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The contribution of the *C*-terminal sequence to the catalytic activity of GST2, a human Alpha-class glutathione transferase

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A plasmid vector was constructed that encodes the expression in *Escherichia coli* of a truncated form of GST2, a human Alpha-class glutathione transferase. The truncated enzyme, GST2del210, has 12 residues deleted from the *C*-terminus and has the last two residues of the new *C*-terminal mutated from aspartic acid and glutamic acid to histidine and glycine respectively. GST2del210 has substantially diminished specific activity with either 1-chloro-2,4-dinitrobenzene or cumene hydroperoxide as substrate. The affinity of the truncated enzyme for a GSH–agarose matrix was also diminished, but sufficient interaction remained to enable affinity purification. Inhibition of GST2del210 by bromosulphophthalein was not altered. In contrast, this truncated form was not inhibited by *S*-pentylglutathione, a competitive inhibitor of the wild-type GST2 isoenzyme. The results show that the *C*-terminal segment of the Alpha-class glutathione transferases may form a component of the hydrophobic substrate-binding site. In contrast, this region appears not to be directly involved in GSH binding and is not absolutely essential for catalytic activity.

INTRODUCTION

The glutathione transferases (GST, EC 2.5.1.18) are a family of enzymes that conjugate GSH to a wide variety of xenobiotic compounds, including many known carcinogens and clinically useful drugs (Chasseaud, 1979; Suzukake *et al.*, 1983; Dulik *et al.*, 1986; Robertson *et al.*, 1986; Evans *et al.*, 1987). There is now considerable evidence that the GSTs contribute to a major pathway for the metabolism and detoxication of xenobiotics, and several reports have indicated that over-expression of GST may be involved in the development of resistance to therapeutic drugs in certain tumours (cf. Hayes & Wolf, 1988).

Mannervik (1985) proposed that the cytosolic GSTs from mammals could be grouped into at least three evolutionary classes, termed Alpha, Mu and Pi. The isoenzymes within each class share similarities in substrate- and inhibitor-specificities and strong amino acid sequence homology. Thus they may be expected to have substantial similarity in their tertiary structure. Between these classes there are notable differences in their catalytic properties, and there is less than 30% identity between the amino acid sequences (Mannervik & Danielson, 1988). These differences have led to the prediction that there may be significant variations in the three-dimensional structures of the different classes (Cowan *et al.*, 1989). GSTs from each class have been crystallized and subjected to preliminary X-ray-diffraction studies (Sesay *et al.*, 1987; Schäffer *et al.*, 1988; Cowan *et al.*, 1989), but their three-dimensional structures have yet to be resolved.

Despite extensive study, little is known about the structure of the GST active site. Hoesch & Boyer (1989) have utilized a photoaffinity labelling technique in an attempt to localize the active site in two rat Alpha-class GSTs. Their study indicated the *C*-terminal portion to be a component of the active site in each case. In the present investigation we have deleted this region from a homologous human Alpha-class isoenzyme termed GST2 type 1 or GSTϵ and investigated the effect of this deletion on the enzyme's catalytic activity.

MATERIALS AND METHODS

Plasmid construction

The construction of a plasmid termed pTacGST2, which directs the expression of human GST2 in *Escherichia coli*, has been previously described by Board & Pierce (1987). This enzyme is fully active and consists of the 221 residues encoded by the nucleotide sequence excluding the *N*-terminal methionine. A truncated form of GST2 in which residues 211–222 of the *C*-terminus have been deleted and residues 209 and 210 have been mutated to histidine and glycine respectively was engineered. The plasmid pTacGST2 has two *Nco*I cleavage sites in positions 425 and 619 bases from the A of the initiating ATG codon. Partial digestion with *Nco*I followed by a fill-in reaction with the Klenow fragment of DNA polymerase I and blunt-end ligation with T_4 DNA ligase resulted in recircularized plasmids, which were transformed into *E. coli* JM103 (Fig. 1). Selection of clones that had lost either the 619 bp site or the 425 bp site was carried out by analysis of restriction digests of plasmid DNA on agarose gels. The fill-in reaction inserts four bases (CATG) at the cleavage site, and therefore in the case of the 619 bp site changes the reading frame of the GST2 cDNA after the codon encoding Met-208. This results in the insertion of histidine and glycine at positions 209 and 210 before premature termination at a TGA codon. The resulting truncated enzyme was termed GST2del210, and its sequence at the *C*-terminus relative to GST2 is shown in Table 1.

Modification of the alternative 425 bp *Nco*I site changed the reading frame and resulted in the mutation of 14 residues following His-143 before termination at a TGA codon. This modified enzyme had no catalytic activity and was not studied further.

Enzyme activity

The GST activity was determined with GSH and 1-chloro-2,4-dinitrobenzene as substrates as previously described by Habig *et al.* (1974). Glutathione peroxidase activity was determined by

Abbreviation used: GST, glutathione transferase.

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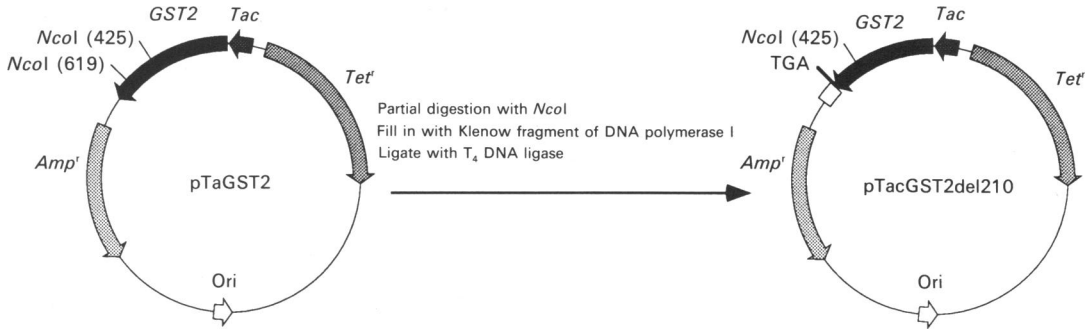

Fig. 1. Schematic diagram of the plasmids pTacGST2 and pTacGST2del210, showing the relative positions of the two *Nco*I sites subjected to partial digestion, blunt-ending and re-ligation

The black filled box indicates the position and direction of the GST2 cDNA. The position of the TGA (stop) codon in the new reading frame is indicated.

Table 1. *C*-Terminal of normal and truncated GST2

Isoenzyme	Residue no.		
	200	210	220
GST2	PGSPRKPPMDEKSLEEARKIFRF		
GST2del210	PGSPRKPPMHG		

the method described by Beutler (1974) except that 0.1 M-sodium phosphate buffer, pH 7, was used in place of Tris/HCl and that cumene hydroperoxide was used as a substrate.

Enzyme purification

Normal GST2 and GST2del210 were expressed in *E. coli* cultures and purified by affinity chromatography largely as previously described by Board & Pierce (1987). Overnight cultures of either pTacGST2 or pTacGST2del210 were subcultured at a dilution of 1:100 and grown in 500 ml of Luria broth with vigorous shaking to an A_{600} of 0.2. Isopropyl β-D-thiogalactopyranoside was added to a final concentration of 0.1 mM and the culture was grown on for a further 3 h to allow expression of the cloned protein. The bacteria were then pelleted by centrifugation (1500 *g* for 10 min) and washed in 150 mM-NaCl/50 mM-Tris/HCl buffer, pH 7.5. The cells were resuspended in distilled water and sonicated (Branson B_{12} instrument; 50 W). The cell debris was pelleted by centrifugation at 10000 *g* for 15 min, and the supernatant was dialysed against 50 mM-sodium phosphate buffer, pH 7.2. The cell extract was then passed through a 5 cm × 2 cm column of GSH–agarose (Simons & Vander Jagt, 1977) equilibrated in 50 mM-sodium phosphate buffer, pH 7.2. The column was extensively washed in the same phosphate buffer, and then the bound enzyme was eluted with 5 mM-GSH in 50 mM-sodium phosphate buffer, pH 7.2.

Electrophoresis

Denaturing SDS/PAGE was carried out by the method of Laemmli (1970) in 12.5% gels. The molecular-mass markers were Rainbow markers (Amersham International).

RESULTS

The procedure used afforded the expression of two alternative truncated GST2 proteins. The truncated protein resulting from the modification of the 425 bp *Nco*I site was detectable by immunoblot analysis, but showed no detectable GST activity and was not further studied.

The second truncated isoenzyme, expressed by pTacGST2 del210, was catalytically active and was purified by affinity chromatography on GSH–agarose. Denaturing SDS/PAGE confirmed its substantially diminished subunit size (Fig. 2). The calculated size of this protein excluding the initiating methionine residue is 23944 kDa compared with 25500 kDa for the normal GST2 isoenzyme expressed in *E. coli*.

Although GST2del210 could be purified by affinity chromatography on GSH–agarose, its ability to bind to the affinity matrix was considerably diminished when compared with the normal GST2. This decreased binding was quantified during purification of the isoenzyme from *E. coli* extracts. When 7 mg of the wild-type GST2 was added, only 6% of the enzyme was not bound to the column. In contrast, when 5 mg of GST2del210 was added to the column, 70% passed through unbound. In this experiment, even if the total amount of enzyme protein added was comparable, the total activity with 1-chloro-2,4-dinitrobenzene differed significantly because of a large decrease in the specific activity of the GST2del210 mutant. The decrease

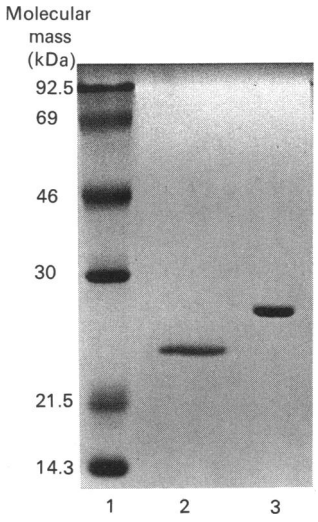

Fig. 2. SDS/PAGE of purified recombinant human GST

Lane 1, molecular-mass markers; lane 2, GST2del210; lane 3, GST2.

Table 2. Specific GST and glutathione peroxidase activities of GST2 and GST2del210 with 1-chloro-2,4-dinitrobenzene and cumene hydroperoxide

Enzyme activity is expressed as μmol/min per mg of protein at 30 °C.

Isoenzyme	GST activity with 1-chloro-2,4-dinitrobenzene	Glutathione peroxidase activity with cumene hydroperoxide
GST2	78	8.3
GST2del210	0.4	0.06

Table 3. Inhibition of GST2 and GST2del210

The I_{50} value is the concentration of inhibitor giving 50% inhibition of enzyme activity at pH 6.5 with 1 mM-1-chloro-2,4-dinitrobenzene.

	I_{50}	
Isoenzyme	*S*-Pentylglutathione (μM)	Bromosulphophthalein (μM)
GST2	8	19
GST2del210	> 500	22

in specific activity was evident with either 1-chloro-2,4-dinitrobenzene or cumene hydroperoxide as substrate (Table 2).

The sensitivity of GST2del210 to compounds known to inhibit the normal enzyme was evaluated with GSH and 1-chloro-2,4-dinitrobenzene as substrates, as shown in Table 3. The inhibition of activity by bromosulphophthalein is not substantially altered by the truncation of the *C*-terminus. In contrast, the inhibitory effect of *S*-pentylglutathione is largely removed by this modification.

DISCUSSION

Little is known about the structure of the active site of the GSTs. So far no specific residues involved in the catalytic activity or substrate binding have been unambiguously identified. There are two main Alpha-class GSTs expressed in human liver, termed either GST2 type 1 and GST2 type 2, or B_1B_1 and B_2B_2, which differ in their glutathione peroxidase activity and their sensitivity to inhibition by non-substrate-binding ligands such as haem and bromosulphophthalein (Stockman *et al.*, 1987; Suzuki *et al.*, 1987). These isoenzymes are encoded by different genes and differ in sequence in 11 residues (Board & Pierce, 1987; Rhoads *et al.*, 1987). It is not yet clear which of these residues contribute to the differential catalytic characteristics of the two isoenzymes. In other studies of Alpha-class GSTs Bhargava & Dasgupta (1988) have shown by photoaffinity techniques that the non-substrate-binding site for bromosulphophthalein may involve a tryptophan-containing peptide in the *N*-terminal region. Site-directed mutagenesis of conserved arginine residues in GST2 suggest that they may, directly or indirectly, contribute to the binding of GSH (Stenberg *et al.*, 1991). In studies of the human Mu-class isoenzyme GST ψ, modification of histidine residues by treatment with diethyl pyrocarbonate has inactivated the enzyme (Awasthi *et al.*, 1987). This inactivation can be blocked by GSH, suggesting that the modified histidine residue is at or near the GSH-binding site. The exact position of the histidine residue and the specificity of the reaction are still open to question. However, mutation of His-15 in this enzyme into Ala-15 by site-directed mutagenesis has been shown to result in a substantial decrease in catalytic activity (Widersten *et al.*, 1990). Despite these studies, the exact regions involved in forming the catalytic centre remain undefined.

Mannervik (1985) has discussed the requirements and properties of the active site and has suggested that each subunit includes a G site that binds GSH and an H site that interacts with the hydrophobic substrates. It is reasonable to expect that residues from different regions of the linear amino acid sequence may interact to form these sites in the native fully folded dimeric protein.

The photoaffinity labelling experiments of Hoesch & Boyer (1989) have implicated the *C*-terminal region as a component of the active site of two rat Alpha-class GSTs. In the experiments described here we have further examined the role of this region by constructing a plasmid that expresses a truncated form of GST2, a human Alpha-class GST. The truncated enzyme, GST2del210, has 12 residues deleted from its *C*-terminus, a region that is equivalent to the peptides identified as components of the active site by photoaffinity labelling of the rat Alpha-class isoenzymes. The method used to create this truncated isoenzyme also results in the insertion of histidine and glycine residues at positions 209 and 210. Thus, although the altered catalytic properties of GST2del210 probably result substantially from the deletion, the mutation of the two *C*-terminal residues of the truncated isoenzyme may also make a contribution to the observed effects. It is clear from these experiments that, although the specific activity of GST2del210 is substantially diminished, it is not entirely devoid of GST or glutathione peroxidase activity. Thus the *C*-terminal region is not an absolute requirement for catalytic activity. Although it appears to have diminished affinity, the GST2del210 isoenzyme binds to a GSH–agarose affinity matrix, and thus it can be concluded that the *C*-terminal region does not contain the primary components of the GSH-binding site. The diminished affinity for this matrix may just reflect conformational changes in the region.

The inhibition characteristics of the GST2del210 isoenzyme are also informative. Bromosulphophthalein is known to bind to a site other than the active site (Bhargava *et al.*, 1978; Vander Jagt *et al.*, 1982; Boyer, 1986), and since its I_{50} is unaltered by the truncation of the *C*-terminal region it can be concluded that this region does not contribute to the non-substrate-ligand-binding site. In addition, this finding shows that this deletion has not altered the overall conformation of the molecule sufficiently to influence this binding site indirectly. In contrast, the large decrease in sensitivity to *S*-pentylglutathione, a product analogue and a competitive inhibitor of GST (Mannervik, 1985), indicates that the *C*-terminal region of GST2 may interact with the hydrophobic region of the product.

Thus the results of the present study confirm the interpretation of the photoaffinity labelling experiment of Hoesch & Boyer (1989) that the *C*-terminal segment of Alpha-class GSTs may form a component of the hydrophobic region or H site. These experiments also show that this region is not directly involved in GSH binding (G site) and is not absolutely essential for catalytic activity. The apparent lowered affinity for the GSH–agarose affinity matrix may reflect impairment of the proposed conformational change induced upon binding of GSH derivatives to the enzyme (cf. Mannervik & Danielson, 1988). Further investigations utilizing site-directed mutagenesis of the *C*-terminal segment of GST2 may help define the critical residues involved in binding of *S*-substituted GSH derivatives, which may reflect interaction with hydrophobic regions of the active site (H site).

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