RESEARCH COMMUNICATION

Glutathione transferase Zeta catalyses the oxygenation of the carcinogen dichloroacetic acid to glyoxylic acid

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Dichloroacetic acid (DCA), a common drinking-water contaminant, is hepatocarcinogenic in rats and mice, and is a therapeutic agent used clinically in the management of lactic acidosis. DCA is biotransformed to glyoxylic acid by glutathione-dependent cytosolic enzymes *in vitro* and is metabolized to glyoxylic acid *in vivo*. The enzymes that catalyse the oxygenation of DCA to glyoxylic acid have not, however, been identified or characterized. In the present investigation, an enzyme that catalyses the glutathione-dependent oxygenation of DCA was purified to homogeneity (587-fold) from rat liver cytosol. SDS/PAGE and HPLC gel-filtration chromatography showed that the purified enzyme had a molecular mass of 27–28 kDa. Sequence analysis showed that the N-terminus of the purified protein was blocked. An internal sequence of 30 amino acid

residues was obtained that matched the recently discovered human glutathione transferase Zeta well [Board, Baker, Chelvanayagam and Jermini (1997) *Biochem. J.* **328**, 929–935]. Western-blot analysis showed that the purified rat-liver enzyme cross-reacted with rabbit antiserum raised against recombinant human glutathione transferase Zeta. The apparent K_m and V_{max} values of the purified enzyme with DCA as the variable substrate were 71.4 μ M and 1334 nmol/min per mg of protein, respectively; the K_m for glutathione was 59 μ M. Both the purified rat-liver enzyme and the recombinant human enzyme showed high activity with DCA as the substrate. These results demonstrate that the glutathione-dependent oxygenation of DCA to glyoxylic acid is catalysed by a Zeta-class glutathione transferase.

INTRODUCTION

Dichloroacetic acid (DCA) is a common drinking-water contaminant that arises from the chlorination of humic or fulvic acids in raw water supplies [1]. It is estimated that 170 million individuals in the U.S.A. consume chlorinated drinking water [2]. DCA is hepatocarcinogenic in male B6C3F1 mice and Fischer 344 rats [3–5]. Although the mechanism of its tumorigenicity has not been elucidated, DCA is not mutagenic in a range of bacterial and mammalian test systems and does not induce peroxisome proliferation [6,7]. DCA is a metabolite of trichloroethylene, trichloroacetic acid and chloral hydrate [8–10]. DCA, which activates pyruvate dehydrogenase secondarily to its inhibition of pyruvate dehydrogenase kinase, is used clinically in the management of lactic acidosis and has been proposed for use as a neuroprotective agent [11,12].

[¹⁴C]DCA is metabolized to chloroacetic acid, glyoxylic acid, glycolic acid, oxalic acid, glycine, hippuric acid and carbon dioxide [9,13,14]. The biotransformation of DCA to glyoxylic acid is catalysed by hepatic cytosolic enzymes that require glutathione for maximal activity [15,16].

In the present paper, we report that the glutathione-dependent oxygenation of DCA to glyoxylic acid is catalysed by the recently discovered Zeta-class glutathione transferases (GSTs) [17].

EXPERIMENTAL

Materials

SP-Sepharose Fast Flow, Phenyl-Sepharose high performance and Sephacryl S-200 were purchased from Pharmacia (Piscataway, NJ, U.S.A.). Macro-Prep ceramic hydroxyapatite, Trans-Blot PVDF membranes, nitrocellulose membranes, Bio-

Rad Immunoblot assay kits, Bio-Sil size-exclusion chromatography HPLC gel-filtration columns and protein standards were obtained from Bio-Rad (Hercules, CA, U.S.A.). Centriprep-10, Centricon-10, model 8050 ultrafiltration cells and YM-10 membranes were purchased from Amicon (Beverly, MA, U.S.A.). Dialysis tubing (molecular-mass cut-off 12000–14000 Da) was obtained from Spectrum Medical Laboratories (Houston, TX, U.S.A.). Recombinant human GST Zeta (GSTZ1-1) and rabbit antiserum raised against this protein were prepared as described previously [17]. Other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Assays

The spectrometric method of Vogels and Van Der Drift [18] was modified to quantify glyoxylic acid formation. The enzyme preparation was incubated with 0.5 mM DCA/1 mM glutathione in 1 ml of 0.1 M phosphate buffer (pH 7.4) at 37 °C. DCA was added to start the reaction, and 50 μ l of trifluoroacetic acid was added to stop the reaction after 20 min of incubation. The reaction mixtures were placed on ice for 10 min and then centrifuged for 5 min to remove precipitated proteins. A sample (0.85 ml) of the supernatant was transferred to a clean test tube and neutralized with 0.5 ml of 1 M NaOH. To each tube, 0.3 ml of 0.8 M phosphate buffer (pH 6.8) and 0.4 ml of phenylhydrazine hydrochloride solution (100 mg in 15 ml of water) were added, and the contents were mixed. The mixtures were kept at room temperature for 10 min and then placed on ice for 5 min. Chilled, concentrated HCl (1 ml) and 0.4 ml of potassium ferricyanide solution (500 mg in 15 ml of water) were added to each sample. The samples were thoroughly mixed and then kept at room temperature for exactly 15 min; the absorbance was

Abbreviations used: DCA, dichloroacetic acid; GST, glutathione transferase; GSTZ1-1, recombinant human GST Zeta.

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measured at 535 nm against water. Blanks lacked DCA. Glyoxylic acid concentrations were quantified with a standard curve prepared under the same conditions. Incubation mixtures containing 0.4 mg of cytosolic protein and 4–32 nmol glyoxylic acid in a final volume of 1 ml showed that the recovery was $85.8 \pm 7.1\%$ ($n = 6$). Recovery of glyoxylic acid from incubation mixtures containing purified enzyme was $94.9 \pm 3.7\%$ ($n = 4$). Glyoxylic acid formation was not detected in the control incubation mixtures, which contained DCA and glutathione but lacked enzyme (results not shown). Glutathione peroxidase activity, with *t*-butyl hydroperoxide as the substrate, was measured as described by Beutler [19]. Reaction mixtures that lacked enzyme were used to correct for non-enzymic peroxidase activity.

Purification of rat-liver GST Zeta

Enzyme activity, with DCA as the substrate, and protein concentrations [20] were measured at each step. Rat-liver cytosol was prepared from male Fischer 344 rats (225–250 g; Charles River Laboratories, Wilmington, MA, U.S.A.). The protocol for animal use was reviewed and approved by the University Committee on Animal Resources. The rats were anaesthetized with diethyl ether and then killed by cardiac exsanguination. The liver was removed, rinsed with 20 mM phosphate buffer (pH 7.4) containing 1.15% KCl, 2 mM EDTA, 100 μ M PMSF and 5 μ M dithiothreitol (buffer A), and homogenized in 4 volumes (w/v) of buffer A. The cytosolic fraction was isolated as described previously [21], and was dialysed overnight with three buffer changes against 20 volumes of 50 mM sodium acetate buffer (pH 5.0) containing 10% glycerol, 2 mM EDTA, 100 μ M PMSF and 5 mM β -mercaptoethanol (buffer B). The dialysed cytosol was centrifuged at 2000 *g* for 5 min, and the supernatant was loaded on to an SP-Sepharose column (2.6 $\times$ 60 cm) that had been equilibrated with buffer B. The column was eluted with one bed volume of buffer B, then with 500 ml of a gradient of 0–250 mM NaCl in buffer B, and then with 250 mM NaCl in buffer B at 5 ml/min. Fractions (5 ml) were collected and assayed, and those containing the highest activities were pooled. The pooled fractions from the SP-Sepharose column were concentrated to about 10 ml (Amicon ultrafiltration cell, YM10 membrane) and centrifuged. The supernatant was applied to a Sephacryl S-200 column (2.6 $\times$ 60 cm) that had been equilibrated with 10 mM potassium phosphate buffer (pH 6.8) containing 10% glycerol and 5 mM β -mercaptoethanol (buffer C). The column was eluted with buffer C at 1 ml/min. Fractions (3 ml) were collected and assayed, and those containing the highest activities were pooled. The pooled fractions from the Sephacryl S-200 column were loaded on to a hydroxyapatite column (1.6 $\times$ 16 cm) that had been equilibrated with buffer C. The column was eluted (approx. 1.5 ml/min) with two bed volumes of buffer C and then with a 150 ml gradient of 10–150 mM potassium phosphate in buffer C. Fractions (3 ml) were collected and assayed and those with the highest activities were pooled. The pooled fractions from the hydroxyapatite column were concentrated by centrifugal ultrafiltration (Centriprep-10) and diluted in 50 mM potassium phosphate buffer (pH 6.8) containing 10% glycerol, 1.0 M ammonium sulphate, 2 mM EDTA and 5 mM β -mercaptoethanol (buffer D).

The enzyme preparation was loaded on to a Phenyl-Sepharose high-performance column (1.6 $\times$ 8 cm), which was eluted (0.8 ml/min) with two bed volumes of buffer D, then with 50 ml of a linear gradient from 100% buffer D to 100% buffer C and finally with 100% buffer C. Fractions (2 ml) containing the highest activities were concentrated by centrifugal ultrafiltration

(Centricon-10) and individually assayed for purity by SDS/PAGE. The fractions with the highest purities were pooled for protein and enzyme assays and were then diluted with 20 mM piperazine/HCl (pH 9.0), 10% glycerol, 2 mM EDTA and 5 mM β -mercaptoethanol (buffer E) and applied to a Mono Q FPLC column (HR5/5) that had been equilibrated with buffer E. The column was eluted with 10 ml of buffer E, then with 20 ml of a linear gradient of 0–250 mM NaCl in buffer E and finally with 20 ml of a linear gradient of 250–1000 mM NaCl in buffer E. Fractions (1 ml) were collected and assayed. Fractions with the highest activities were concentrated by centrifugal ultrafiltration (Centricon-10), diluted with buffer C, concentrated again by centrifugal ultrafiltration and analysed by SDS/PAGE.

Kinetic studies

The kinetics of the glutathione-dependent oxygenation of DCA was studied with the enzyme-activity assay described above. Reaction mixtures contained 0.25 μ g of purified enzyme protein and 0.05–1 mM DCA when the K_m and V_{max} for the oxygenation of DCA at a fixed glutathione concentration of 1 mM were studied. To measure the K_m for glutathione, a fixed concentration of 0.5 mM DCA was used, and glutathione concentrations ranged from 0.05–1 mM. The K_m and V_{max} were calculated with the EZ-Fit computer program, version 2.0 (Perrella Scientific, Inc., Springfield, PA, U.S.A.).

HPLC gel filtration

HPLC gel-filtration analysis was carried out with a Bio-Sil SEC HPLC gel-filtration column eluted at 1 ml/min with 100 mM phosphate buffer (pH 7.4) as the mobile phase. The molecular mass of the purified protein was estimated with Bio-Rad protein standards.

SDS/PAGE

PAGE was performed in the presence of SDS in a Bio-Rad Protein II unit as described previously by Laemmli [22], and the molecular mass of the purified protein was estimated by comparison with protein standards. The gel was stained with Coomassie Blue R-250.

Immunoblot analysis

The purified enzyme and cytosolic fractions were resolved by 10% SDS/PAGE and electrophoretically transferred to nitrocellulose membranes. The membranes were blocked with 3% gelatin in Tris-buffered saline [20 mM Tris and 0.5 M sodium chloride (pH 7.5)] for 30 min. The membranes were incubated with a 1:1000 dilution of rabbit anti-GSTZ1-1 antiserum for 1 h at room temperature with gentle shaking in Tris-buffered saline containing 0.05% Tween-20 and 1% gelatin. The Bio-Rad Immunoblot assay kit containing goat anti-rabbit IgG antibody-alkaline phosphatase conjugate was used as the secondary antibody for detection of immunoconjugates.

Amino acid sequencing

The purified protein was analysed by SDS/PAGE (10% gel), electrophoretically transferred to a PVDF membrane with a Bio-Rad Transblot apparatus, and stained briefly with Coomassie Blue. Initial attempts to determine the N-terminal sequence failed because the protein was N-blocked. The purified protein was degraded with cyanogen bromide, and *o*-phthalaldehyde was

used to block the peptides, except the N-terminal prolyl peptide that had formed. The internal sequence was obtained from the N-terminal prolyl peptide (Proseq Microsequencing Co., Salem, MA, U.S.A.).

RESULTS

Preliminary studies showed that dialysed rat-liver cytosol catalysed the glutathione-dependent oxygenation of DCA to glyoxylic acid (results not shown). To characterize the enzyme catalysing the oxygenation of DCA, the protein was purified by SP-Sepharose cation-exchange chromatography, Sephacryl S-200 gel-filtration chromatography, hydroxyapatite chromatography, hydrophobic-interaction chromatography and Mono Q anion-exchange FPLC (Table 1). The enzyme obtained by hydrophobic-interaction chromatography was 60–80% pure by SDS/PAGE. These impurities were removed by Mono Q anion-exchange FPLC.

The enzyme was purified 587-fold to apparent homogeneity from rat-liver cytosol (Table 1). The estimated molecular mass of the purified enzyme was 28.2 kDa by SDS/PAGE (Figure 1) and

Table 1 Purification of the rat-liver enzyme that catalyses the oxygenation of DCA to glyoxylic acid

The rat-liver enzyme was prepared as described in the Experimental section. Activity was measured with DCA as the substrate.

Step	Protein (mg)	Specific activity (nmol/min per mg of protein)	Total activity (nmol/min)	Yield (%)	Purification (fold)
Cytosol	1406	1.83	4408	100	1
SP-Sepharose	70.8	28.8	2039	4603	15.7
Sephacryl S-200	13.3	62.1	827	18.8	34
Hydroxyapatite	0.75	381	289	6.5	209
Phenyl-Sepharose	0.12	753	88.8	2	411
Mono Q FPLC	0.041	1075	44.1	1	587

Figure 1 SDS/PAGE of the purified enzyme from rat-liver cytosol

The enzyme was prepared and analysed as described in the Experimental section. Lane 1, purified enzyme (1 μ g); and lane 2, protein markers. The proteins were resolved on a 10% gel and stained with Coomassie Blue SR-250.

Figure 2 Sequence alignment of an internal peptide from the purified rat-liver enzyme with amino acids 131–158 of human GST Zeta

The enzyme was prepared and analysed as described in the Experimental section.

27.2 kDa by HPLC gel-filtration chromatography. Kinetic analysis showed that the apparent K_m and V_{max} values of the purified enzyme with DCA as the substrate were $71.4 \pm 5.1 \mu M$ and 1334 ± 181 nmol/min per mg of protein, respectively; the K_m for glutathione was $59.0 \pm 3.4 \mu M$ (mean $\pm$ S.D., $n = 3$).

Initial attempts to obtain the amino acid sequence of the purified protein showed that the N-terminus was blocked. Accordingly, the protein was degraded with cyanogen bromide, and an internal sequence of 30 amino acid residues was obtained. A search of GenBank revealed that the residues 3–30 of the isolated peptide showed 85% sequence identity with amino acids 131–158 of the recently discovered human GST Zeta (accession no. U86529; [17]; Figure 2). Identities ranging from 40–60% were found with GSTs from *Triticum aestivum* (wheat; accession no. AF002211), *Dianthus caryophyllus* (carnation; accession no. L05915) and *Caenorhabditis elegans* (accession no. Z66560) (results not shown).

To confirm the identity of the purified rat-liver enzyme as a Zeta-class GST, immunoblot analysis was carried out with rabbit antiserum raised against GSTZ1-1. The purified protein cross-reacted with anti-GST Zeta antibodies (Figure 3). Moreover, the antibodies reacted with a protein in rat-liver cytosol, but only when 50 μ g protein was used, indicating the concentration of GST Zeta in rat-liver cytosol is low (Figure 3).

Both the purified rat-liver enzyme and GSTZ1-1 showed activity with DCA as the substrate which was higher than glutathione peroxidase activity with t-butyl hydroperoxide as the substrate (Table 2).

DISCUSSION

The results show that human and rat Zeta-class GSTs catalyse the glutathione-dependent oxygenation of DCA to glyoxylic acid.

An enzyme that catalyses the oxygenation of DCA was purified 587-fold from rat-liver cytosol. The purified protein was N-blocked and had a molecular mass of about 27–28 kDa. An internal sequence of 30 amino acid residues was obtained from the purified rat-liver enzyme. A search of the GenBank CDS database showed that amino acids 3–30 of the rat liver protein matched well (85%) with the recently described human GST Zeta [17]. Lower identities were found with Zeta-class GSTs from wheat, carnation and *C. elegans*. Western-blot analysis showed that the purified rat-liver enzyme cross-reacted with rabbit antiserum raised against GSTZ1-1, which confirmed the similarity of the purified rat-liver enzyme with GSTZ1-1. Anti-human GST Zeta antibodies cross-reacted with a cytosolic protein, but only when relatively high amounts of protein, i.e. 50 μ g/lane, were loaded on to the gel, indicating that GST Zeta is present in low concentrations in rat-liver cytosol. Hence, these results show that the purified rat liver enzyme is an orthologue of human GST Zeta.

Zeta-class GSTs represent a newly identified evolutionary class of GSTs that are present in a range of phylogenetic groups [17]. Human GST Zeta has been cloned and expressed in *Escherichia coli*, and substrate-selectivity studies showed that

Figure 3 Immunoblot analysis of rat-liver cytosol and the purified enzyme from rat-liver cytosol

The blot was prepared and probed with anti-human GST Zeta antibodies, as described in the Experimental section. Lane 1, protein markers; lane 2, rat-liver cytosol (50 μ g); lane 3, rat-liver cytosol (10 μ g); lane 4, purified enzyme (1 μ g); and lane 5, purified enzyme (0.5 μ g).

Table 2 Catalytic activities of the purified rat-liver enzyme and recombinant human GST Zeta

The enzyme preparations were prepared and assayed as described in the Experimental section. The data are presented as means $\pm$ S.D., $n = 3$.

Substrate	Activity (nmol/min per mg of protein)	
	Purified rat-liver enzyme	Recombinant human GSTZ1-1
Dichloroacetic acid	1110 $\pm$ 60.3	1400 $\pm$ 19.4
t-Butyl hydroperoxide	657 $\pm$ 48.3	170 $\pm$ 4*

* Data from Table 1 of reference 17.

human GSTZ1-1 has peroxidase activity with cumene hydroperoxide and t-butyl hydroperoxide as substrates, but little activity with other known substrates for GSTs, including 1-chloro-2,4-dinitrobenzene, ethacrynic acid and 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole [17]. In contrast, both the purified rat-liver and recombinant human Zeta-class GSTs showed high and similar activities with DCA as the substrate.

The GSTZ1-1 is a homodimer with 24.2 kDa subunits [17]. In the present studies, SDS/PAGE and HPLC gel-filtration chromatography showed that the purified rat-liver enzyme had a molecular mass of 27.2–28.2 kDa. Size variations in human-muscle GST Zeta have also been observed [17]. HPLC gel-filtration chromatography of the rat-liver enzyme only showed the presence of monomeric GST Zeta, even with buffers of low salt concentration, indicating a weak association between the subunits of the rat-liver Zeta-class GST.

The knowledge that GST Zeta catalyses the glutathione-dependent oxygenation of DCA has important implications for carcinogen risk assessment, presuming that the observed hepatocarcinogenicity of DCA in rats and mice is associated with GST Zeta-dependent bioactivation of DCA. The present studies show similar rates of biotransformation of DCA with the purified rat-

liver and recombinant human enzymes, and previous studies have shown that the elimination half-lives for DCA are similar in rats and humans [16]. Further studies are needed to examine rates of DCA bioactivation in tissues from humans and from rats and mice, in which DCA is carcinogenic.

Studies designed to elaborate the mechanism of the GST Zeta-catalysed oxygenation of DCA are warranted. Preliminary studies with [13 C]DCA indicate that [13 C]glyoxylic acid is the only observable stable metabolite formed. Also, glutathione is required, but not consumed, for the oxygenation of DCA. These results are consistent with a reaction mechanism that involves displacement of chloride by glutathione to afford *S*-(α -chlorocarboxymethyl)glutathione as an intermediate; solvolysis would give the hemithioacetal *S*-(α -hydroxycarboxymethyl)-glutathione, which may eliminate glutathione to give glyoxylic acid. Similarly, *S*-chloromethylglutathione has been identified as an intermediate in the GST Theta-catalysed oxygenation of dichloromethane [23].

In summary, the results show that the glutathione-dependent oxygenation of DCA to glyoxylic acid is catalysed by the Zeta-class GSTs.

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