Mutagenesis of the active site of the human Theta-class glutathione transferase GSTT2-2: catalysis with different substrates involves different residues

Kian-Leong TAN*, Gareth CHELVANAYAGAM*, Michael W. PARKER† and Philip G. BOARD‡

*Molecular Genetics Group, Division of Molecular Medicine, John Curtin School of Medical Research, Australian National University, G.P.O. Box 334, Canberra, ACT 2601, and †The Ian Potter Foundation Protein Crystallography Laboratory, St Vincent's Institute of Medical Research, 41 Victoria Parade, Fitzroy, Victoria 3065, Australia

The role of serine-11 in the catalytic mechanism of recombinant human GSTT2-2 was examined by site-directed mutagenesis. Amino acid sequence comparison of the Theta-class isoenzymes has identified a conserved serine residue in the N-terminal domain [Wilce, Board, Feil and Parker (1995) *EMBO J.* **14**, 2133–2143]. This conserved serine has been implicated in the activation of the enzyme-bound glutathione [Board, Coggan and Parker (1995) *Biochem. J.* **311**, 247–250]. Mutating the equivalent serine (residue 11) of GSTT2-2 to Ala, Thr or Tyr abolished the catalytic properties of GSTT2-2 with cumene hydroperoxide and ethacrynic acid as second substrate. However, with 1-menaphthyl sulphate (MSu) as the second substrate, the specific activity of the S11A mutant was doubled, while the S11T mutant retained half the wild-type activity and the S11Y mutant was inactive. The role of Ser-11 in catalysis seems to vary with different second substrates. In the substitution reaction with MSu, GSTT2-2

activity appears to depend on the size of the Ser-11 replacement rather than the presence of a side-chain hydroxy group. In addition, the reaction rate appears to be a function of pH, and there is no non-enzymic reaction even at high pH. We demonstrated that a reaction between MSu and an alternative thiol such as L-cysteine or 2-mercaptoethanol can take place in the presence of S-methylglutathione and GSTT2-2. We propose that the catalytic activity of GSTT2-2 with MSu is preceded by a conformational or charge modification to the enzyme upon the binding of glutathione or S-methylglutathione. This is followed by the binding of MSu and the subsequent removal of the sulphate group, giving rise to the carbonium ion of 1-methylnaphthalene as the electrophile that reacts with the nucleophilic species. The reaction mechanism of GSTT2-2 with MSu may represent a novel function of GSTT2-2 as a glutathione-dependent sulphotase.

INTRODUCTION

Mammalian cytosolic glutathione transferases (GSTs) can be grouped into four distinct classes, termed Alpha, Mu, Pi and Theta, and a consensus nomenclature for the human isoenzymes has been agreed [1]. Amino acid sequence similarity within a class tends to be around 70% or greater, while inter-class sequence similarity is usually less than 30%. The most conserved region lies within the N-terminal half of the protein where the common glutathione binding domain is located, while the C-terminal region shows greater diversity in each isoenzyme. The diversity in the C-terminal sequence is likely to account for the broad specificity for the electrophilic substrate.

The structure and function of GSTs have been studied by various approaches. Examples of the many studies in this area include chemical modification [2,3], site-directed mutagenesis [4–7], construction of deletion mutants and chimaeric enzymes [8,9], photoaffinity labelling [10,11], X-ray diffraction studies [12–16] and NMR spectroscopy [17]. In general, these studies have suggested that the catalytic mechanism of GSTs depends on the ability of the isoenzymes to lower the pK_a of the thiol group of glutathione (GSH), resulting in the formation of a glutathione thiolate anion (GS^-) which acts as a nucleophile to attack the electrophilic centres of xenobiotic or endogenous substrates [18,19]. Studies of the three-dimensional structure of the Alpha-, Mu- and Pi-class isoenzymes supported by site-directed muta-

genesis have attributed this activation role predominantly to an N-terminal domain tyrosine residue which is conserved throughout all cytosolic GST classes. In lowering the pK_a of the thiol group of enzyme-bound glutathione, the hydroxy group of this tyrosine may function as a general base in abstracting the $S\gamma$ hydrogen [20] and subsequently stabilizing GS^- by hydrogen bonding ($Tyr-OH \cdots SG$) [5]. Site-directed mutagenesis of this tyrosine residue to phenylalanine decreased the 1-chloro-2,4-dinitrobenzene (CDNB)-conjugating activity of the mutant enzyme to less than 2% of that of wild-type enzymes of the Alpha, Mu and Pi classes [4–6].

Recently, the molecular cloning and expression of a cDNA encoding the human Theta-class isoenzyme GSTT2-2 has been described by Tan and co-workers [21,22]. The protein sequence as translated from the cDNA, as well as N-terminal amino acid sequencing [21,23], revealed the existence of a phenylalanine at position 9 and the absence of a conserved tyrosine residue near the N-terminal domain. This observation suggested the possibility that GSTT2-2 may utilize another residue in the catalytic mechanism. Recent studies of a Theta-like GST from *Lucilia cuprina* [16,24] have identified a serine residue that is conserved throughout all Theta-class isoenzymes and appears to be within hydrogen bonding distance of the glutathionyl sulphur. These studies have also shown that the conserved tyrosine in the N-terminal domain (Tyr-5) of the *Lucilia cuprina* GST is positioned away from the glutathionyl sulphur and, unlike its counterparts

in the Alpha, Mu and Pi classes, is unlikely to participate in the catalytic mechanism. Site-directed mutagenesis of Ser-9 in the *Lucilia cuprina* GST has confirmed its importance in catalysis [24]. To confirm that the equivalent serine residue plays an important catalytic role in the mammalian Theta-class GSTs, for which there is no crystal structure, we have mutated Ser-11 of GSTT2 to Ala, Thr or Tyr and evaluated the catalytic properties of the mutant enzymes with several substrates.

EXPERIMENTAL

Materials

Compounds used in the preparation of culture broth and buffers were purchased from Difco Laboratories (Detroit, MI, U.S.A.), Sigma Chemical Co. (St. Louis, MO, U.S.A.) and Ajax Chemical Co. (Sydney, Australia). Restriction endonucleases and their buffers were products of Pharmacia. *Taq* polymerase was purchased from Promega. A Sequenase® Version 2.0 DNA sequencing kit was obtained from USB. Oligonucleotides for PCR and site-directed mutagenesis were synthesized on an Applied Biosystems 380B DNA synthesizer (Applied Biosystems Inc., Foster City, CA, U.S.A.) by the Australian National University Biomolecular Resource Facility. [α -³²P]dATP for DNA sequencing was purchased from Brestec (Adelaide, Australia). Nitrocellulose membrane for dot blots was purchased from Bio-Rad Laboratories. Substrates used in the GST assays were obtained from the following sources: cumene hydroperoxide and 1-(chloromethyl)naphthalene from Aldrich Chemical Co.; reduced glutathione, ethacrynic acid and L-cysteine from Sigma; glutathione reductase from Boehringer Mannheim; 2-mercaptoethanol from BDH Chemicals Ltd. 1-Menaphthyl sulphate (MSu) was synthesized by the method of Clapp and Young [25]. S-Methylglutathione was synthesized by the reaction of reduced glutathione with methyl iodide at pH 9 in an ethanolic solution.

Enzyme assays

Glutathione peroxidase activity was determined by the procedure described by Beutler [26]. The determination of GST activity with MSu was essentially as described by Gillham [27], using a molar absorption coefficient for S-(1-menaphthyl)glutathione of $3.9 \text{ mM}^{-1} \cdot \text{cm}^{-1}$. Glutathione transferase assays using ethacrynic acid were previously described in detail by Mannervik and Widersten [28]. The reaction between L-cysteine or 2-mercaptoethanol and MSu in the presence of S-methylglutathione and GSTT2-2 was measured spectrophotometrically at 298 nm in 0.1 M Tris/HCl, pH 8.3, containing 10 mM S-methylglutathione, 20 mM L-cysteine (or 30 mM 2-mercaptoethanol) and 0.25 mM MSu, at 37 °C. The molar absorption coefficient for S-(1-menaphthyl)-L-cysteine is $4.8 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ [27], while that for S-(1-menaphthyl)-2-mercaptoethanol ($2.1 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ at 298 nm) was determined by allowing 50 nmol of MSu to react to completion with an excess of 2-mercaptoethanol in the presence of GSTT2-2. Sulphatase activity was determined by measuring sulphate production by a turbidimetric procedure after reaction with BaCl_2 in a gelatin solution [29].

Site-directed mutagenesis

A *Bgl*II–*Hind*III fragment of pRBKL3 [22] was subcloned into an M13 vector digested with *Bam*HI and *Hind*III to generate a single-stranded DNA template for site-directed mutagenesis. Oligonucleotides for site-directed mutagenesis of the N-terminal Ser-11 were: S11A, 5' GCTGGGCTGGGCCACCAAGGTC 3'; S11T, 5' GCTGGGCTGGGTCACCAAGGTC 3'; and S11Y, 5'

GCTGGGCTGGTACCAAGGTC 3'. Site-directed mutagenesis was performed using a Sculptor® *in vitro* mutagenesis kit (Amersham). The resulting mutations were confirmed by DNA sequencing using a Sequenase® Version 2.0 DNA sequencing kit after subcloning the *Sac*II and *Hind*III fragment into pRB269 [30]. The plasmids bearing the Ser-11 → Ala, Ser-11 → Thr and Ser-11 → Tyr mutations were given the names pRBS11A, pRBS11T and pRBS11Y respectively.

Construction of pQE expression vectors

A 354 bp fragment was amplified from a GSTT2 cDNA cloned in pRBKL3 using a forward primer QET2 (5' GCGGATCCGT-ACCCGCGGTATGGGCCTAGAGCTG 3') and a reverse primer HTB3 (5' ATACCAAAGGTGCCACGG 3'). Primer QET2 contained additional *Bam*HI and *Sac*II restriction sites, while primer HTB3 was expected to amplify a region spanning an *Sph*I site within the GSTT2 cDNA sequence. PCR was performed on a capillary thermal cycler (Corbett Research) in a 20 μ l reaction containing 200 μ M dNTPs, 10 pmol of each primer, 1.5 units of *Taq* polymerase (Promega) and 1 $\times$ *Taq* polymerase buffer (Promega). PCR conditions were as follows: a hot start (94 °C/10 s); 20 cycles of 94 °C/10 s, 56 °C/30 s, 72 °C/60 s; 10 cycles of 94 °C/10 s, 56 °C/30 s, 72 °C/80 s; and an extra extension cycle of 72 °C for 2 min. The amplified product was digested with *Bam*HI and *Sph*I and cloned into the same restriction sites in the QIAexpress System pQE30 (QIAGEN). The resulting plasmid, pQE130, was digested with *Sac*II and *Hind*III and ligated with a *Sac*II–*Hind*III fragment from pRBKL3, pRBS11A, pRBS11T or pRBS11Y to generate the wild-type and mutant pQE expression vectors. The pQE vector incorporates a tract of six histidine residues at the N-terminal end of the expressed protein to facilitate purification. The construction of the pQE(GSTT2) expression vectors is shown in Figure 1, and the expression constructs were termed pQEHT1, pQES11A, pQES11T and pQES11Y.

Protein expression and purification

For the production of recombinant protein, the wild-type or mutant expression plasmids were transformed into *Escherichia coli* host strain TG1 and were each grown in a 10 litre fermenter. Standard Luria Broth supplemented with 100 μ g/ml ampicillin was used as the growth medium. Isopropyl β -D-thiogalactoside was added to a final concentration of 0.1 mM when the density of the cells reached an A_{600} between 0.7 and 0.9. Bacterial cells were harvested by centrifugation and resuspended in buffer A (50 mM sodium phosphate, 300 mM NaCl, 10 % glycerol at pH 6.0). The cells were lysed by passage through a Ribi cell disruptor and stored frozen in 50 ml aliquots at –20 °C if not used immediately.

The wild-type and mutant GSTT2 isoenzymes were purified by immobilized metal ion chromatography. A 50 ml sample of cell lysate was diluted with buffer A to 80 ml and centrifuged at 2600 g for 20 min. The supernatant was mixed with 20 ml of a 50 % slurry of nickel-nitrilotriacetic acid resin (Ni-NTA; QIAGEN) pre-equilibrated in buffer A, and allowed to mix overnight on a rotating mixer at 4 °C. The resin was washed twice in buffer A by centrifugation and then packed into a Pharmacia XK16 column. The resin was washed with a continuous flow of buffer A at a rate of 1.0 ml/min at 4 °C until the A_{280} of the flow-through fraction was less than 0.01. The column was then mounted on to a Pharmacia FPLC system and developed with three linear imidazole gradients of 0–50 mM, 50–100 mM and 100–200 mM, pH 8.2, formed over a period of 20 min each. Each gradient was interspersed by elution for

Figure 1 Construction of the pQE expression vector

A forward primer QET2 with additional *Bam*HI and *Sac*II restriction sites and a reverse primer HTB3 were used to amplify a 5' coding region of pRBKL3. The amplified product (354 bp) was digested with *Bam*HI and *Sac*II and cloned into the same restriction sites in the QIAexpress System pQE30. The resulting plasmid pQE130 was digested with *Sac*II and *Hind*III and ligated with the *Sac*II–*Hind*III fragment from pRBKL3, pRBS11A, pRBS11T or pRBS11Y.

40 min with a constant imidazole concentration equivalent to the concentration achieved at the end of each gradient. The protein concentration was monitored by absorbance at 280 nm. Fractions containing the recombinant protein were detected by dot blotting on nitrocellulose and probed with rabbit polyclonal antibodies raised against recombinant GSTT2-2 [22] and anti-(rabbit IgG)–alkaline phosphatase as previously described by Board [31]. Fractions that showed a positive signal with the dot blot were pooled and concentrated using a Centricon-30 ultrafiltration apparatus (Amicon Inc.). To eliminate minor contaminating proteins the concentrated samples were loaded on to a Mono Q HR 5/5 FPLC column and developed with two linear NaCl gradients as previously described [22]. Protein concentrations were determined using the method described by Bradford [32].

Determination of kinetic parameters and pH profiles

Kinetic parameters for the wild-type and mutant enzymes were obtained with cumene hydroperoxide and MSu as the second substrates. The values of $V_{\max}$ and K_m were determined from the rate saturation curves of GSH and the second substrates when the concentration of one was fixed while varying the other. Table

Table 1 Substrate concentrations used in determining the kinetic parameters

Substrate	Expt.	Second substrate concn. (mM)	GSH concn. (mM)
Cumene hydroperoxide	1	0.35	0.10–15
	2	0.07–0.56	2.0
Ethacrynic acid	1	0.04–3.0	0.25
	2	0.2	0.1–2.0
MSu	1	0.05	0.1–4.0
	2	0.003–0.04	5.0

1 shows the different ranges of substrate concentrations used in determining the kinetic parameters. The value of k_{cat}/K_m was determined as $V/[S][E]_{\text{tot}}$ at low substrate concentrations when the rate saturation curve degenerates to first-order with respect to the concentration of the varied substrate [33]. The molecular mass of the enzyme used in these calculations was 54978 Da.

Activity–pH profiles for the wild-type and mutant recombinant proteins were determined with cumene hydroperoxide or MSu as the second substrate, using a previously described range of buffers [34]. Preliminary tests showed that the glutathione reductase added to the assay of glutathione peroxidase activity with cumene hydroperoxide was not rate limiting at any pH used in this study. The non-enzymic reaction rate at each pH was subtracted from the total recorded reaction rate and the results were expressed as a percentage of the maximum enzymic rate.

RESULTS

Protein expression in the pQE expression system

The pQE expression system offers a convenient means for the purification of recombinant enzymes. The extra six histidine residues tagged on to the N-terminus of the recombinant protein enable it to be rapidly purified by immobilized metal ion affinity chromatography. The His₆-tagged GSTs were eluted from the column between 100 and 200 mM imidazole, at pH 8.2. However, there were a few minor contaminating proteins in the region of 23 kDa and between 30 and 66 kDa. The minor contaminants were removed using a Mono Q FPLC column; the His₆-tagged proteins passed straight through while the contaminants were retained. The existence of contaminating proteins that co-elute with recombinant His₆-tagged proteins was also reported by

Table 2 Comparison of specific activities of wild-type and Ser-11 mutant enzymes

Wild-type recombinant GSTT2-2 was expressed in both the pQE system and the ubiquitin fusion system (Ubq). The mean $\pm$ S.D. were calculated from at least three replicates; n.d., not detectable.

Enzyme	Specific activity ($\mu\text{mol/min per mg of protein}$)		
	MSu	Cumene hydroperoxide	Ethacrynic acid
Wild type (pQE)	0.2367 ± 0.017	5.256 ± 0.041	0.1594 ± 0.010
Wild type (Ubq)	0.3167 ± 0.028	6.885 ± 0.482	0.290 ± 0.020
S11A	0.3517 ± 0.021	n.d.	n.d.
S11T	0.1792 ± 0.010	n.d.	n.d.
S11Y	n.d.	n.d.	n.d.

Figure 2 pH profiles of recombinant GSTT2-2 with MSu and cumene hydroperoxide as substrates, and effect of pH on non-enzymic reaction rates

Comparison of pH profiles between wild-type GSTT2-2 expressed in the pQE system ($\blacktriangle$) and the Ubiquitin-GSTT2 fusion system ($\square$) [22] with (A) cumene hydroperoxide or (B) menaphthyl sulphate as substrate. Activities of the recombinant enzymes are expressed as percentages of the highest activity obtained. (C) Non-enzymic rates of reaction between GSH and either MSu ($\blacktriangle$) or cumene hydroperoxide ($\square$) as substrate, measured as absorbance (OD) change/min.

other groups [35]. This method of protein expression and purification was adopted because it was anticipated that some mutant proteins would be devoid of GST activity and could be difficult to purify by the multiple ion-exchange and gel-filtration

Table 3 Kinetic parameters for recombinant GSTT2-2 with cumene hydroperoxide (Cu-OOH) as second substrate

Enzyme form	$V_{\max}^{\text{GSH}}$ ($\mu\text{mol/min}$ per mg)	K_m^{GSH} (mM)	$k_{\text{cat}}/K_m^{\text{GSH}}$ ($\text{mM}^{-1} \cdot \text{s}^{-1}$)	$K_m^{\text{Cu-OOH}}$ (mM)	$k_{\text{cat}}/K_m^{\text{Cu-OOH}}$ ($\text{mM}^{-1} \cdot \text{s}^{-1}$)
Wild type	8.3 ± 0.35	4.3 ± 0.48	1.3 ± 0.01	1.2 ± 0.58	7.7 ± 0.16

Table 4 Kinetic parameters for recombinant GSTT2-2 and its mutant forms with MSu as second substrate

Results are expressed as means $\pm$ S.D.

Enzyme form	$V_{\max}^{\text{GSH}}$ ($\mu\text{mol/min}$ per mg)	K_m^{GSH} (mM)	$k_{\text{cat}}/K_m^{\text{GSH}}$ ($\text{mM}^{-1} \cdot \text{s}^{-1}$)	K_m^{MSu} (mM)	$k_{\text{cat}}/K_m^{\text{MSu}}$ ($\text{mM}^{-1} \cdot \text{s}^{-1}$)
Wild type	0.27 ± 0.019	0.62 ± 0.138	0.24 ± 0.010	0.0048 ± 0.0026	28.92 ± 0.424
S11A	0.38 ± 0.014	0.37 ± 0.049	0.48 ± 0.011	0.0027 ± 0.0001	137.49 ± 7.301
S11T	0.22 ± 0.010	1.10 ± 0.070	0.13 ± 0.012	0.0067 ± 0.0013	19.93 ± 0.231

steps we have previously used to purify recombinant GSTT2-2 [22].

Kinetic studies and pH profiles

Wild-type recombinant GSTT2-2 expressed in the pQE system was compared with the same recombinant protein expressed in a co-translationally cleaved ubiquitin fusion protein system previously described by Tan and Board [22]. The GSTT2-2 expressed in the latter system has no additional N-terminal residues. However, both recombinant proteins showed essentially the same specific activity towards cumene hydroperoxide, ethacrynic acid and MSu (Table 2). This shows that the extra residues on the N-terminus of the recombinant GSTT2-2 do not interfere significantly with the activity and function of the enzyme. Furthermore, pH curves of GST activity with cumene hydroperoxide or MSu showed a similar profile for each enzyme (Figures 2A and 2B). With this confirmation, we proceeded to study the catalytic characteristics of the wild-type and mutant proteins expressed in the pQE system. The specific activities of each mutant enzyme with cumene hydroperoxide, ethacrynic acid or MSu as a substrate are shown in Table 2. The S11Y mutant was inactive with all substrates. In comparison, while the S11A and S11T mutants were inactive with cumene hydroperoxide and ethacrynic acid, they were both active with MSu as a substrate. In particular, the S11A mutant had a higher specific activity than that of the wild-type enzyme. Several kinetic parameters for the wild-type enzyme with cumene hydroperoxide as the second substrate are shown in Table 3, and the same values for the wild-type enzyme and the active Ser-11 mutants with MSu as a substrate are shown in Table 4. Mutating Ser-11 to Ala or Thr did not seem to impair the affinity of the enzyme for GSH, as shown by the apparent K_m values of the Ser-11 mutants, with MSu as the second substrate.

The GSH peroxidase activity of pQE-expressed GSTT2-2 varies with pH and adopts the same bell-shaped profile as for recombinant GSTT2-2 expressed in the ubiquitin fusion system

Table 5 Activity of MSu with L-cysteine or 2-mercaptoethanol as the alternative thiol substrate

The reaction was determined at pH 8.3, 37 °C, in a 1.0 ml cuvette. The final concentrations of the respective components were as follows: 0.25 mM MSu, 0.1 M Tris/HCl, 10 mM S-methylglutathione and 20 mM L-cysteine or 30 mM 2-mercaptoethanol (2-ME). The reaction rate was monitored by measuring absorbance at 298 nm. n.d., not detectable.

Reaction mixture	Enzymic activity ($\mu\text{mol}/\text{min}$ per mg of protein)
MSu + S-methylglutathione + 2-ME + GSTT2-2	0.3446 ± 0.0249
MSu + S-methylglutathione + L-Cys + GSTT2-2	0.1670 ± 0.0011
MSu + S-methylglutathione + GSTT2-2	n.d.
MSu + S-methylglutathione + 2-ME	n.d.
MSu + S-methylglutathione + L-Cys	n.d.
MSu + 2-ME + GSTT2-2	n.d.
MSu + L-Cys + GSTT2-2	n.d.
MSu + GSH	n.d.

Figure 3 Production of sulphate from MSu by GSTT2-2 in the presence of GSH (□), S-methylglutathione plus 2-mercaptoethanol (◇) and S-methylglutathione alone (○)

[22], with an optimum pH range of 7.6–8.5 (Figure 2A). The pH curve of GSH-conjugating activity with MSu did not show a similar bell-shaped profile for recombinant GSTT2-2 expressed in either expression system (Figure 2B). In this case, the enzyme activity increased as a function of the pH. Similar pH profiles with MSu were also obtained with the S11A and S11T mutants (results not shown). The non-enzymic rates of reaction that were subtracted from the data in Figures 2(A) and 2(B) are shown in Figure 2(C). Clearly, unlike most GST substrates, MSu does not show increased non-enzymic reaction rates at high pH.

Thiol specificity of GSTT2-2

Because the reaction between MSu and GSH does not involve Ser-11, and since its increasing rate with increasing pH resembles a non-enzymic chemical reaction, we investigated the MSu–glutathione conjugation reaction in greater detail. The enzyme shows a high specificity for glutathione as a thiol substrate, and compounds such as L-cysteine or 2-mercaptoethanol are not normally substrates (Table 5). However, as shown in Table 5, the specificity for GSH as the reactive thiol can be relaxed if L-cysteine or 2-mercaptoethanol is added in the presence of non-reactive S-methylglutathione.

GSTT2-2 as a sulphatase

Sulphate production from MSu was determined in the presence or absence of GSH or S-methylglutathione. The data in Figure 3 show the production of sulphate in the presence of GSH or S-methylglutathione plus 2-mercaptoethanol. Sulphate was also released in the presence of S-methylglutathione alone; however, the rate was only one-tenth of that in the presence of GSH. In the absence of a readily available nucleophile it might be expected that the benzylic carbon of MSu would gain a hydroxy group by reaction with water; however, we have not confirmed the presence of this product. It is also possible that, in the absence an alternative nucleophilic species, SO_4^{2-} could reassociate with the transitory carbonium ion of 1-methylnaphthalene, thus limiting the appearance of free SO_4^{2-} . Essentially no sulphate was released in the absence of GSH or S-methylglutathione.

DISCUSSION

The mammalian Theta-class GSTs are distinct from enzymes in the Alpha, Mu and Pi classes because they do not utilize the model substrate CDNB and do not bind to glutathione or S-hexylglutathione affinity matrices. Therefore the purification of the Theta-class GSTs has been more laborious, taking at least four or five chromatographic steps to purify the enzymes to apparent homogeneity [22,23,36]. As a multi-step purification scheme would largely preclude the purification of mutant enzymes with severely diminished activity, we adopted an alternative expression system for this study. The QIAexpress system adds six histidine residues to the N-terminus of the recombinant protein, which facilitates its purification on an immobilized Ni matrix. Comparison of the wild-type His-tagged enzyme with the enzyme produced without an N-terminal extension [22] did not indicate any substantial differences in enzymic function. Using the pQE system, we managed to purify wild-type and mutant GSTT2-2 species to apparent homogeneity in two chromatographic steps.

Recently, the crystal structure of a Theta-like GST from *Lucilia cuprina* was solved, and a serine residue at position 9 from the N-terminal domain was found to be within hydrogen-bonding distance of the glutathionyl sulphur atom [16]. Site-directed mutagenesis of this residue to alanine or threonine led to a marked decrease in GSH-conjugating activity with CDNB, confirming the importance of Ser-9 in catalysis [24]. Based on the co-ordinates for the *Lucilia cuprina* GST structure and an alignment of all the Theta-class amino acid sequences, we have constructed a homology model of GSTT2-2 [44]. As shown in Figure 4, the hydroxy group of Ser-11 appears to be within hydrogen-bonding distance of the enzyme-bound glutathionyl sulphur. Replacement of Ser-11 with Ala, Thr or Tyr showed that this serine residue is essential for the GSH peroxidase activity of GSTT2-2, as all three mutant enzymes lost activity completely with cumene hydroperoxide as the second substrate. A similar loss of activity was also observed for all Ser-11 mutants when ethacrynic acid was used as the second substrate (Table 2). Therefore it appears that Ser-11 plays an important role in the catalysis with cumene hydroperoxide and ethacrynic acid, presumably by activating the enzyme-bound GSH to form a reactive glutathione thiolate anion (GS^-), in a similar mechanism to that of the N-terminal domain tyrosine found in the Alpha-, Mu- and Pi-class GSTs.

An interesting contrasting observation was noted when MSu was used as the second substrate. The Ser-11 → Ala mutant showed a marked increase in specific activity, as well as a higher affinity for GSH and MSu. When the side-chain hydroxy group of residue 11 was preserved by replacing Ser-11 with Thr or Tyr,

Figure 4 Schematic stereo illustration of the N-terminal domain of recombinant GSTT2 highlighting the potential interaction between the GSH thiol and the side chain of Ser-11

The figure was generated by the program MOLSCRIPT [43] from the co-ordinates of a homology model of GSTT2 [44] based on the structure of a *Lucilia cuprina* GST [16].

a decrease in specific activity was observed that correlated with the size of the substituting residue. The Ser-11 $\rightarrow$ Thr mutation reduced activity with MSu to 50% of the wild type, while replacement of Ser-11 with a bulky residue like tyrosine abolished the conjugating activity of GSH with MSu completely. These data suggest that the hydroxy group side chain of Ser-11 is of little consequence in the reaction with MSu and that the size of the side chain on residue 11 is particularly significant. A favourable conformation of tyrosine substituted in position 11 in the homology model suggests that this mutation would occupy part of the GSH binding site. This may well explain the inactivity of the mutant enzyme with cumene hydroperoxide, ethacrynic acid and MSu. The k_{cat} value for MSu in the Ser $\rightarrow$ Ala mutant was 3-fold higher (0.3712 s^{-1}) than that of the wild type, while the k_{cat} value for the Ser $\rightarrow$ Thr mutant was comparable with that of the wild type ($\sim 0.1362 \text{ s}^{-1}$). This suggests that the high k_{cat}/K_m value for MSu for the S11A mutant is due to a faster product release as well as a higher affinity for MSu as the second substrate. It is possible that the elimination of the side-chain hydroxy group at residue 11 facilitates the entry of substrates as well as the release of products.

The conjugation reaction between GSH and MSu may employ a different reaction mechanism and clearly involves different residue(s) in the active site. Our data suggest that the reaction between GSH and MSu is not purely enzymic, as the increase in reaction rate with increasing pH appears more like a non-enzymic chemical reaction between a thiolate anion and a reactive electrophile. In most cases, GST substrates are electrophiles that will react non-enzymically with the glutathione thiolate anion at high pH. This is clearly not the case with MSu, as even at high pH, when glutathione should be fully deprotonized, MSu does not react non-enzymically with GSH (Figure 2C). However, GSH-conjugating activity with MSu increases as a function of pH in the presence of GSTT2-2 (Figure 2B). These observations suggest that the enzyme modifies the MSu substrate to a form that is reactive with GS^- , which is increasingly present at elevated pH values approaching the pK_a (~ 9) of glutathione in aqueous solution [37].

Gillham [38] reported the purification of a rat liver GST which has GSH-conjugating activity with MSu. This rat GST is likely to be the rat Yrs-Yrs isoenzyme [36], an orthologue of human GSTT2-2 and the only rat liver GST known to utilize MSu as a substrate. Gillham suggested that the reaction between GSH and MSu follows an Ordered Bi Bi mechanism in which GSH is first added to the enzyme before MSu. The order of product release

is SO_4^{2-} followed by *S*-(1-menaphthyl)glutathione. If the reaction of GSTT2-2 also follows an Ordered Bi Bi mechanism, then GSH has to be bound with the enzyme before MSu. We propose that GSH must be added first to the enzyme to induce a conformational change. This is followed by the entry of the second substrate, MSu. The SO_4^{2-} group of MS is then removed by the enzyme in a mechanism similar to the action of a sulphatase. Removal of SO_4^{2-} releases the newly formed 1-menaphthyl carbonium ion which reacts with the thiolate anion of GSH to form *S*-(1-menaphthyl)glutathione. The increase in the reaction rate between MSu and GSH at elevated pH can therefore be explained by the reaction between the carbonium ion and the GS^- species, which predominates at high pH. The reaction between the carbonium ion and GS^- may take place in free solution since Ser-11, which stabilizes the bound GSH as a thiolate anion, appears not to take part in the reaction.

In order to further investigate the function of GSTT2-2 as a GST and as a sulphatase, we utilized 1-(chloromethyl)naphthalene as an alternative substrate to MSu. As a GST, GSTT2-2 should catalyse the removal of the chloride group on the methyl side chain and give rise to *S*-(1-menaphthyl)glutathione, in the same fashion as the reaction between MSu and GSH. However, there was no detectable activity with this substrate, suggesting that the removal of the sulphate group on the methyl side chain of MSu is a specific reaction. Similarly, Gillham [27] demonstrated that a rat liver GST which utilized a range of aralkyl sulphate esters as substrates, including benzyl sulphate and 1-menaphthyl sulphate, did not utilize non-sulphate ester substrates such as benzyl orthophosphate, 1-menaphthyl acetate or 1-menaphthyl alcohol.

To study the reaction between MSu and GSH in greater detail, we replaced GSH with its analogue, *S*-methylglutathione, and used L-cysteine or 2-mercaptoethanol as alternative thiol substrates. As shown in Table 5, there was no detectable activity in the absence of *S*-methylglutathione, L-cysteine, 2-mercaptoethanol or GSTT2-2. However, if *S*-methylglutathione is added in combination with either L-cysteine or 2-mercaptoethanol in the presence of MSu and GSTT2-2, the formation of MSu-thiol conjugates can be detected spectrophotometrically. As shown in Figure 3, GSTT2-2 is able to catalyse the release of SO_4^{2-} from MSu in the presence of GSH. Significantly, this reaction also takes place in the presence of *S*-methylglutathione and a suitable nucleophile to react with the carbonium ion of 1-methylnaphthalene. Principato et al. [39] reported a relaxed thiol substrate specificity of rat GST4-4 utilizing 2-mercaptoethanol as an

alternative thiol substrate to CDNB in the presence of *S*-methylglutathione. They proposed a glutathione-induced conformational change in the enzyme, rendering a relaxed thiol specificity for any sterically acceptable thiol substrate(s).

Our data suggest that the reaction between MSu and GSH requires a conformational change or a change in the electrostatic environment of the active site induced by the binding of GSH or *S*-methylglutathione. An example of a similar substrate-induced modification is provided by the relatively flexible $\alpha 2$ helix of the Pi-class GSTs, which appears to undergo a conformational change upon binding of GSH in the G-site [40,41]. Furthermore, the three-dimensional structural analysis of human GSTA1-1 has suggested that the $\alpha 9$ helix, which is otherwise disordered in the apo-form, forms a lid over the H-site upon binding of the hydrophobic substrate [15]. While the standard reaction between GSH and MSu catalysed by GSTT2-2 results in the production of *S*-(1-menaphthyl)glutathione, the reaction between *S*-methylglutathione and MSu does not result in the formation of any glutathione conjugate. In this case, the unconjugated products released from the enzyme will be the carbonium ion and sulphate, which may rapidly reassociate in the absence of a nucleophile. In view of the results obtained in the present study, we propose that the route for inactivation of aralkyl sulphate esters by GSTT2-2 and rat Yrs-Yrs [36] involves binding of glutathione, desulphation, formation of a carbonium ion, conjugation with glutathione or a thiol substrate and the subsequent breakdown of the conjugated product in the mercapturic acid pathway [25]. Although we have shown *in vitro* that there is a relaxed specificity for the nucleophilic substrate in the presence of *S*-methylglutathione, it is highly probable that GSH acts as the nucleophile *in vivo* as it is likely to be the most abundant nucleophile in the intracellular environment.

Studies of the Alpha- and Mu-class GSTs have shown that residues other than the conserved tyrosine can contribute significantly to catalysis [7,42]. In the case of GSTT2-2, residue(s) other than Ser-11 must be responsible for its apparent sulphatase activity with MSu. Although our homology model of GSTT2-2 predicts the position of GSH, we cannot be certain about the position or orientation of MSu in the active site. This unfortunately precludes the reliable identification of residues that could contribute to the sulphatase activity. Resolution of this question will require the solution of the GSTT2-2 crystal structure with MSu as a ligand.

We are grateful to Dr. Gordon Barlin and Mr. Steve Ireland for the preparation of MSu. K.-L.T. received scholarships from the Australian National University and the AusAid scholarship scheme.

REFERENCES

- Mannervik, B., Awasthi, Y. C., Board, P. G., Hayes, J. D., Di, I. C., Ketterer, B., Listowsky, I., Morgenstern, R., Muramatsu, M., Pearson, W. R., Pickett, C. B., Sato, K., Widersten, M. and Wolf, C. R. (1992) *Biochem. J.* **282**, 305–306
- Awasthi, Y. C., Bhatnagar, A. and Singh, S. V. (1987) *Biochem. Biophys. Res. Commun.* **143**, 965–970
- Meyer, D. J., Xia, C., Coles, B., Chen, H., Reinemer, P., Huber, R. and Ketterer, B. (1993) *Biochem. J.* **293**, 351–356
- Stenberg, G., Board, P. G. and Mannervik, B. (1991) *FEBS Lett.* **293**, 153–155
- Liu, S., Zhang, P., Ji, X., Johnson, W. W., Gilliland, G. L. and Armstrong, R. N. (1992) *J. Biol. Chem.* **267**, 4296–4299
- Kolm, R. H., Sroga, G. E. and Mannervik, B. (1992) *Biochem. J.* **285**, 537–540
- Bjornstedt, R., Stenberg, G., Widersten, M., Board, P. G., Sinning, I., Jones, T. A. and Mannervik, B. (1995) *J. Mol. Biol.* **247**, 765–773
- Board, P. G. and Mannervik, B. (1991) *Biochem. J.* **275**, 171–174
- Zhang, P., Liu, S., Shan, S. O., Ji, X., Gilliland, G. L. and Armstrong, R. N. (1992) *Biochemistry* **31**, 10185–10193
- Hoesch, R. M. and Boyer, T. D. (1989) *J. Biol. Chem.* **264**, 17712–17717
- Barycki, J. J. and Colman, R. F. (1993) *Biochemistry* **32**, 13002–13011
- Reinemer, P., Dirr, H. W., Ladenstein, R., Schaffer, J., Gally, O. and Huber, R. (1991) *EMBO J.* **10**, 1997–2005
- Reinemer, P., Dirr, H. W., Ladenstein, R., Huber, R., Lo, B. M., Federici, G. and Parker, M. W. (1992) *J. Mol. Biol.* **227**, 214–226
- Ji, X., Zhang, P., Armstrong, R. N. and Gilliland, G. L. (1992) *Biochemistry* **31**, 10169–10184
- Sinning, I., Kleywegt, G. J., Cowan, S. W., Reinemer, P., Dirr, H. W., Huber, R., Gilliland, G. L., Armstrong, R. N., Ji, X., Board, P. G., Olin, B., Mannervik, B. and Jones, T. A. (1993) *J. Mol. Biol.* **232**, 192–212
- Wilce, M. C. J., Board, P. G., Feil, S. C. and Parker, M. W. (1995) *EMBO J.* **14**, 2133–2143
- Penington, C. J. and Rule, G. S. (1992) *Biochemistry* **31**, 2912–2920
- Chen, W. J., Graminski, G. F. and Armstrong, R. N. (1988) *Biochemistry* **27**, 647–654
- Graminski, G. F., Kubo, Y. and Armstrong, R. N. (1989) *Biochemistry* **28**, 3562–3568
- Karshikoff, A., Reinemer, P., Huber, R. and Ladenstein, R. (1993) *Eur. J. Biochem.* **215**, 663–670
- Tan, K. L., Webb, G. C., Baker, R. T. and Board, P. G. (1995) *Genomics* **25**, 381–387
- Tan, K. L. and Board, P. G. (1996) *Biochem. J.* **315**, 727–732
- Hussey, A. J. and Hayes, J. D. (1992) *Biochem. J.* **286**, 929–935
- Board, P. G., Coggan, M. and Parker, M. W. (1995) *Biochem. J.* **311**, 247–250
- Clapp, J. J. and Young, L. (1970) *Biochem. J.* **118**, 765–771
- Beutler, E. (1975) *Red Blood Cell: A Manual of Biochemical Methods*, pp. 71–73 (2nd edn.), Grune and Stratton, New York
- Gillham, B. (1971) *Biochem. J.* **121**, 667–672
- Mannervik, B. and Widersten, M. (1995) in *Advances in Drug Metabolism in Man* (Pacific, G. M. and G.N.F., eds.), 1st edn., pp. 407–459, Commission of the European Communities, Luxembourg
- Dodgson, K. S. (1960) *Biochem. J.* **78**, 312–319
- Baker, R. T., Smith, S. A., Marano, R., McKee, J. and Board, P. G. (1994) *J. Biol. Chem.* **269**, 25381–25386
- Board, P. G. (1984) *Ann. Hum. Genet.* **48**, 223–228
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Mannervik, B., Alin, P., Guthenberg, C., Jonsson, H., Tahir, M. K., Warholm, M. and Jornvall, H. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 7202–7206
- Beutler, E., Mathai, C. K. and Smith, J. E. (1968) *Blood* **31**, 131–150
- Hengen, P. N. (1995) *Trends Biochem. Sci.* **20**, 285–286
- Hiratsuka, A., Sebata, N., Kawashima, K., Okuda, H., Ogura, K., Watabe, T., Satoh, K., Hatayama, I., Tsuchida, S., Ishikawa, T. and Sato, K. (1990) *J. Biol. Chem.* **265**, 11973–11981
- Jung, G., Breitmaier, E. and Voelter, W. (1972) *Eur. J. Biochem.* **24**, 438–445
- Gillham, B. (1973) *Biochem. J.* **135**, 797–804
- Principato, G. B., Danielson, U. H. and Mannervik, B. (1988) *FEBS Lett.* **231**, 155–158
- Wilce, M. C. J. and Parker, M. W. (1994) *Biochim. Biophys. Acta* **1205**, 1–18
- Lo Bello, M., Pastore, A., Petruzzelli, R., Parker, M. W., Wilce, M. C. J., Federici, G. and Ricci, G. (1993) *Biochem. Biophys. Res. Commun.* **194**, 804–810
- Johnson, W. W., Liu, S., Ji, X., Gilliland, G. L. and Armstrong, R. N. (1993) *J. Biol. Chem.* **268**, 11508–11511
- Kraulis, J. P. (1991) *J. Appl. Crystallogr.* **24**, 946–950
- Chelvanayagam, G., Wilce, M. C. J., Parker, M. W., Tan, K. L. and Board P. G. (1996) *Proteins, in the press*