (CDO) and *S*-carboxymethyl-L-cysteine (SCMC) *S*-oxygenase activity in-vitro. Each data point is the mean ± s.d. of six experiments. One experiment is an enzyme assay carried out in duplicate with blanks. A total of four rats were used in this experiment. Using one-way analysis of variance, the effects of NAD^+ on CDO and SCMC *S*-oxidase activity was significantly different ($P < 0.01$). Individual results were analysed using Tukey's test and those marked ** were found to be significantly different ($P < 0.01$). ●, CDO; ▲, SCMC *S*-oxidase. B. Effects of varying Fe^{2+} concentration on rat in-vitro. Each data point is the mean ± s.d. of six experiments. One experiment is an enzyme assay carried out in duplicate with blanks. A total of four rats were used in this experiment. Using one-way analysis of variance the effects of Fe^{2+} on CDO and SCMC *S*-oxidase activity were significantly different ($P < 0.01$). Individual results were analysed using Tukey's test and those marked ** were found to be significantly different ($P < 0.01$). ●, CDO; ▲, SCMC *S*-oxidase. C. Effects of varying hydroxylamine concentration on rat hepatic cysteine dioxygenase and *S*-carboxymethyl-L-cysteine *S*-oxygenase activity in-vitro. Each data point is the mean ± s.d. of six experiments. One experiment is an enzyme assay carried out in duplicate with blanks. A total of four rats were used in this experiment. Using one-way analysis of variance the effects of hydroxylamine on CDO and SCMC *S*-oxidase activity was significantly different ($P < 0.01$). Individual results were analysed using Tukey's test and those marked ** were found to be significantly different ($P < 0.01$). ●, CDO; ▲, SCMC *S*-oxidase.

an increase in enzyme activity over the Fe^{2+} concentration range of 0.1–0.5 mM, with maximal enzyme activity being reached for both enzymes at 0.5 mM Fe^{2+} and no further increases being seen above this concentration. These results show that both enzymes required Fe^{2+} (Figure 2B) (see below). SCMC *S*-oxygenase activity was independent of hydroxylamine (a pyridoxal phosphate dependent enzyme inhibitor) concentration over the range 0–10.0 mM, whereas CDO activity showed a linear increase over the concentration range of 0.0–2.0 mM hydroxylamine and a slow decrease in activity from 2–10.0 mM (Figure 2C).

Cation requirements

The cations Mg^{2+}, Ca^{2+}, Fe^{3+} and Cu^+ showed no effect on rat hepatic cytosol fraction CDO and SCMC *S*-oxygenase activity at 0.1 and 1.0 mM concentrations. However, Cu^{2+} caused almost 89.5% inhibition at 0.1 mM concentration and >99.5% inhibition of CDO activity at 1.0 mM concentration. In contrast, these same concentrations of Cu^{2+} resulted in a 1.2- and 1.46-fold increase in SCMC *S*-oxygenase activity and a 1.17-fold increase in activity at 10.0 mM concentration. Only Fe^{2+} caused an increase in both CDO and SCMC *S*-oxygenase activity (see above; Table 1 and Figure 2B). This requirement for Fe^{2+} was further investigated by chelation studies (Table 2). The Fe^{2+} chelator 2,2′ dipyridyl was found to be a highly effective inhibitor of both CDO and SCMC *S*-oxygenase activity in-vitro. However, CDO activity was far more sensitive to 2,2′ dipyridyl inhibition than SCMC *S*-oxygenase (Table 2). The Fe^{3+} chelator deferoxamine was also found to be a highly effective inhibitor of both enzymes with CDO again showing the greatest response to the action of the chelator. Finally, only CDO showed any

Table 1 Effects of various cations on rat hepatic cysteine dioxygenase (CDO) and *S*-carboxymethyl-L-cysteine (SCMC) *S*-oxygenase activity in-vitro

Cation	Concentration (mM)	CDO activity (% control)	SCMC *S*-oxygenase activity (% control)
Control		100.0	100.0
Mg^{2+}	0.1	100.0 ± 2.0	99.5 ± 1.3
	1.0	98.4 ± 2.3	101.7 ± 2.5
Ca^{2+}	0.1	99.7 ± 3.2	96.7 ± 4.5
	1.0	100.2 ± 2.5	98.3 ± 3.0
Fe^{2+}	0.1	205.3 ± 6.3	112.7 ± 5.6
	1.0	810.4 ± 15.4	125.0 ± 7.8
Fe^{3+}	0.1	101.3 ± 3.5	99.0 ± 5.1
	1.0	97.6 ± 2.1	102.8 ± 3.7
Cu^{+}	0.1	98.3 ± 3.6	100.5 ± 4.0
	1.0	102.4 ± 6.1	98.1 ± 2.8
Cu^{2+}	0.1	10.5 ± 2.3	120.2 ± 5.4
	1.0	< 0.5%**	146.8 ± 7.3*

Results are the mean ± s.d. of six experiments. One experiment is an enzyme assay carried out in duplicate with blanks. A total of four rats were used in this experiment. Statistical analysis using two-way analysis of variance resulted in $P > 0.05$ for the effects of the various cations on CDO and SCMC *S*-oxidase activity. *$P < 0.05$, **$P < 0.01$, Tukey's test.

Table 2 Effects of various chelators on rat hepatic cysteine dioxygenase (CDO) and *S*-carboxymethyl-L-cysteine (SCMS) *S*-oxygenase activity in-vitro

Cation	Concentration (mM)	CDO activity (% control)	SCMC *S*-oxygenase activity (% control)
Control		100.0	100.0
2,2′ dipyridyl	0.1	8.2 ± 1.3**	66.0 ± 2.4*
	1.0	< 0.5**	41.5 ± 4.0*
Deferoxamine	0.1	20.0 ± 5.5**	44.7 ± 5.1*
	1.0	< 0.5**	1.2 ± 0.5**
EDTA	0.1	5.0 ± 3.5**	103.0 ± 6.0
	1.0	< 0.5**	99.1 ± 4.2
EGTA	0.1	6.6 ± 4.2**	100.3 ± 5.6
	1.0	< 0.5**	103.6 ± 7.0

Results are the mean ± s.d. of six experiments. One experiment is an enzyme assay carried out in duplicate with blanks. A total of four rats were used in this experiment. Statistical analysis resulted in $P < 0.05$, two-way analysis of variance test for the effects of various chelators on CDO activity and SCMC S-oxidase activity. *$P < 0.05$, **$P < 0.01$, Tukey's test.

significant inhibition with EDTA and EGTA (95.0 and 94.4% inhibition at 0.1 mM), whereas SCMC *S*-oxygenase was unaffected at the tested concentrations (Table 2).

Sulphydryl/disulphide modification

The effects of the sulphydryl modifying cation, Hg^{2+}, showed that both CDO and SCMC *S*-oxygenase were inhibited by > 99.5% at 1.0 mM concentration. This was also the case when Cd^{2+} was used. The sulphydryl alkylating reagent *N*-ethylmaleimide, however, produced opposite effects in the two enzyme assays. The compound was an inhibitor of CDO activity (44.4% and 86.4% inhibition at 1.0 and 10.0 mM, respectively) but an activator of SCMC *S*-oxygenase activity (1.8-, 3.6- and 4.2-fold activation at 0.1, 1.0 and 10.0 mM, respectively). The disulphide reducing reagent, DTT, was inhibitory to both CDO and SCMC *S*-oxygenase activity at 1.0 mM (14.3% and 14.7% inhibition, respectively) and 10.0 mM (25.5% and 71.9% inhibition, respectively). Finally, the effects of the two substrates (L-cysteine and SCMC), D-cysteine and glutathione were investigated. L-Cysteine showed activator properties in the SCMC *S*-oxygenase assays with 1.38- and 2.03-fold activation at 1.0 and 10.0 mM concentrations. Similar results were seen with D-cysteine, which caused 1.35- and 1.98-fold activation of SCMC *S*-oxygenase activity. The tripeptide glutathione was also found to be an activator of SCMC *S*-oxygenase activity, showing 1.51- and 2.15-fold activation at 1.0 and 10.0 mM concentrations. When the CDO assay was investigated, SCMC was found to be an inhibitor of CDO activity at 0.1, 1.0 and 10.0 mM concentrations, causing 8.5%, 39.3% and 57.7% inhibition, respectively. D-Cysteine was also found to be inhibitory to CDO activity, causing 14.4%, 48.7% and 65.3% inhibition at 0.1, 1.0 and 10.0 mM concentrations, respectively. Similar results were seen with glutathione, which resulted in 21.8%, 37.3% and 58.5% inhibition at 0.1, 1.0 and 10.0 mM concentrations, respectively (Table 3).

Other compounds known to affect CDO/SCMC *S*-oxygenase activity

None of the three remaining nicotinamide-adenine dinucleotides ($NADH(H^+)$, $NADP^+$, $NADPH(H^+)$) showed any effect on SCMC *S*-oxygenase activity at 0.1, 1.0 or 10.0 mM concentrations. However, $NADH(H^+)$ was shown to activate CDO activity at 1.0 (1.25-fold activation) and 10.0 mM (1.74-fold activation). Both $NADP^+$ and $NADPH(H^+)$ were inhibitors of CDO activity at all concentrations investigated. The enzyme superoxide dismutase, which decomposes the superoxide anion to hydrogen peroxide and oxygen, was found to inhibit both CDO and SCMC *S*-oxygenase at 100 and 1000 units mL^{-1} activity, causing 10.0% and 54.3% inhibition in the CDO assays and 24.4% and 95.7% inhibition in the SCMC *S*-oxygenase assays. However, catalase, an enzyme that decomposes hydrogen peroxide to water and oxygen, gave opposite results in the two enzyme assays. CDO activity was unaffected by the presence of either 100 or 1000 units mL^{-1} of catalase. SCMC *S*-oxygenase was activated by 1.37-fold (100 units mL^{-1} catalase) and 2.43-fold by the presence of 1000 units mL^{-1} of catalase in the enzyme assay (Table 4).

S-Oxidation activity correlation studies

When the CDO and SCMC *S*-oxygenase activities in hepatic cytosol fractions from 20 female Wistar rats were

Table 3 Effects of various sulphydryl modifying reagents on rat hepatic cysteine dioxygenase (CDO) and *S*-carboxymethyl-L-cysteine (SCMS) *S*-oxygenase activity in-vitro

Compound	Concentration (mM)	CDO activity (% control)	SCMC *S*-oxygenase activity (% control)
Control		100.0	100.0
$Hg(NO_3)_2$	0.1	11.6 ± 2.5**	25.3 ± 5.8**
	1.0	< 0.5**	< 0.5**
$CdCl_2$	1.0	< 0.5**	< 0.5**
N-ethylmaleimide	0.1	95.7 ± 2.6	180.6 ± 12.0**
	1.0	55.6 ± 5.3**	365.8 ± 15.5**
	10.0	13.6 ± 8.6**	421.7 ± 11.4**
DTT	0.1	95.2 ± 6.1	97.5 ± 3.8
	1.0	85.7 ± 9.5	85.3 ± 4.0
	10.0	74.5 ± 3.4*	28.1 ± 7.0**
L-Cysteine	0.1	ND	108.4 ± 6.5
	1.0	ND	138.4 ± 10.3*
	10.0	ND	203.6 ± 13.1**
SCMC	0.1	91.5 ± 4.2	ND
	1.0	60.7 ± 8.5*	ND
	10.0	42.3 ± 11.3**	ND
D-Cysteine	0.1	85.6 ± 6.9	110.0 ± 8.6
	1.0	51.3 ± 8.6**	135.9 ± 9.1*
	10.0	34.7 ± 5.7**	198.3 ± 7.2**
Glutathione	0.1	78.2 ± 6.1*	114.2 ± 5.6
	1.0	62.7 ± 7.5*	151.3 ± 8.4*
	10.0	41.5 ± 8.0*	215.3 ± 7.6**

Results are the mean ± s.d. of six experiments. One experiment is an enzyme assay carried out in duplicate with blanks. A total of four rats were used in this experiment. ND, not determined. Statistical analysis resulted in $P < 0.05$, two-way analysis of variance test of the effects of various sulphydryl modifying agents on CDO and SCMC *S*-oxidase activity. *$P < 0.05$, **$P < 0.01$, Tukey's test.

Table 4 Effects of various compounds on rat hepatic cysteine dioxygenase (CDO) and *S*-carboxymethyl-L-cysteine (SCMS) *S*-oxygenase activity in-vitro

Cation	Concentration (mM or units)	CDO activity (% control)	SCMC *S*-oxygenase activity (% control)
Control		100.0	100.0
$NADH(H^+)$	0.1	98.5 ± 5.3	102.0 ± 5.2
	1.0	125.0 ± 8.4	97.3 ± 7.3
	10.0	174.6 ± 10.3**	100.0 ± 2.8
$NADP^+$	0.1	84.3 ± 4.2	103.7 ± 6.0
	1.0	42.7 ± 6.1*	102.1 ± 4.5
	10.0	16.7 ± 3.5**	97.8 ± 5.7
$NADPH(H^+)$	0.1	74.2 ± 8.5*	99.9 ± 6.0
	1.0	35.2 ± 4.2*	96.5 ± 4.3
	10.0	20.3 ± 6.9**	104.7 ± 7.2
SKF525A	1.0	99.1 ± 5.5	101.9 ± 5.9
Superoxide	100	90.0 ± 4.0	75.6 ± 7.1*
dismutase	1000	45.7 ± 6.2*	4.3 ± 2.5**
Catalase	100	98.6 ± 3.8	137.8 ± 2.8*
	1000	102.7 ± 4.3	243.1 ± 9.8**

Results are the mean ± s.d. of six experiments. One experiment is an enzyme assay carried out in duplicate with blanks. A total of four rats were used in this experiment. Statistical analysis resulted in $P < 0.05$, two-way analysis of variance test for the effects of various compounds on CDO and SCMC *S*-oxidase activity. *$P < 0.05$, **$P < 0.01$, Tukey's test.

assayed there was no linear relationship found between the two-enzyme activities in-vitro (Figure 3).

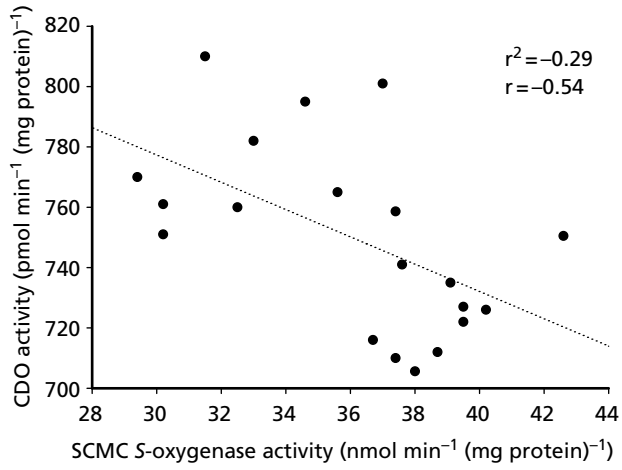

Figure 3 Correlation of hepatic cysteine dioxygenase (CDO) and *S*-carboxymethyl-L-cysteine (SCMC) *S*-oxygenase activity in 20 female Wistar rats in-vitro.

Discussion

The substrate and cofactor requirements for rat CDO are well known (Bagley et al 1995), but similar data for SCMC *S*-oxygenase are limited, with only one brief publication to date (Waring et al 1986). The present study has shown that these cytosolic activities shared many similar properties and had some common cofactor requirements. However, certain obvious disparities have been uncovered and it is these differences that suggest that the two investigated activities may have arisen from different enzyme proteins. The nicotinamide-adenine dinucleotide cofactors (NAD^+, $NADH(H^+)$) were shown to increase CDO activity, while the phosphorylated cofactors ($NADP^+$, $NADPH(H^+)$) had an inhibitory effect. None of the nicotinamide-adenine dinucleotide cofactors appeared to have any effect on SCMC *S*-oxygenase activity at the corresponding concentrations. Ferrous ions (Fe^{2+}) also greatly increased the activity of CDO while having only a slight effect on SCMC *S*-oxygenase. Similarly, the presence of hydroxylamine (a pyridoxal cofactor inhibitor) increased CDO activity but not that of SCMC *S*-oxygenase. However, caution is required in the interpretation of this latter finding as aminotransferase enzymes that normally remove the product of CDO activity, L-cysteine sulphinic acid, are inhibited by hydroxylamine, leading to the potential

accumulation of L-cysteine sulphinic acid (Bagley et al 1995). As would be predicted from its avid requirement for Fe^{2+} (Bagley et al 1995), the presence of the ion chelators, 2,2′-dipyridyl (specific Fe^{2+} chelator), EDTA and EGTA (non-specific chelators), decreased CDO activity while showing a less pronounced effect on SCMC *S*-oxygenase activity. Moreover, there were instances, as with the presence of Cu^{2+} ions, *N*-ethylmaleimide, D-cysteine and glutathione, where CDO activity decreased while SCMC *S*-oxygenase activity increased. These latter responses, which were in opposite directions, lend strong support to the hypothesis that different cytosolic enzymes undertake these two *S*-oxygenation activities.

A few insights into the workings of the biological catalyst undertaking SCMC *S*-oxidation may be gleaned from the present study. Inhibition of activity by Hg^{2+} ions indicated that a sulphydryl group was essential for enzyme activity. The thiol-containing compounds, L-cysteine, D-cysteine and glutathione, were all significant activators, this effect not being isomer-specific and probably resulting from sulphydryl group modification by disulphide bond formation. Indeed, inhibition of activity was observed following the use of the disulphide reducing reagent, DTT, although coincident chelation of Fe^{2+} by DTT could also be occurring. However, the use of *N*-ethylmaleimide, a sulphydryl alkylating reagent, increased SCMC *S*-oxidation activity (CDO activity decreased), suggesting that alkylation of the thiol group(s) was necessary for this activation. Protein *S*-thiolation is a known mechanism of activation and inhibition of a number of enzymes (Zeigler 1985; Ward et al 2000; Kuhn 2001; Borges et al 2002). Inhibition of activity by the enzyme superoxide dismutase had led to the proposal that the superoxide anion may be oxidizing species utilized by SCMC *S*-oxidase (Waring et al 1986). Inhibition by superoxide dismutase was also observed in the present study, but the presence of catalase enzyme was shown to increase SCMC *S*-oxidation activity. Thus, it can be proposed that the observed inhibition of SCMC *S*-oxygenase activity by superoxide dismutase was the result of an increased concentration of hydrogen peroxide produced by this enzyme and not necessarily the decreased level of superoxide anion radicals.

Now that it has been revealed that the enzyme activity responsible for the *S*-oxidation of SCMC is probably not CDO as previously suspected, more work needs to be undertaken in characterising, isolating and identifying the SCMC *S*-oxidase and understanding its various roles in health and disease.

Conclusion

Parallel incubation studies undertaken with hepatic cytosolic fractions prepared from female Wistar rats have shown that the detailed characteristic of CDO activity and SCMC *S*-oxygenase activity differ in several measurable aspects. The previously anecdotally accepted concordance of these two *S*-oxygenation activities is now in doubt. It is probable that the production of L-cysteine sulphinic acid from the amino acid L-cysteine, and SCMC sulphoxide from the mucoactive drug SCMC are undertaken by two distinct and separate enzyme systems residing within the cytosolic fraction of the cell.

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