Evidence for an essential serine residue in the active site of the Theta class glutathione transferases

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A consistent feature of the Alpha-, Mu- and Pi-class glutathione transferases (GSTs) is the presence near the N-terminus of a tyrosine residue that contributes to the activation of glutathione. While this residue appears to be conserved in many Theta-class GSTs, its absence in some suggested that the Theta-class GSTs may have a significantly different structure or catalytic mechanism. The elucidation of the crystal structure of the Theta-class GST from the Australian sheep blowfly, *Lucilia cuprina*, has

indicated that a serine residue rather than a tyrosine residue can form a hydrogen bond with the glutathionyl sulphur atom. The present studies show that mutation of Ser-9 to alanine substantially inactivates the *L. cuprina* GST, confirming its importance in the reaction mechanism. As this serine is conserved in all Theta-class enzymes reported so far, it seems that an active-site serine is a significant factor that distinguishes the Theta-class GSTs from members of the Alpha-, Mu- and Pi-class isoenzymes.

INTRODUCTION

The glutathione transferases (GSTs) form a large family of enzymes responsible for the conjugation of glutathione to a wide range of electrophilic compounds, including many carcinogens and some therapeutic drugs. The mammalian cytosolic GST family can be subdivided into four evolutionary classes on the basis of their amino acid sequences. The classes known as Alpha, Mu, Pi and Theta show substantial sequence similarity within classes (50–80%) and about 30% or less between classes. Genetically determined deficiency of the Mu-class enzyme GSTM1 [1,2] has been associated with various forms of cancer [3–7]. In contrast, elevated expression of Alpha-, Mu- and Pi-class GSTs has been observed in certain cancers and in patients resistant to chemotherapy [8–10]. Since some anti-cancer drugs are known substrates for GSTs, inhibitors that target specific GST classes or isoenzymes may have considerable therapeutic potential. In order to design such inhibitors, detailed knowledge of the structure of the isoenzymes and their catalytic mechanism is essential. Recent studies of the crystal structures of the Alpha-, Mu- and Pi-class enzymes have shown that these three classes share a similar topological pattern [11]. Each enzyme is a dimer of similar or identical subunits that fold into two domains. In general, the N-terminal domain is responsible for binding glutathione in the active site and contains a highly conserved tyrosine residue. The tyrosine appears to play a significant role in catalysis by stabilizing the glutathione as a thiolate anion through hydrogen bonding from its hydroxy group [11].

Although considerably less is known about the structure and function of the Theta-class GSTs, there is mounting evidence that the Theta-class GSTs may have a significantly different structure to that of the Alpha, Mu and Pi classes. Theta-class isoenzymes have distinct substrate preferences and generally show little or no activity with 1-chloro-2,4-dinitrobenzene (CDNB), which is a significant substrate for the Alpha-, Mu- and Pi-class isoenzymes. In addition, most Theta-class GSTs show low or negligible capacity to bind to immobilized glutathione or S-hexylglutathione affinity matrices [12,13]. A computational

model of the active site of the Theta-class enzymes has predicted that they contain a serine residue instead of the N-terminal tyrosine implicated in the activation of glutathione in the Alpha-, Mu- and Pi-class enzymes [14]. In a mutagenic study of the Theta-like GST from *Escherichia coli*, conversion of a tyrosine at position 5 to phenylalanine failed to significantly alter the enzyme's activity [15]. Most recently, it has been shown that there are no tyrosine residues in the N-terminal sequence of GSTT2, a human Theta-class GST [13,16]. The absence of a tyrosine residue in the N-terminal sequence strongly supports the suggestion that the Theta-class GSTs may be structurally distinct and may utilize a different catalytic mechanism.

As yet the crystal structure of a mammalian Theta-class GST has not been solved. However, we have recently cloned, crystallized and solved the crystal structure of a GST from the Australian sheep blowfly (*Lucilia cuprina*) [17–19]. In the N-terminal region this isoenzyme has sequence similarity with members of the mammalian Theta-class enzymes, and it appears to be a member of a broader group of Theta-like enzymes found in many diverse species [17,19]. Although the *Lucilia* GST folds with a similar topological pattern to that of the other GST classes, and contains a tyrosine residue at position 5, the structural data show that the hydroxy group of tyrosine 5 is 13.9 Å from the sulphur atom of glutathione and could not participate in the hydrogen bonding that is a feature of the active sites in the Alpha-, Mu- and Pi-class GSTs. In contrast, the crystallographic data show that the hydroxy group of a serine residue at position 9 is within hydrogen-bonding distance of the glutathionyl sulphur. In addition, the crystal structure indicated that Tyr-113 could also potentially form hydrogen bonds with the glutathionyl sulphur through an adjacent water molecule. This residue may therefore also play an important role in catalysis.

In order to confirm the potential roles of Ser-9 and Tyr-113 in the active site of the *Lucilia* Theta-class GST, we have now changed the residues at positions 9 and 113 by site-directed mutagenesis and have expressed the mutated enzymes in *E. coli* for further characterization.

MATERIALS AND METHODS

GST from *L. cuprina* was expressed in *E. coli* as previously described [17]. Mutations in the cDNA sequence were made using the DNA Sculptor kit from Amersham Australia. The mutations S9A, S9T and Y113F were made with the following oligonucleotides: S9A, 5'-TTG CCC GGT GCC GCT CCC TGC-3'; S9T, 5'-TTG CCC GGT ACC GCT CCC TGC-3'; Y113F, 5'-GAC TAC TAC TTC CCA CAA ATC-3'. The recombinant enzymes were purified by affinity chromatography on glutathione-agarose [17]. The S9A and S9T mutants showed a considerably reduced affinity for glutathione-agarose and did not bind in the presence of high concentrations of bacterial protein. Bacterial lysates containing S9A or S9T were dialysed extensively against 50 mM Tris, pH 7.2, and then passed through a DE52 ion-exchange column equilibrated in the same buffer. The enzymes were slightly retarded by the column and were detected in the eluted fractions by immunoassay with rabbit antiserum raised against the normal recombinant enzyme. The partially purified enzymes were then applied to glutathione-agarose columns, and after washing in 50 mM Tris, pH 7.2, the enzymes were eluted with 50 mM Tris, pH 9.6. The purified preparations were concentrated on an Amicon UM10 filter and were shown to be homogeneous on SDS/PAGE. GST activity was determined with CDNB as a substrate at 30 °C as previously described [20].

RESULTS

An alignment of representative N-terminal sequences from the Alpha-, Mu- and Pi-class GSTs with those from Theta-class

enzymes from humans and several other species is shown in Figure 1. Although an N-terminal tyrosine residue that makes a significant contribution to catalysis in the Alpha-, Mu- and Pi-class GSTs is conserved in most Theta-class isoenzymes, it is evident from the human GSTT2 and fish sequences that it is not essential in the Theta class. In contrast, a serine at position 9 in the *Lucilia* GST is conserved in all Theta-like isoenzymes, from many species, including the mammalian Theta-class isoenzymes.

The positions of Tyr-5, Ser-9 and Tyr-113 with respect to the sulphur atom of glutathione bound in the active site of the *Lucilia* GST are shown in Figure 2. The proximity of the Ser-9 hydroxy group suggests that it could play a similar role to that of the N-terminal tyrosine residues conserved in the Alpha, Mu and Pi classes. In addition, Tyr-113 may also potentially influence the reactivity of glutathione by hydrogen bonding through a water molecule close to the active site. To test these possibilities we mutated Ser-9 to alanine or threonine, and Tyr-113 to phenylalanine. As shown in Table 1, both substitutions at position 9 resulted in a substantially incapacitated enzyme. The kinetic parameters V_{max} , K_m and k_{cat}/K_m were determined for the normal enzyme and the S9A and S9T mutant forms with CDNB as the electrophilic substrate. The mutation S9A resulted in a substantial reduction in specific activity, as reflected in the V_{max} . The K_m values for GSH and CDNB were both elevated, and the values of k_{cat}/K_m were significantly diminished. The increased K_m for GSH and decreased catalytic capacity are consistent with the crystallographic analysis suggesting the importance of a hydrogen bond between the hydroxy group of Ser-9 and the sulphur of glutathione. The mutation S9T also resulted in a large reduction in enzyme activity; however, the

Residue no. ...				1	2	3	4	5	6	7		8	9	10	11	12	13	14	15	16	17	18	19	20
Secondary structure ...																								
Blowfly					M	D	F	Y	Y	L	P	G	S	A	P	C	R	S	V	L	M	T	A	K
Fruit fly					M	V	D	F	Y	Y	L	P	G	S	P	C	R	S	V	I	M	T	A	K
Fish			M	A	K	D	M	T	L	L	W	G	S	P	P	C	W	R	V	M	I	V	L	E
Plant				A	P	M	K	L	Y	G	A	V	M	S	W	N	L	T	R	C	A	T	A	L
Bacteria						M	K	L	Y	Y	T	P	G	S		C	S	L	S	P	H	I	V	L
Human Theta GSTT1						M	G	L	E	L	Y	L	D	L	V	S	Q	P	C	R	A	V	Y	I
Human Theta GSTT2						M	G	L	E	L	F	L	D	L	V	S	Q	P	S	R	A	V	Y	I
Human Alpha GSTA1			M	A	E	K	P	K	L	H	Y	F	N	A	R	G	R	M	E	S	T	R	W	L
Human Mu GSTM1				M	P	M	I	L	G	Y	W	D	I	R	G	L	A	H	A	I	R	L	L	
Human Pi GSTP1				M	P	P	Y	T	V	Y	F	P	V	R	G	R	C	A	A	L	R	M	L	

Figure 1 Alignment of GST N-terminal sequences showing the secondary structure elements of the *L. cuprina* enzyme

The sequences were obtained from the SWISS-PROT or GenBank databases, and the database entry code is given in brackets: blowfly is *L. cuprina* GST1-1 (LUCGLTR), fruit fly is *Drosophila melanogaster* DMGST1-1 (GT1-DROME), fish is *Pleuronectes platessa* (GT-PLLEPL), plant is maize GST1 (GT2-MAIZE), bacteria is *Proteus mirabilis* GSTB1-1 (GT-PROM), human Theta GSTT1 (GSTT1-HUMAN), human Theta GSTT2 (HUMGST2A), human Alpha GSTA1 (HUMGST2), human Mu GSTM1 (GTM1-HUMAN), human Pi GSTP1 (GTP-HUMAN). The sequence numbering is based on the *L. cuprina* sequence. Only conserved tyrosine and serine residues have been boxed.

Figure 2 Stereo view of the active site of *L. cuprina* GST generated by the program MOLSCRIPT [22]

Table 1 Kinetic parameters for normal and mutant recombinant *L. cuprina* GST

The $V_{\max}$ and K_m values were determined from the rate-saturation curves where GSH or CDNB concentrations were fixed at 1 mM. The k_{cat}/K_m values were derived from measurements at low substrate concentrations where the rate-saturation curve degenerates to first-order with respect to concentration of the varied substrate [23]. Values are means $\pm$ S.D.

Enzyme	$V_{\max}^{\text{GSH}}$ ($\mu\text{mol}/\text{min}$ per mg)	K_m^{GSH} (mM)	$k_{\text{cat}}/K_m^{\text{GSH}}$ ($\text{s}^{-1} \cdot \text{mM}^{-1}$)	K_m^{CDNB} (mM)	$k_{\text{cat}}/K_m^{\text{CDNB}}$ ($\text{s}^{-1} \cdot \text{mM}^{-1}$)
Normal	20.1 ± 1.72	0.51 ± 0.093	23.2 ± 0.60	0.15 ± 0.029	60.1 ± 6.26
S9A	0.07 ± 0.021	6.65 ± 2.16	0.0078 ± 0.0003	1.22 ± 1.033	0.66
S9T	0.48 ± 0.155	1.16 ± 0.635	0.207 ± 0.019	0.95 ± 0.258	16.25

reduction was not as large as occurred in the S9A mutant. The K_m values for GSH and CDNB were also elevated for the S9T mutant, but not as substantially as for the S9A mutant form. The relatively large standard errors associated with the determination of the kinetic parameters for the mutant enzymes reflect the low activity of the enzymes in relation to the high non-enzymic reaction rate.

When purified in the presence of glutathione, substantial activity ($25 \mu\text{mol}/\text{min}$ per mg) was detected with the Y113F mutant form. However, when this form was prepared for kinetic analysis in the absence of glutathione, we detected a substantial reduction in specific activity and a failure to obey Michaelis-Menten kinetics. The replacement of glutathione resulted in a slow activation that suggests a conformational change. This aspect is under further investigation.

DISCUSSION

The absence of a tyrosine residue in the N-terminal region of the human Theta-class GSTT2 isoenzyme strongly suggested either that the structure of the Theta-class enzymes would prove to be radically different from that of the Alpha, Mu and Pi classes or that in the Theta class catalysis is dependent on another residue [16]. There is strong evidence in the Alpha class that, in addition to Tyr-9, Arg-15 makes a substantial contribution to the electrostatic field in the active site [21]. It seemed possible that a similar mechanism may be utilized by the Theta-class isoenzymes. The solution of the crystal structure of the *L. cuprina* Theta-like GST [19] has demonstrated that the overall fold of the Theta-class GSTs is similar to the topology of the Alpha, Mu and Pi classes, but has pointed to a significant difference in the active site, with a serine residue hydroxy group being within hydrogen-bonding distance of the glutathione sulphur atom. The present study has confirmed that Ser-9 is essential for activity in the *Lucilia* GST, with the S9A mutant enzyme having less than 0.5% of normal activity. The present data also suggest that, although the active-site residue is different, the reaction mechanism in the Theta class is likely to be similar to that in the other classes, as serine has a side-chain hydroxy group like that of tyrosine. The involvement of the hydroxy group in the reaction is also supported by the fact that the S9T mutation maintains a hydroxy group on the side chain and had notably higher activity than the S9A mutant. Modelling of the S9T mutant form shows that the hydroxy group could occupy several conformations that would not favour the formation of a hydrogen bond to the glutathionyl sulphur, and the data suggest that adoption of those conformations lowers activity. Recent studies of the Alpha-class isoenzyme GSTA1-1 have indicated that the hydroxy group of Tyr-9 remains in an undissociated form and contributes to catalysis by stabilizing the glutathione as a thiolate anion by hydrogen

bonding [21]. Because of its high $\text{p}K_a$, the hydroxy group of serine is even less likely to dissociate than a tyrosine residue. At this stage it is unclear if the glutathionyl sulphur becomes ionized in the *Lucilia* GST. Although Tyr-113 could potentially influence the active site by forming hydrogen bonds through water to the glutathionyl sulphur atom, the relatively high activity of the Y113F mutant suggests that this influence is minimal.

One surprising finding of this study was that mutation of Ser-9 to either alanine or threonine substantially diminished the enzyme's affinity for the glutathione-agarose affinity matrix. The loss of affinity for GSH is consistent with the elevated K_m values. The reason for this change in affinity is not clear, as the structural data suggest that there are several more important residues responsible for binding and aligning glutathione in the active site [19]. However, as the normal *L. cuprina* GST only shows a relatively weak affinity for glutathione-agarose [17], any reduction in contacts between GSH and the enzyme appear to be important. In this sense it is significant that the Y113F mutant showed normal affinity for glutathione-agarose and essentially normal activity.

The observation that Ser-9 in the *L. cuprina* GST is absolutely conserved throughout all the mammalian Theta-class GSTs and in Theta-like enzymes from numerous other species suggests that this active-site residue is a general feature that distinguishes the Theta-class GSTs from the other evolutionary classes and appears to mark a major evolutionary bifurcation.

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REFERENCES

- Board, P. G. (1981) *Am. J. Hum. Genet.* **33**, 36–43
- Board, P. G. (1981) *FEBS Lett.* **135**, 12–14
- Seidegård, J. and Pero, R. W. (1986) *Carcinogenesis* **7**, 751–753
- Strange, R. C., Matheroo, B., Faulder, G. C. et al. (1991) *Carcinogenesis* **12**, 25–28
- Zhong, S., Howie, A. F., Ketterer, B. et al. (1991) *Carcinogenesis* **12**, 1533–1537
- Strange, R. C., Fryer, A. A., Matheroo, B. et al. (1992) *Biochim. Biophys. Acta* **1139**, 222–228
- Zhong, S., Wyllie, A. H., Barnes, D., Wolf, C. R. and Spurr, N. K. (1993) *Carcinogenesis* **14**, 1821–1824
- Linsmeyer, M. E., Jefferson, S., Wolf, M., Cooper, I. A., Board, P. G. and Woodcock, D. M. (1992) *Br. J. Cancer* **65**, 471–475
- Kelley, M. K., Engqvist-Goldstein, Å., Montali, J. A., Wheatley, J. B., Schmidt, D. E., Jr. and Kauvar, L. M. (1994) *Biochem. J.* **304**, 843–848
- Behane, K., Hao, X.-Y., Egyházi, S., Hansson, J., Ringborg, U. and Mannervik, B. (1993) *Cancer Res.* **53**, 4257–4261
- Wilce, M. C. J. and Parker, M. W. (1994) *Biochim. Biophys. Acta* **1205**, 1–18
- Meyer, D. J., Coles, B., Pemble, S. E., Fraser, G. M. and Ketterer, B. (1991) *Biochem. J.* **274**, 409–414
- Hussey, A. J. and Hayes, J. D. (1992) *Biochem. J.* **296**, 929–935
- Blocki, F. A., Ellis, L. B. M. and Wackett, L. P. (1993) *Protein Sci.* **2**, 2095–2102

- 15 Nishida, M., Kong, K.-H., Inoue, H. and Takahashi, K. (1994) *J. Biol. Chem.* **269**, 32536–32541
- 16 Tan, K. L., Webb, G. C., Baker, R. T. and Board, P. G. (1995) *Genomics* **25**, 381–387
- 17 Board, P., Russell, R. J., Marano, R. J. and Oakeshott, J. G. (1994) *Biochem. J.* **299**, 425–430
- 18 Wilce, M. C. J., Feil, S. C., Board, P. G. and Parker, M. W. (1994) *J. Mol. Biol.* **236**, 1407–1409
- 19 Wilce, M. C. J., Board, P. G., Feil, S. C. and Parker, M. W. (1995) *EMBO J.* **14**, 2133–2143
- 20 Habig, W. H. and Jakoby, W. B. (1981) *Methods Enzymol.* **77**, 398–405
- 21 Björnstedt, R., Stenberg, G., Widersten, M., Board, P. G., Sinning, I., Jones, T. A. and Mannervik, B. (1995) *J. Mol. Biol.* **247**, 765–773
- 22 Kraulis, J. P. (1991) *J. Appl. Crystallogr.* **24**, 946–950
- 23 Danielson, U. H. and Mannervik, B. (1985) *Biochem. J.* **231**, 263–267

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