Heterologous expression, purification and characterization of rat class Theta glutathione transferase T2-2

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Rat glutathione transferase (GST) T2-2 of class Theta (rGST T2-2), previously known as GST 12-12 and GST Yrs-Yrs, has been heterologously expressed in *Escherichia coli* XL1-Blue. The corresponding cDNA was isolated from a rat hepatoma cDNA library, ligated into and expressed from the plasmid pKK-D. The sequence is the same as that of the previously reported cDNA of GST Yrs-Yrs. The enzyme was purified using ion-exchange chromatography followed by affinity chromatography with immobilized ferric ions, and the yield was approx. 200 mg from a 1 litre bacterial culture. The availability of a stable recombinant rGST T2-2 has paved the way for a more accurate characterization of the enzyme. The functional properties of the recombinant rGST T2-2 differ significantly from those reported earlier for the enzyme isolated from rat tissues. These differences

probably reflect the difficulties in obtaining fully active enzyme from sources where it occurs in relatively low concentrations, which has been the case in previous studies. 1-Chloro-2,4-dinitrobenzene, a substrate often used with GSTs of classes Alpha, Mu and Pi, is a substrate also for rGST T2-2, but the specific activity is relatively low. The K_m value for glutathione was determined with four different electrophiles and was found to be in the range 0.3 mM–0.8 mM. The K_m values for some electrophilic substrates were found to be in the micromolar range, which is low compared with those determined for GSTs of other classes. The highest catalytic efficiency was obtained with menaphthyl sulphate, which gave a k_{cat}/K_m value of $2.3 \times 10^6 \text{ s}^{-1} \cdot \text{M}^{-1}$ and a rate enhancement over the uncatalysed reaction of 3×10^{10} .

INTRODUCTION

Glutathione transferases (GSTs) catalyse the conjugation of glutathione (GSH) with a variety of electrophilic compounds. In mammals the cytosolic GSTs have been grouped into four different classes: Alpha, Mu, Pi and Theta, based upon sequence similarities [1,2].

The GSTs seem to be serving mainly as parts of the cellular detoxification system. GSTs may have evolved to provide protection against an oxygen-containing environment and the resulting reactive oxygen metabolites. Class Theta GSTs show more sequence similarity with plant and prokaryotic GSTs than do class Alpha, Mu and Pi GSTs. They have therefore been proposed to represent the ancestral forms from which the members of the other classes have descended [3]. A GST, showing 36% N-terminal sequence identity with rat GST T2-2, has also been found in mitochondria [4].

In comparison with other GSTs, class Theta enzymes have proved difficult to isolate and characterize. Following some early work [5,6] they have only relatively recently been more extensively studied and recognized as a separate class of mammalian GSTs. Class Theta GSTs are reported not to bind to the affinity matrices based on immobilized GSH derivatives used for purifying enzymes of the other three classes [7,8] and their activity with the substrate 1-chloro-2,4-dinitrobenzene (CDNB), commonly used when purifying GSTs, is significantly lower than those measured with the Alpha, Mu and Pi class enzymes [2,9].

Class Theta GSTs have approx. 7% overall amino acid sequence identity with members of the other three classes [3]. The conserved region giving the 7% identity is centred around amino acid 70 in rGST T2-2.

The tertiary structure has been determined for one Alpha class

[10], two Mu class [11,12] and three Pi class [13–16] GSTs. Their overall chain-fold is essentially the same. The crystal structure of a GST from the Australian sheep blowfly *Lucilia cuprina* has recently been published [17], and the tertiary structure resembles those of the other GSTs. The *Lucilia* enzyme has been proposed to be a class Theta GST, based on primary structure similarities with mammalian class Theta GSTs and an important difference in the active site as compared with the class Alpha, Mu and Pi GSTs. This difference involves the tyrosine close to the N-terminus, catalytically important in previously reported GST structures. The phenolic hydroxy group of the tyrosine residue is interacting with the sulphur of GSH and thereby stabilizes its thiolate form. In the *Lucilia* structure the tyrosine in this position is pointing away from the active site rather than being directed toward GSH. Ser-9 was proposed as the residue activating GSH in the *Lucilia* GST, and a recent study lends support to this assumption [18].

So far, two distinct Theta GSTs have been identified in man, GST T1-1 and GST T2-2 [2,19]; three in the rat, designated GSTs 5-5, 12-12 or Yrs-Yrs, and 13-13 [2,4,9,20]; and one in the mouse [21]. A new nomenclature is proposed (cf. [22]) and according to this the rat enzymes GST 12-12 (or GST Yrs-Yrs) and GST 5-5 should be called rGST T2-2 and rGST T1-1 respectively. Throughout this paper we will use this new nomenclature. Two isoforms have been reported for the subunit Yrs, i.e. Yrs and Yrs'. Yrs' probably derives from a post-translational modification of Yrs [21,23]. The two identified human class Theta GSTs have been cloned [24,25] as well as their rat counterparts [3,26]. Rat GST T1-1 (GST 5-5) has been expressed in *Salmonella typhimurium* [27].

This paper describes the heterologous expression, purification and characterization of rGST T2-2. The enzyme has been cloned

Abbreviations used: CDNB, 1-chloro-2,4-dinitrobenzene; EPNP, 1,2-epoxy-3-(4-nitrophenoxy)propane; GST, glutathione transferase (EC 2.5.1.18); i.d., internal diameter; IMAC, immobilized metal ion affinity chromatography; MS, 1-menaphthyl sulphate; NBC, 4-nitrobenzyl chloride; NPG, 3-(4-nitrophenyl)glycidol.

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and sequenced before [26] and it has been purified from liver [2,9], but heterologous expression has not been reported. The functional properties of the recombinant enzyme differ significantly from those reported earlier for the enzyme isolated from rat tissues. These differences probably reflect the difficulties in obtaining fully active enzyme from sources in which it occurs in relatively low concentrations.

EXPERIMENTAL

All chemicals used were of the highest purity available. Enzymes and reagents for recombinant DNA work were from Boehringer-Mannheim (Mannheim, Germany) and Promega Corp. (Madison, WI, U.S.A.).

Molecular cloning

Two μl [10^{11} plaque-forming units (p.f.u.)/ml] of a rat hepatoma cDNA library [28] was treated with RNase A and DNase I at 37 °C for 30 min. Phages were precipitated with an equal volume of 20 % poly(ethylene glycol) and 2 M NaCl on ice for 1 h. After phenol/chloroform extraction the DNA was precipitated with 1 vol. of propan-2-ol and dissolved in water.

The PCR was carried out in a total volume of 100 μl in 10 mM Tris/HCl, pH 8.3, 50 mM KCl, 1 mM 2-mercaptoethanol, 1 % (w/v) gelatin, 1 mM MgCl_2 , 0.2 mM of each dNTP, various amounts (containing a number of cDNA molecules 2–10 times the library size of approx. 10^6 individuals) of the rat hepatoma cDNA, 2.5 units of AmpliTaq DNA Polymerase (Perkin-Elmer, Cetus Instruments, Emeryville, CA, U.S.A.) and 0.6 mM each of the primers (5'-GAATTC ATG GGT TTG GAG CTC TAC) and (5'-GAATTC TTA GGG AAT CCT GGC AAT TCG) (custom-synthesized by Operon Technologies Inc., Alameda, CA, U.S.A.). The reaction was started with 10 min at 95 °C and followed by 30 cycles of 1 min at 94 °C, 2 min at 60 °C and 3 min at 72 °C; 10 min at 72 °C ended the reaction.

PCR products of approx. 750 bp were recovered from a 1 % (w/v) agarose gel after electrophoresis and purified by chromatography on a Gene Clean silica matrix according to the manufacturer's recommendations (BIO 101, La Jolla, CA, U.S.A.). Fragments were ligated into the *EcoRI* restriction sites of pGEM-3Zf(-) vectors (Promega Corp., Madison, WI, U.S.A.) used to transform competent *E. coli* XL1-Blue cells (Stratagene, La Jolla, CA, U.S.A.). To make sequencing of the entire cDNA possible, the fragments were cleaved with *XbaI* and subcloned into the *EcoRI* and *XbaI* cloning sites of pGEM-3Zf(-). Double-stranded DNA sequencing of four different clones was performed with a slightly modified protocol of Sanger's dideoxynucleotide method involving Sequenase® (US Biochemical Corp., Cleveland, OH, U.S.A.). The fragments were ligated into the *EcoRI* cloning site of the pKK-D vector [29], and the resulting expression clone was called pKRT2. Competent *E. coli* XL1-Blue cells were transformed with the expression constructs and then distributed on LB-ampicillin agar plates. Colonies were cultured overnight and the plasmids purified using Magic minipreps DNA purification resin (Promega). Restriction analysis was performed using *EcoRI* and *HindIII* to verify that the cDNA was in the correct orientation for expression.

Heterologous expression of rGST T2-2

Bacteria were grown in Erlenmeyer flasks containing 500 ml of medium (20 g tryptone, 15 g yeast extract, 5 g NaCl and 10 g glycerol dissolved in 1 l of water) containing ampicillin (50 μg per ml). Two flasks were each inoculated with 5 ml of an overnight culture of *E. coli* XL1-Blue containing the pKRT2

plasmid and incubated at 37 °C in a rotary shaker. When D_{600} of the medium reached 0.2, isopropyl β -D-thiogalactopyranoside was added to a final concentration of 0.2 mM to induce expression of rGST T2-2. The flasks were incubated overnight (approx. 15 h) and the cells were harvested by centrifugation at 2500 *g* for 15 min. The cells were resuspended in 20 mM Tris/HCl buffer, pH 8, sonicated (4 $\times$ 20 s) and centrifuged at 30000 *g* for 20 min. This procedure was repeated twice and the pooled lysate was centrifuged at 120000 *g* for 60 min. The pH was adjusted to 7.5 using 1 M Tris base.

Purification

Ion-exchange chromatography was performed on a column [6.2 cm $\times$ 3.2 cm internal diam. (i.d.)] packed with DEAE-Sepharose 6B (Pharmacia Biotech, Uppsala, Sweden) and equilibrated with 20 mM Tris/HCl, pH 7.5 (buffer A). The loading and washing was carried out at a flow rate of approx. 1.8 ml/min and a temperature of 4 °C. The column was developed with 1.0 M NaCl in buffer A and flow-through fractions containing GST activity were pooled (DEAE pool).

Immobilized ferric ion affinity chromatography (Fe-IMAC) was performed on a column (4.0 cm $\times$ 3.2 cm i.d.) of chelating Sepharose FF (Pharmacia Biotech), charged with filter-sterilized 20 mM FeCl_3 at 2 ml/min flow rate. Excess metal ions were removed with deionized water. The flow rate was increased to 4 ml/min and the column equilibrated with five column vols. of 20 mM Pipes, pH 6.5 (buffer B). The pH of the DEAE pool containing rGST T2-2 was adjusted to pH 6.5 by adding approx. 75 μl of 1.0 M Pipes, pH 6.5, per ml. The sample was loaded on to the column and the non-adsorbed material eluted with buffer B. Elution of bound non-GST proteins was effected with 1.0 M NaCl in buffer B. Rat GST T2-2 was eluted by an increase in pH from 6.5 to 7.5 in buffer B.

The purity at each stage of the purification was checked by size-exclusion chromatography on a Superose 6B column using the FPLC system and by electrophoresis in the Phast system (Pharmacia Biotech) using Phast Gel gradient 8-25 and Phast gel SDS buffer strips. The gels were stained with Coomassie Brilliant Blue. The purity was also checked by SDS/PAGE [30], using a 12.5 % (w/v) polyacrylamide gel and a 16 cm $\times$ 20 cm Protean II system (Bio-Rad, Richmond, CA, U.S.A.). The protein bands were visualized by silver staining [31].

Immunoreactivity was checked by dot-blot and Western blot analyses with polyclonal antiserum raised against rGST T1-1 [2].

Activity assays and binding studies

All enzyme activity assays were performed at 30 °C. The activities towards CDNB, 1,2-epoxy-3-(4-nitrophenoxy)propane (EPNP), 4-nitrobenzyl chloride (NBC); 1,2-dichloro-4-nitrobenzene and ethacrynic acid were measured as described by Habig and Jakoby [32]. Other substrates used were 1-menaphthyl sulphate (MS) [5], cumene hydroperoxide [33], (2*R*,3*R*)- and (2*S*,3*S*)-3-(4-nitrophenyl)glycidol (NPG) [34] and 4-hydroxyhept-2-enal and 4-hydroxyoct-2-enal [35]. Phenethyl isothiocyanate (0.125 mM) conjugation to GSH was measured spectrophotometrically at 270 nm in the presence of 2.5 % (v/v) dimethylformamide [36]. Additional assay conditions are given in the legends to the Tables.

MS, CDNB, NBC and EPNP were chosen as electrophilic substrates for determination of K_m , k_{cat} and k_{cat}/K_m values. Six to ten different substrate concentrations were used and GSH was held constant at saturating concentrations of 5 mM or 10 mM. Kinetic parameters were also established by varying the GSH concentration (0.05 mM–5.0 mM) at saturating concentrations

of electrophiles (in most cases 0.5 mM). Three to five replicates of rate determinations were made at each concentration. The k_{cat} and k_{cat}/K_m values were calculated on an active-site basis using a subunit molecular mass of 27300 Da. Kinetic data were analysed by use of the SIMFIT program [37].

The dissociation constant, K_s , for rGST T2-2 with MS was determined at 20 °C by measuring the quenching of the intrinsic tryptophan fluorescence of the enzyme upon ligand binding. Two different enzyme active-site concentrations, 0.2 μM and 0.5 μM , were used and the concentration of MS in the cuvette was increased in steps of 0.2 μM . The measurements were performed in 50 mM triethanolamine/HCl, pH 8.3, in an Aminco spectrofluorimeter using $\lambda_{\text{excitation}} = 280 \text{ nm}$ and $\lambda_{\text{emission}} = 340 \text{ nm}$. Binding parameters were determined as described by Gutfreund [38].

RESULTS

Expression and purification

Heterologous expression of rGST T2-2 was accomplished in *E. coli* XL1-Blue. After sonication and centrifugation of the bacteria, the resulting soluble fraction was loaded on to a DEAE-Sephacrose column. Rat GST T2-2 was hardly retarded at all and most of the enzyme appeared in the flow-through peak (Figure 1). Two fractions containing the major part of the activity were pooled and used for further purification.

IMAC on immobilized Fe(III) ions was the final step in the purification. Frontal analysis was used to determine the capacity of the gel as approx. 5 mg of rGST T2-2 per ml of chelating

Figure 1 Purification of recombinant rGST T2-2 on a DEAE-Sephacrose 6B column

The pH of the crude *E. coli* lysate containing the recombinant protein was adjusted to 7.5 using 1 M Tris base. The lysate was then subjected to anion-exchange chromatography on a DEAE-Sephacrose 6B column (6.2 cm $\times$ 3.2 cm i.d.) equilibrated with 20 mM Tris/HCl, pH 7.5. Fractions were collected in 10 ml aliquots at a flow rate of 1.8 ml/min. Rat GST T2-2 activity was essentially unretained and collected in the flow-through fractions 3 and 4. These were pooled for further purification. Bound proteins were eluted with 20 mM Tris/HCl, pH 7.5, containing 1 M NaCl; the change of eluent is indicated by an arrow. GST activity (■) was measured with MS as electrophilic substrate and protein concentration (○) was estimated by determination of absorbance at 260 and 280 nm.

Figure 2 Purification of recombinant rGST T2-2 on a Fe(III)-chelating Sepharose FF column

The pH of the pooled fractions from the anion-exchange chromatography was adjusted to 6.5 by adding 1 M Pipes-NaOH, pH 6.5, and the resulting sample used for IMAC on immobilized Fe(III) ions. The column (4.0 cm $\times$ 3.2 cm i.d.) was packed with chelating Sepharose FF and charged with 5 column volumes of filter-sterilized FeCl_3 . Excess Fe(III) ions were removed with deionized water and the column was equilibrated with 20 mM Pipes, pH 6.5. Fractions of 10 ml were collected at a flow rate of 4 ml/min. The column was rinsed with 20 mM Pipes, pH 6.5, containing 1 M NaCl, as indicated in the Figure. This resulted not only in elution of undesired proteins but also in delayed isocratic elution of rGST T2-2 (fractions 35–61). Finally, the column was developed with 20 mM Pipes, pH 7.5 containing 1 M NaCl which gave a sharp rGST T2-2 peak. The enzyme is pure both in the fractions of the delayed elution at pH 6.5 and in those of the elution at pH 7.5. GST activity (■) and protein concentration (○) were determined as described in Figure 1.

Table 1 Purification scheme for rGST T2-2

Protein concentration was estimated by determination of absorbance at 260 and 280 nm. Activity was measured with MS as electrophilic substrate and GSH at a non-saturating concentration, hence the low specific activity in the pure Fe-IMAC pool (cf. Table 2).

Purification step	Total protein (mg)	Total activity ($\mu\text{mol} \cdot \text{min}^{-1}$)	Specific activity ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)	Purification (-fold)	Yield (%)
Lysate	1300	520	0.4	1	100
DEAE pool	490	550	1.1	2.8	106
Fe-IDA pool	200	420	2.1	5.3	81

Sephacrose. Rat GST T2-2 started to emerge slowly during the washing step, 1 M NaCl in 20 mM Pipes (pH 6.5), and could be recovered by prolonged elution. In order to obtain the enzyme in a more concentrated form the elution was carried out at pH 7.5. The enzyme was pure as isolated by either elution procedure, except that the very last fractions obtained by the latter procedure contained some contaminating proteins (Figure 2).

The yield of recombinant rGST T2-2 after purification was approx. 200 mg from 1 litre of culture medium. The amount of rGST T2-2 in the crude lysate was 250 mg as estimated by the specific activity of the purified enzyme. The purification factor of 5.3 for obtaining homogeneous enzyme (Table 1) indicates an

Figure 3 Analytical size-exclusion chromatography of rGST T2-2 at different stages of the purification

Superose 6B HR 30/10 was equilibrated with 20 mM Tris/HCl, pH 7.5, containing 0.25 M NaCl and 200 μ l of sample was loaded at 0.4 ml/min flow rate. (a) Crude lysate; (b) DEAE pool; (c) rat GST T2-2 eluted from Fe(III)-chelating Sepharose FF. The absorbance at 280 nm [arbitrary units (AU)] is plotted against the elution volume (ml). The arrow marks the elution position of rGST T2-2.

abundance close to 20 % of the soluble protein of the *E. coli* cells. Instability and loss of activity were reported for rGST T2-2 when purified from liver extract [2]. Recombinant rGST T2-2 has been stored in 20 mM Pipes, pH 6.5, 1 M NaCl at 4 °C for more than a year and most of its activity was still retained.

Table 1 shows the purification scheme for rGST T2-2. The enzyme was pure as judged both from silver-stained SDS/

polyacrylamide gels (results not shown) and from analytical size-exclusion chromatography (Figure 3). The enzyme displayed immunoreactivity with antibodies raised against the related rGST T1-1 (results not shown).

The molecular mass of the rGST T2-2 subunit is 27311 Da and its isoelectric point is 7.98, both calculated from the amino acid sequence deduced from the cDNA structure [26]. The enzyme purified from liver had an apparent molecular mass of 26 kDa on SDS/PAGE and an isoelectric point of 7.9 [9]. The molecular mass of the recombinant enzyme was 27.5 kDa determined by SDS/PAGE and the isoelectric point was 7.9, in good agreement with the published values (results not shown).

By quantitative amino acid analysis of a total hydrolysate of rGST T2-2 a specific absorption coefficient of $1.22 \text{ l} \cdot \text{g}^{-1} \cdot \text{cm}^{-1}$ at 280 nm was determined.

Catalytic properties of the purified enzyme

The specific activities for rGST T2-2 with different substrates are given in Table 2. NBC and MS are good substrates for the enzyme, and CDNB, cumene hydroperoxide, EPNP and (2*R*,3*R*)-NPG also serve as substrates for rGST T2-2. However, no activity could be detected with ethacrynic acid, although such has been reported [9,23].

The K_m^{GSH} value for rGST T2-2 varies between 0.3 mM and 0.8 mM, depending on the second substrate (Table 3). These values are thus in the same range or slightly higher than those determined for other GSTs [39]. K_m^{NBC} , K_m^{EPNP} and especially K_m^{MS} are very low, i.e. in the micromolar range. The $k_{\text{cat}}/K_m^{\text{GSH}}$ values are low compared with those for the electrophiles owing to the much lower K_m values of the latter substrates. The $k_{\text{cat}}/K_m^{\text{MS}}$ value is high ($2.3 \times 10^6 \text{ s}^{-1} \cdot \text{M}^{-1}$ at pH 7.5) not only as compared with other substrates tested, but also on an absolute

Table 2 Specific activities for recombinant rGST T2-2 with different substrates compared with other preparations of Theta class GSTs

Experimental conditions were as detailed. The GSH concentration was 10 mM except with the following substrates: Δ^5 -androsterone-3,17-dione where it was 0.1 mM; cumene hydroperoxide, 1.0 mM; phenethyl isothiocyanate, 1.0 mM; 1,2-dichloro-4-nitrobenzene, 5 mM; ethacrynic acid, 5 mM and the 4-hydroxyalkenals, 0.5 mM. Activity with Δ^5 -androsterone-3,17-dione was measured in triethanolamine/HCl, pH 8.3; MS in sodium phosphate, pH 7.5, 1 mM EDTA; 1,2-dichloro-4-nitrobenzene in sodium phosphate, pH 8.0, 1 mM EDTA; cumene hydroperoxide in sodium phosphate, pH 7.0, 1 mM EDTA; and all other substrates in sodium phosphate, pH 6.5, 1 mM EDTA. All reactions were performed at 30 °C in a final volume of 1 ml. N.D., no detectable activity.

Substrate	Concn (mM)	Specific activity ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)			
		rGST T2-2			
		Recombinant (this work)	GST Yrs-Yrs*	hGST T2-2†	rGST T1-1‡
Δ^5 -Androstene-3,17-dione	0.068	ND	—	—	—
CDNB	0.50	0.7 ± 0.1	≤ 0.1	ND	< 0.5
Cumene hydroperoxide	1.5	2.4 ± 0.3	1.76	6.9 ± 0.12	41
1,2-Dichloro-4-nitrobenzene	1.0	ND	ND§	—	—
EPNP	0.50	0.9 ± 0.1	ND	ND	180
Ethacrynic acid	0.25	ND	0.25	—	—
4-Hydroxyhept-2-enal	0.1	0.06 ± 0.02	—	—	—
4-Hydroxyoct-2-enal	0.1	0.12 ± 0.02	—	—	—
MS	0.075	4.3 ± 0.3	—	0.497 ± 0.019	—
Phenethylisothiocyanate	0.125	0.03 ± 0.02	—	—	—
NBC	0.50	3.7 ± 0.2	ND¶	ND	86
(2 <i>R</i> ,3 <i>R</i>)-NPG	0.50	0.34 ± 0.03	—	—	—
(2 <i>S</i> ,3 <i>S</i>)-NPG	0.50	ND	—	—	—

* Hiratsuka et al. [9].

† Hussey and Hayes [19].

‡ Meyer et al. [2].

§ Less than 2 nmol/min per mg.

|| Less than 10 nmol/min per mg.

¶ Less than 5 nmol/min per mg.

Table 3 Kinetic parameters for rGST T2-2 with different substrates

To determine kinetic constants for GSH, the concentration was varied between 0.050 mM and 5.0 mM or 0.10 mM and 10 mM at a constant electrophile concentration of 0.50 mM. Kinetic constants for the electrophiles were determined by varying their concentrations in the following ranges: MS 0.125 μ M–10 μ M (pH 8.3) and 1 μ M–450 μ M (pH 7.5); CDNB 25 μ M–500 μ M; NBC 1 μ M–500 μ M and EPNP 5.0 μ M–500 μ M. In these cases the GSH concentration was 10 mM (NBC and MS, pH 7.5) or 5 mM. All experiments were performed at 30 °C in 0.1 M sodium phosphate, 1 mM EDTA, except at pH 8.3 where 50 mM triethanolamine/HCl was used as the buffer.

Substrate variable (electrophilic substrate)	pH	K_m (μ M)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)
MS	8.3	0.8 ± 0.2	0.72 ± 0.06	900 ± 200
MS	7.5	0.8 ± 0.2	1.8 ± 0.1	2300 ± 300
GSH (MS)	7.5	740 ± 60	1.6 ± 0.1	2.2 ± 0.2
GSH (MS)	7.0	610 ± 60	1.2 ± 0.1	2.0 ± 0.2
GSH (MS)	6.5	540 ± 50	1.08 ± 0.04	2.0 ± 0.2
GSH (MS)	6.0	330 ± 30	1.20 ± 0.03	3.6 ± 0.3
CDNB	6.5	55 ± 15	0.26 ± 0.02	4.6 ± 1.0
GSH (CDNB)	6.5	310 ± 20	0.35 ± 0.01	1.1 ± 0.1
NBC	6.5	7 ± 1	1.4 ± 0.1	190 ± 30
GSH (NBC)	6.5	520 ± 50	1.7 ± 0.1	3.2 ± 0.3
EPNP	6.5	8 ± 3	0.29 ± 0.02	35 ± 10

scale [40]. The second-order rate constant for the non-enzymic reaction (in 0.1 M sodium phosphate, pH 7.5, 30 °C) was determined as $7 \times 10^{-8} s^{-1} \cdot M^{-1}$, and the rate enhancement afforded by the enzyme [$k_{cat}/(K_m \cdot k_2)$] was accordingly 3×10^{10} .

By measuring fluorescence quenching, a K_s value of 2 μ M for the binding of MS to rGST T2-2 was determined.

DISCUSSION

Purification

The high isoelectric point of rGST T2-2 suggested a cation exchanger as a first step of purification, but experiments resulted in loss of activity and an anion exchanger was employed instead. In order to bind most of the rGST T2-2 to the DEAE Sepharose-6B column, a pH above 9 would have to be used and this was outside the functional range for the gel. Using a buffer of pH 7.5 practically no rGST T2-2 bound, but other proteins were adsorbed and removed.

Various immobilized metal ions were tested for their ability to adsorb rGST T2-2 without causing loss of its catalytic activity. Fe(III) ions were found to be the best choice, with respect to both recovery and capacity. Rat GST T2-2 expressed similar affinity towards immobilized Fe(III) ions as other GST isoenzymes [41]. Fe-IMAC gave a pure rGST T2-2 pool and the yield was about 80 %. The gradual elution during washing of the column indicates that the adsorption of rGST T2-2 is partly due to electrostatic interactions with the adsorbent.

The successful purification of rGST T2-2 in high yield did not call for further tests of alternative procedures such as dye-affinity chromatography, previously used for class Theta GSTs [2,9].

Kinetics

Although cDNAs encoding several mammalian class Theta GSTs have been cloned, functional data have been obtained almost exclusively from enzymes purified from tissues [2,4,9,19,21,23,39] and not from heterologously expressed proteins. The availability in this study of large quantities of a stable recombinant class Theta GST has made possible more accurate and extensive

functional characterization than before. Major differences in specific activities were found in comparison with those previously reported and novel kinetic data are presented. Thus, the notion that class Theta GSTs in general have high K_m values for GSH [39], and that they lack activity with CDNB as electrophilic substrate does not apply for rGST T2-2. On the other hand, the high activity with MS, originally used in the identification of the GST activity [5] that is now assigned to the Theta class, is a salient feature of rGST T2-2.

The activity of rGST T2-2 is not restricted to benzylic sulphate esters [9], but includes uncharged compounds such as NBC (Table 2). Related aralkyl isothiocyanates, which have been shown to be excellent substrates for certain GSTs of the Mu and Pi classes [36,42,43] are not among the best substrates for rGST T2-2. A possible explanation is that the chemistry of the thiol addition to an organic isothiocyanate is different from the nucleophilic displacement reaction of the benzylic sulphate esters and the halogen compounds. The stereochemical requirements for the reaction to occur are not the same for the two classes of reactions. Interestingly, the enzyme has activity with (2*R*,3*R*)-NPG but not with (2*S*,3*S*)-NPG, demonstrating significant stereoselectivity with this pair of chiral compounds. High stereoselectivity of rGST T2-2 has previously been observed in the catalysis of substitution reactions involving chiral bromocarboxylic acids and their urea derivatives [44].

CDNB, a good and often used substrate for classes Alpha, Mu and Pi, serves as a substrate also for rGST T2-2, but the specific activity is comparatively low. Since MS is difficult to get access to, CDNB is a good alternative when monitoring the purification. The major drawback is the high rate of the uncatalysed reaction. NBC on the other hand has a negligible non-enzymic reaction rate at pH 6.5 and low substrate concentrations and is an even better choice.

GST Yrs-Yrs and GST Yrs'-Yrs' were reported to have specific activities of 0.25 and 2.5 μ mol $\cdot$ min $^{-1} \cdot$ mg $^{-1}$, respectively, with ethacrynic acid, but no activity was detectable with the recombinant enzyme in the present study. Nevertheless, the specific activities of rGST T2-2 determined, with the exception of ethacrynic acid, were higher or even considerably higher than the values published previously [9,23]. They also differ from the activities of rGST T1-1 published [2], which with several substrates seem to be much higher than those of rGST T2-2 (Table 2). The enzymes used in the previous investigations were purified from liver and according to Meyer et al. [2] the stability of rGST T2-2 was very low leading to a poor recovery. Such lability could explain the low activities reported by Hiratsuka et al. [9]. Meyer et al. did not publish any rGST T2-2 activities because of the instability of the enzyme. In contrast, kinetic characterization of the stable recombinant enzyme presented no difficulties.

The K_m^{GSH} values obtained, assuming Michaelis-Menten kinetics, were in the range of 0.3 mM–0.8 mM (Table 3). The values are in the same order of magnitude as the published value of 1.34 mM for GSH using 5-hydroxymethylchrysene sulphate as second substrate [9]. Thus, the K_m^{GSH} values for rGST T2-2 are lower than the K_m^{GSH} values reported for rGST T1-1, which are 5.9 mM with 4-nitrophenethyl bromide as electrophilic substrate and 4.2 mM with NBC [39].

The K_m values of the electrophiles seem to be low in general. In particular the K_m^{MS} value is very low, 0.8 μ M, giving rise to a fairly large k_{cat}/K_m^{MS} value in the order of $10^6 s^{-1} \cdot M^{-1}$. The K_m^{MS} value is similar to the K_s^{MS} value of 2 μ M, obtained by fluorescence measurements and, if it is also assumed that the product of the catalysed reaction binds tightly, it is likely that the release of product is a rate-contributing step. The k_{cat} values for rGST T2-2 are in the range of 0.2–1 s^{-1} and thus quite modest compared

with those found for rGST T1-1, which are about two orders of magnitude higher using 4-nitrophenethyl bromide and NBC as electrophiles [39]. The $k_{\text{cat}}^{\text{MS}}$ value at pH 8.3 is lower than the $k_{\text{cat}}^{\text{MS}}$ value at pH 7.5, but this difference is somewhat uncertain since accurate activity values are difficult to obtain at the low substrate concentrations used at pH 8.3. However, the K_{m}^{MS} value is not significantly different at the two pH values. It is noteworthy that MS, which was used in the original studies of the enzyme [5], is the substrate giving the highest catalytic efficiency of those tested in the present investigation. The $k_{\text{cat}}/K_{\text{m}}^{\text{MS}}$ value of $2.3 \times 10^6 \text{ s}^{-1} \cdot \text{M}^{-1}$, determined at pH 7.5, corresponds to a rate enhancement of 3×10^{10} afforded by rGST T2-2. Similar catalytic efficiency and rate enhancement has previously been reported for rGST T1-1 with 4-nitrophenethyl bromide as substrate [39]. However, the high catalytic efficiency with the latter enzyme is primarily due to a relatively high k_{cat} value, whereas the high value obtained with rGST T2-2 and MS is due to a low K_{m}^{MS} value.

In conclusion, this paper sheds new light on the properties of the class Theta GSTs. In particular the low K_{m} values for some of the electrophiles and the high binding affinity for MS are noteworthy properties of rGST T2-2. However, further studies are required to find out if these features are characteristic also for the human counterpart, hGST T2-2, and the other cytosolic class Theta enzymes from rat and man, rGST T1-1 and hGST T1-1. It also remains to elucidate the catalytic mechanism of the class Theta GSTs, e.g. whether they make use of an active-site serine as does the *Lucilia* GST [18], or a tyrosine as do the members of the other three GST classes, for the stabilization of the thiolate of GSH. Such questions can be approached by mutagenesis as well as by structural studies of the recombinant rGST T2-2.

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