The role of tyrosine-9 and the C-terminal helix in the catalytic mechanism of Alpha-class glutathione S-transferases

Claire S. ALLARDYCE*†[1], Paul D. McDONAGH*†[2], Lu-Yun LIAN†, C. Roland WOLF‡ and Gordon C. K. ROBERTS*†[3]

*Centre for Mechanisms of Human Toxicity, University of Leicester, PO Box 138, Lancaster Road, Leicester LE1 9HN, U.K., †Biological NMR Centre, Department of Biochemistry, University of Leicester, Leicester LE1 9HN, U.K., and ‡Biomedical Research Centre, Ninewells Hospital and Medical School, University of Dundee, Dundee, Scotland, U.K.

Glutathione S-transferases (GSTs) play a key role in the metabolism of drugs and xenobiotics. To investigate the catalytic mechanism, substrate binding and catalysis by the wild-type and two mutants of GST A1-1 have been studied. Substitution of the 'essential' Tyr^9 by phenylalanine leads to a marked decrease in the k_{cat} for 1-chloro-2,4-dinitrobenzene (CDNB), but has no affect on k_{cat} for ethacrynic acid. Similarly, removal of the C-terminal helix by truncation of the enzyme at residue 209 leads to a decrease in k_{cat} for CDNB, but an increase in k_{cat} for ethacrynic acid. The binding of a GSH analogue increases the affinity of the wild-type enzyme for CDNB, and increases the rate of the enzyme-catalysed conjugation of this substrate with the small thiols 2-mercaptoethanol and dithiothreitol. This suggests that GSH binding produces a conformational change which is transmitted to the binding site for the hydrophobic substrate, where it alters both the affinity for the substrate and the catalytic-centre activity ('turnover number') for conjugation, perhaps by increasing the proportion of the substrate bound productively. Neither of these two effects of GSH analogues are seen in the C-terminally truncated enzyme, indicating a role for the C-terminal helix in the GSH-induced conformational change.

Key words: 1-chloro-2,4-dinitrobenzene, ethacrynic acid, conjugation, substrate activation.

INTRODUCTION

The GSH S-transferases (GSTs) are a ubiquitous family of enzymes that catalyse the conjugation of GSH with a wide range of generally hydrophobic compounds [1,2]. This reaction plays an important part in the metabolism and subsequent elimination of xenobiotics. Mammalian cytosolic GSTs can be divided into at least five classes (Alpha, Mu, Pi, Sigma and Theta) on the basis of sequence comparisons, gene structure and substrate specificity [2,3]. The sequence identity between classes is relatively low, about 30 % on the basis of structural alignment, but these GSTs nonetheless share a similar folding topology [4]. They all exist as homo- or hetero-dimers of $\approx$ 25 kDa subunits. Crystal structures are available for representatives of each of the five classes of cytosolic GST, alone and/or in complexes with substrates or products [5–13]. Each subunit can be described as having two domains: domain I has the more conserved sequence and contains the GSH-binding site ('G-site'), whereas domain II forms the majority of the binding site for the hydrophobic substrates ('H-site') and shows greater variability between isoenzymes, presumably reflecting differences in substrate specificity. For example, the H-site of Pi-class GSTs is comparatively open, while GSTs of the Mu and Alpha classes have a characteristic loop or helix respectively, which protrudes over the H-site [6,13]. In Alpha-class GSTs, including the human GST A1-1 which is the subject of the present work, the C-terminal helix covers the substrate bound in the H-site (Figure 1) and is thought to dictate a preference for more hydrophobic compounds [13,14]. The C-terminal helix of the Alpha-class enzymes is also important for catalysis [15], but its role in the mechanism remains to be established.

The catalytic mechanism of GSTs is not fully understood, but is thought to involve activation of the thiol group of GSH, promoting its nucleophilic attack on an electrophilic centre of the hydrophobic substrate [16,17]. A conserved amino acid residue having a hydroxy group [either a tyrosine residue, as in GST A1-1 (Figure 1) or, less commonly, a serine residue], has been shown to be important in catalysis [18–20], probably by contributing to the activation of the GSH thiol.

In the present paper we report kinetic and mutagenesis experiments aimed at understanding the role of the hydroxy group of Tyr^9 and the C-terminal region of Alpha-class GSTs in the conjugation of two substrates ethacrynic acid and 1-chloro-2,4-dinitrobenzene (CDNB). The data confirm the importance of the hydroxy group of Tyr^9 in the mechanism of CDNB conjugation, but show this is not true for all substrates: ethacrynic acid conjugation proceeds independently of this functional group. Evidence is also presented that a GSH-induced structural change in the C-terminus of the protein influences the binding of the hydrophobic substrate and has an important effect on the kinetic properties of the enzyme.

EXPERIMENTAL

Mutagenesis

Oligonucleotide-directed mutagenesis was carried out by a modification [21] of the method of Kunkel [22], using the plasmid pEMGST [21,23], derived from pGWL11 [24]; details of all PCR

Abbreviations used: ANS, 8-anilinonaphthalene-1-sulphonate; CDNB, 1-chloro-2,4-dinitrobenzene; DTT, dithiothreitol; GST, glutathione S-transferase; GST ΔCT, GST truncated at residue 209; SMeG, *S*-methylGSH; Y9F, $Tyr^9 \rightarrow Phe$; γGlu, γ-glutamyl.

[1] Present address: Department of Chemistry, University of York, Heslington, York, U.K.
[2] Present address: Seattle Biomedical Research Foundation, Seattle, WA, U.S.A.
[3] To whom correspondence should be addressed (e-mail gcr@le.ac.uk).

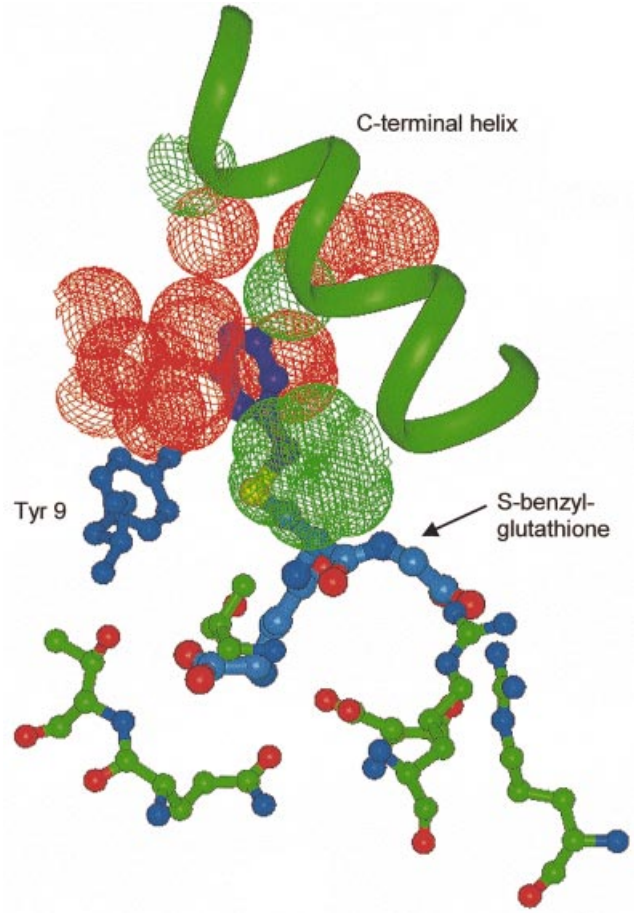

Figure 1 Relationship of the C-terminal helix to the *S*-benzylglutathione-binding site

The C-terminal helix is shown in green, with the side-chain atoms of residues (Ser^{212}, Ala^{216}, Phe^{220}) which contribute to the H-site shown as green surfaces. Other side-chains contributing to the H-site (Phe^{10}, Ala^{12}, Arg^{13}, Gly^{14}, Met^{208}) are shown as red surfaces. Residues involved in hydrogen-bonding to the GSH are shown in a 'ball-and-stick' representation, coloured according to atom type; Tyr^{9} is shown in dark blue ball-and-stick. The *S*-benzylglutathione ligand is shown in ball-and-stick representation with the benzyl group purple, the sulphur atom yellow and the GSH carbons pale blue, nitrogen atoms dark blue and oxygen atoms red. The co-ordinates used are from PDB entry 1guh [8].

procedures and primers used are given in [21]. The C-terminally truncated mutant GST ΔCT, lacking residues 210–222, was produced by oligonucleotide-directed mutagenesis, rather than by a restriction-enzyme deletion method [20], so that the only changes to the protein sequence were the deletion of these residues.

Protein expression and purification

Wild-type and mutant GST was produced by the fermentation of *Escherichia coli* DL39 transformed with the appropriate plasmid in 2xYT (yeast/tryptone) medium (Oxoid Ltd, Basingstoke, U.K.). Expression was induced by addition of isopropyl β-D-thiogalactoside when the cells had reached an A_{595} of 1.0; maximal yields of enzyme were obtained by continuing growth for 6 h after induction. The cells were separated from the broth by centrifugation and resuspended in 50 mM Tris/HCl, pH 7.2, containing 1 mM dithiothreiotol (DTT). Cells were broken using a French press and the cell debris removed by centrifugation. The supernatant was loaded on to an 8.3 cm × 1.5 cm GSH–agarose column (Sigma), prepared according to the manufacturer's guidelines and equilibrated at 20 ml/h with 50 mM Tris/HCl, pH 7.2, containing 1 mM DTT. The column was washed with 100 ml of the same buffer or until all unbound proteins were removed, as monitored by the absorbance of the eluate at 280 nm. GST was eluted with 0.1 M sodium carbonate/0.5 M NaCl, pH 10.5, containing 1 mM DTT; the eluent was collected into 5 ml of 0.1 M phosphate buffer, pH 6.5. The buffer was changed to water by ultrafiltration with a YM10 Diaflo membrane (Amicon Ltd.). The affinity-purification procedure typically had a yield of 95%. For the wild-type enzyme and the Y9F (Tyr^{9} → Phe) mutant enzyme, 150 (±15) mg of purified protein was produced/litre of broth; for the somewhat less stable truncated GST the yield was 100 mg/litre. All samples were freeze-dried and stored at −20 °C; there was no noticeable change in activity before and after freeze-drying. The specific activity with CDNB as substrate determined for several preparations of wild-type GST A1-1 was 680 (±72) $\mu mol \cdot s^{-1} \cdot mg^{-1}$, in good agreement with the published value of 660 $\mu mol \cdot s^{-1} \cdot mg^{-1}$ [17]. MS analysis of the wild-type GST (found 25495 Da; calculated 25504 Da) and GST ΔCT (found 24207 Da; calculated 24195 Da) indicated that a single species of the expected molecular mass was present in each sample.

Ligands

GSH, γGlu-Cys, Cys-Gly, Ala-Gly and the other ligands studied were of the highest purity commercially available. 1,4-Dinitrophenylglutathione was synthesized non-enzymically by mixing excess GSH with CDNB in water, maintaining the pH at approx. 8. The products were isolated using reverse-phase HPLC, loading the mixture at 1 ml/min in 20 mM ammonium acetate and eluting the product with a 30 min gradient of 0–80% acetonitrile in the same buffer. γGlu-Ala-Gly was produced enzymically using γ-glutamyltransferase (Sigma) and the substrates Ala-Gly and L-γ-glutamyl *p*-nitroanilide. The *p*-nitroanilide was added in excess and the reaction allowed to proceed until all the dipeptide had been consumed. The products were isolated by HPLC using a C_{18} reverse-phase column. The mixture was loaded in water containing 1% (v/v) trifluoroacetic acid at 1 ml/min. The γGlu-Ala-Gly was retarded by the reverse-phase column, but was eluted with the load buffer. The *p*-nitroanilide was eluted with a gradient of 0–80% acetonitrile in 1% (v/v) trifluoroacetic acid over 10 min. There was an overall 75% yield of γGlu-Ala-Gly from the dipeptide. MS and NMR spectroscopy confirmed the identity and purity of all of the ligands.

Enzyme-activity assays

GST activity was measured spectrophotometrically using modifications of the methods described by Habig et al. [25] in 50 mM phosphate buffer, pH 6.5. Absorption coefficients for the conjugates of CDNB and the various thiol compounds used in the assays were estimated by non-enzymically conjugating a known amount of the CDNB to excess thiol compound and monitoring the absorbance at the wavelength used in the kinetic assays (340 nm). This was repeated at least three times with different amounts (0.01–1 mM) of CDNB and the absorption coefficients calculated. Estimated absorption coefficients for the CDNB conjugates were: GSH, 9.5 (±0.4) $mM^{-1} \cdot cm^{-1}$ (in satisfactory agreement with the published value of 9.6 $mM^{-1} \cdot cm^{-1}$ [25]); γGlu-Cys, 6.2 (±0.6) $mM^{-1} \cdot cm^{-1}$; Cys-Gly, 9.7 (±0.2) $mM^{-1} \cdot cm^{-1}$. The absorption coefficients of the CDNB conjugates of DTT and 2-mercaptoethanol were esti-

mated as 8.4 ($\pm$0.3) and 8.2 ($\pm$0.2) $mM^{-1} \cdot cm^{-1}$ respectively by the same method.

All rates were corrected for the spontaneous reaction of GSH (or its analogues) with the hydrophobic substrates. These were generally very low, except in the case of ethacrynic acid. Measurements with this substrate were limited by the rate of the spontaneous reaction at high GSH concentrations; to estimate k_{cat} values, measurements were made at a GSH concentration of 0.25 mM and corrected for incomplete saturation using the measured K_m for GSH. The identities of the conjugates were confirmed by analysis of the contents of the cuvette by reverse-phase HPLC. The samples were loaded at 0.5 ml/min in 20 mM ammonium acetate, and fractions were eluted with a 25 min gradient of 0–80 % acetonitrile in the same buffer. Peaks were identified by comparison of retention times with those of the substrates and the non-enzymically synthesized conjugates and by NMR spectroscopy.

The subunit concentration of GST was calculated from the absorbance of the GST solution at 280 nm. The molar absorption coefficient for the truncated (ΔCT) and wild-type proteins was determined by amino acid hydrolysis as 24526 and 25100 $M^{-1} \cdot cm^{-1}$ respectively [26]. For the Y9F mutant enzyme the absorption coefficient was estimated as 23700 $M^{-1} \cdot cm^{-1}$ by correcting the wild-type value for the mutation by the method of Gill and von Hippel [27].

Fluorescence-spectroscopic determination of dissociation constants

The equilibrium dissociation constants for the binding of ligands to GST A1-1 were determined by exploiting the intrinsic protein fluorescence (excitation 280 nm, emission 340 nm). Small volumes of ligand solution were titrated into a cuvette containing GST in 50 mM phosphate, pH 6.5. To allow correction for dilution and inner-filter effects, an identical titration was performed in parallel using a tryptophan solution having fluorescence emission intensity equal to that of the protein sample. The maximum quench in fluorescence and the dissociation constant were determined by fitting a quadratic equation to the corrected fluorescence as a function of ligand concentration. Care was taken to use a protein concentration sufficiently low to ensure that accurate estimates of K_d could be made. The number of ligand-binding sites was determined by a similar method, but using a higher protein concentration so that stoichiometric binding was observed.

RESULTS AND DISCUSSION

Binding and conjugation of GSH analogues

GSTs have generally been found to exhibit stringent substrate specificity with respect to the substrate GSH. Binding of GSH leads to a small decrease in the tryptophan fluorescence of GST hA1-1, which can be used to determine the equilibrium dissociation constant for binding. Similar fluorescence quenching was observed on the binding of the two dipeptide 'fragments' of GSH, γGlu-Cys and Cys-Gly, but two GSH analogues lacking a free thiol, *S*-methylglutathione (SMeG) and γGlu-Ala-Gly, did not affect the fluorescence of GST, indicating that the effect of G-site ligands on tryptophan fluorescence is a function of the free thiol group.

The dissociation constants for GSH, γGlu-Cys and Cys-Gly are given in Table 1. All three ligands bind tightly at a single site/subunit, with dissociation constants in the micromolar range; the two fragments bind only 4–8-fold less tightly than GSH. These values are in interesting contrast with the K_m values, determined with CDNB as the hydrophobic substrate, shown in Table 2. The K_m values are again similar for GSH and its two fragments, but, in contrast with the micromolar dissociation constants, the K_m values fall in the millimolar range. In terms of simple Briggs–Haldane kinetics [28], this indicates that, once the enzyme–substrate complex has formed, the formation of the conjugate is much faster than the rate of dissociation.

Although the K_d and K_m values are similar for GSH and its fragments, the catalytic-centre activity for GSH itself is more than 100-fold greater than those for γGlu-Cys and Cys-Gly (Table 2), suggesting that the smaller substrates may be able to bind in non-productive orientations. From examination of the structure of the binding site, the γ-glutamyl subsite is seen to be more constrained, both by hydrogen bonding and sterically, than that for the glycyl moiety, which could account for the difference in k_{cat} between the two fragments. However, it is impossible to determine from the available data whether the decreased rate of conjugation with the dipeptides is due simply to non-productive binding or to the requirement for the intact GSH peptide to induce an 'activating' conformational change of the kind discussed below.

Table 1 Equilibrium dissociation constants for the binding of ligands to wild-type hA1-1 GST

Ligand	K_d (μM) Alone	With 10 mM G-site ligand*
GSH	0.25 $\pm$ 0.04	–
γGlu-Cys	0.95 $\pm$ 0.3	–
Cys-Gly	2.0 $\pm$ 0.6	–
CDNB	2.1 $\pm$ 0.8	0.54 $\pm$ 0.10
Ethacrynic acid	0.82 $\pm$ 0.01	1.6 $\pm$ 0.4
1,2-Dichloro-4-nitrobenzene	n.d.†	0.77 $\pm$ 0.05
ANS	0.74 $\pm$ 0.26	7.2 $\pm$ 3.1
S-Dinitrophenylglutathione	5.6 $\pm$ 0.9	27 $\pm$ 4.3

* G-site ligands used were either GSH or, where GSH and the hydrophobic ligand react, γGlu-Ala-Gly; in the cases of ANS and dinitrophenylglutathione it was established that GSH and γGlu-Ala-Gly have closely similar effects on the binding of hydrophobic ligands.
† n.d., not determined.

Binding and conjugation of hydrophobic substrates

A range of hydrophobic compounds can serve as substrates [2]. This is a reflection of the biological function of the GSTs: the

Table 2 Kinetic constants for substrates of GST hA1-1

Substrate	k_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)
GSH*	0.38 $\pm$ 0.05	58 $\pm$ 9	1.5×10^5
γGlu-Cys*	0.37 $\pm$ 0.03	0.29 $\pm$ 0.04	7.8×10^2
Cys-Gly*	0.34 $\pm$ 0.04	0.057 $\pm$ 0.003	1.7×10^2
CDNB†	0.32 $\pm$ 0.09	58 $\pm$ 9	1.8×10^5
Ethacrynic acid†	0.10 $\pm$ 0.03	0.10 $\pm$ 0.01	4.5×10^2
1,2-Dichloro-4-nitrobenzene‡	1.3 $\pm$ 0.4	0.11 $\pm$ 0.02	8.4×10^1

* In the presence of 1.5 mM CDNB.
† In the presence of 10 mM GSH.
‡ In the presence of 5 mM GSH.

Table 3 Comparison of the kinetic constants and equilibrium ligand binding constants of wild-type and mutant GST hA1-1

	K_d (μM)				K_m (mM)			k_{cat} (s^{-1})	
Enzyme	GSH	CDNB (alone)	CDNB (with γGlu-Ala-Gly)	*S*-Dinitrophenylglutathione	GSH	Ethacrynic acid	CDNB	Ethacrynic acid*	CDNB
Wild-type	0.25 ± 0.04	2.1 ± 0.8	0.54 ± 0.10	5.6 ± 0.9	0.32 ± 0.09	0.10 ± 0.03	0.38 ± 0.05	0.10 ± 0.01	58 ± 9
Y9F	n.d.†	n.d.†	n.d.†	0.28 ± 0.05	0.33 ± 0.03	0.32 ± 0.04	0.59 ± 0.12	0.09 ± 0.01	0.34 ± 0.03
ΔCT	1.7 ± 0.8	2.2 ± 0.7	2.3 ± 1.6	11 ± 1.3	0.37 ± 0.04	0.14 ± 0.06	10 ± 0.8	5.0 ± 0.6	0.39 ± 0.10

* Values extrapolated from those obtained at sub-saturating concentrations of GSH; see the Experimental section.
† n.d., not determined.

broad substrate specificity allows the GSTs to act on the majority of the wide range of xenobiotics to which the organism is exposed. The fluorescence method was used to measure the binding of a range of hydrophobic ligands: the substrates CDNB, dichloronitrobenzene and ethacrynic acid, the product dinitrophenylglutathione and the fluorescent probe 8-anilinonaphthalene-1-sulphonate (ANS). The dissociation constants of all these ligands were in the micromolar range (Table 1), the substrates showing the same contrast between micromolar K_d values and millimolar K_m values (Table 2) discussed above for GSH. Although the K_m values do not differ markedly between the three substrates studied, CDNB is by far the best substrate, the catalytic-centre activities for 1,2-dichloro-4-nitrobenzene and ethacrynic acid being only about 0.2% of that observed for CDNB under the same conditions.

The binding of the hydrophobic ligands to the H-site was also measured in the presence of a G-site ligand – either GSH or γ-Glu-Ala-Gly. In all cases the occupation of the G-site affects the affinity of the enzyme for the hydrophobic ligand, but the nature of the effect differs among the ligands studied. The substrate CDNB binds approximately four times more tightly when the G-site is occupied, whereas the affinities of the enzyme for *S*-dinitrophenylglutathione, ethacrynic acid and ANS are all decreased by the presence of GSH.

The structure of the GSH conjugate *S*-dinitrophenylglutathione suggests that the ligand would occupy both the G- and H-sites, as seen for *S*-benzylglutathione in the crystal structure of this complex [8], and hence would compete with GSH for the G-site. However, the dissociation constant for *S*-dinitrophenylglutathione is increased less than 5-fold in the presence of 10 mM GSH, three orders of magnitude less than the effect expected if binding of the two ligands were mutually exclusive. The simplest explanation of this observation is that, in the presence of GSH, *S*-dinitrophenylglutathione is able to bind to the H-site alone, through its dinitrophenyl moiety, although it is also possible that, in the presence of GSH, it binds at the binding site for hydrophobic ligands outside the active site which has been characterized for several GSTs [29–33]. Ethacrynic acid is a substrate and is therefore expected to bind at the H-site, but its dissociation constant was also increased in the presence of GSH. The crystal structure of the ethacrynic acid complex of GST A1-1 shows this substrate in an apparently non-productive binding mode, partially occupying the G-site [12]. Hence the modest decrease in affinity for ethacrynic acid in the presence of GSH is likely to be due to its displacement from this non-productive binding mode, favouring an alternative, somewhat weaker, mode of binding, perhaps occupying the H-site exclusively.

A similar explanation can be advanced to explain the effects of GSH on ANS binding, if ANS binds to the H-site. It has been reported that ANS binds outside the active site, perhaps at the dimer interface [30,32]; however, in the present work we observe a stoichiometry of two molecules of ANS per dimer, rather than one per dimer as reported previously. A possible reason for this difference is the fact that we do not use GSH or an analogue to elute the enzyme from the affinity column, thus avoiding the possibility of residual GSH remaining bound to the enzyme. The observation of a stoichiometry of two per dimer does not exclude binding in the dimer interface [33], and at present we cannot distinguish between an allosteric effect of GSH on ANS biding in the dimer interface and a partially competitive effect of the kind discussed above for ethacrynic acid.

By contrast, the K_d for CDNB is decreased approx. 4-fold in the presence of saturating concentrations of the G-site ligand. This shows, first, that any non-productive mode of binding involving overlap into the G-site is less important for the CDNB binding to the free enzyme than for ethacrynic acid. Secondly, the affinity of the enzyme is increased when the G-site is occupied, either by direct interactions between the two substrates or by a conformational effect transmitted from the G- to the H-site. GSH binding has been shown to increase the affinity of GST P1-1 for some hydrophobic substrates [34], and in this isoenzyme the effect is thought to be transmitted between the G- and H-sites through helix 2 [35]. In the case of GST A1-1, evidence presented below indicates that the C-terminal region of the protein plays a key role in this effect. It should be noted that occupation of the G-site may increase the affinity of the H-site for ethacrynic acid as well as for CDNB, but this cannot be established because of the complicating effects of the non-productive binding mode discussed above.

Substrate binding and conjugation by mutant proteins

The effect of GSH on the binding of hydrophobic substrates and differences in activity between CDNB and ethacrynic acid were investigated using two mutants of GST A1-1 (see Figure 1). In the first the catalytic tyrosine residue (Tyr^9, Y^9) was replaced with a phenylalanine (F^9) residue to give GST Y9F. The hydroxy group missing in GST Y9F is postulated to be involved in decreasing the pK_a of GSH and hence in promoting conjugation. Similar mutants have previously been investigated [17–19]. The second was a truncated enzyme, GST ΔCT, which has the C-terminal 12 residues (210–222) deleted. These residues make up the flexible amphipathic helix observed lying over the active site in some crystal structures [8,12]. Board and Mannervik [15] have studied a similar truncated GST, although this had residues Asp^{209}–Glu^{210} replaced by His^{209}–Gly^{210} in addition to the deletion of residues 211–222. The ligand dissociation constants and steady-state kinetic parameters for wild-type GST A1-1, GST Y9F and GST ΔCT are given in Table 3.

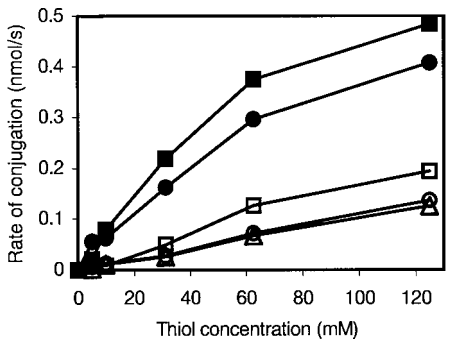

Figure 2 GST-catalysed conjugation of CDNB to small thiols

■ and □, DTT; ○ and ●, 2-mercaptoethanol. □ and ○, the rate of reaction of the thiols with CDNB in the absence of GST. ■ and ●: the rate of reaction in the presence of 1 μM GST and 10 mM γGlu-Ala-Gly. △, Rate of reaction of 2-mercaptoethanol with CDNB in the presence of 1 mM GST, but without γGlu-Ala-Gly.

As with the wild-type protein, the K_d values are in the micromolar range for both mutants, whereas the K_m values are in the millimolar range. The C-terminal truncation in GST ΔCT leads to 7–2-fold decreases in the affinity for both GSH and *S*-dinitrophenylglutathione, whereas the Y9F mutant enzyme binds *S*-dinitrophenylglutathione 20-fold more tightly. The hydroxy group of this tyrosine residue is close to the sulphur atom of *S*-benzylglutathione in its complex with the enzyme [8], and its absence may allow the product to adopt a more favourable binding mode in which interactions of both the GSH and the dinitrophenyl moieties are optimized. The binding of CDNB alone is not affected by the C-terminal truncation, but, interestingly, the increase in CDNB affinity for the wild-type enzyme produced by the presence of a G-site ligand is not observed with GST ΔCT. This strongly suggests that the GSH-induced change in affinity for CDNB involves the C-terminal residues of the protein.

Neither mutation affects the K_m for GSH, but the K_m values for hydrophobic substrates and the k_{cat} values are affected, the nature of the effect depending on both the mutation and the substrate. The Y9F mutation has very little effect on the K_m for CDNB, while decreasing its k_{cat} 170-fold, consistent with the earlier suggestions that the hydroxy group of Tyr[9] plays a role in catalysis, although it is not essential. However, with the poor substrate ethacrynic acid this mutation leads to a modest increase in K_m but no change in k_{cat}; Tyr[9] is not important in the rate-limiting step of catalysis of ethacrynic acid conjugation by GST A1-1. By contrast, the truncation in GST ΔCT has no effect on the K_m for ethacrynic acid, but increases k_{cat} by as much as a factor of 50. However, the opposite effect is observed for CDNB conjugation, where the helix is clearly important for both substrate binding and catalysis, since in GST ΔCT the K_m for CDNB is increased by a factor of 26 and k_{cat} is decreased 150-fold.

The evidence thus indicates that, whereas both the C-terminal helix and the hydroxy group of Tyr[9] play a significant role in the catalysis of CDNB conjugation by GST A1-1, they are not important in ethacrynic acid conjugation by this enzyme. As a result, the two mutations described here produce significant changes in the specificity of the enzyme. In terms of k_{cat}/K_m, CDNB is a better substrate than ethacrynic acid for the wild-type enzyme by a factor of 150, whereas in GST Y9F this ratio is decreased to 2 and in GST ΔCT to 0.001 – a striking reversal of specificity. A possible explanation for these different effects on CDNB and ethacrynic acid conjugation is suggested by the very recent report by Nieslanik et al. [36] of the effects of C-terminal truncation of rat GST A1-1 on the conjugation of ethacrynic acid. They showed that product dissociation is rate-limiting for the wild-type rat enzyme, at least at room temperature and below, but not for a mutant in which residues 209–222 had been removed. Thus, in the case of the human GST A1-1, the different effects observed for the two substrates could be explained if product dissociation is rate-limiting for ethacrynic acid but not for CDNB conjugation, and is increased in GST ΔCT. This is discussed further below under 'Conclusions'.

Conjugation of hydrophobic substrates to small thiols catalysed by GST A1-1

Working with a Mu-class GST, Principato et al. [37] observed that the enzyme catalysed the conjugation of CDNB to 2-mercaptoethanol, provided that a GSH analogue was present. This observation appears to indicate that GSTs might catalyse conjugation by 'activation' of the hydrophobic substrate as well as of GSH, so this reaction was investigated using GST A1-1 and the two mutants described above.

Control experiments showed that addition of GST A1-1 to an incubation mixture containing 2-mercaptoethanol or DTT and CDNB did not increase the rate of conjugation above that observed in the absence of the enzyme. However, if a G-site ligand such as SMeG or γGlu-Ala-Gly was also present, clear catalysis of the conjugation of CDNB with the small thiol compounds was observed (Figure 2). NMR spectroscopy confirmed that the products were identical with the conjugates synthesized non-enzymically [26]. The kinetic constants describing this reaction are presented in Table 4. No corresponding catalysis of the conjugation of ethacrynic acid with 2-mercaptoethanol was observed, although the intrinsic reactivity of ethacrynic acid with thiols is much greater than that of CDNB; this provides further evidence that the rate of reaction under these circumstances is controlled by the enzyme.

Both for the GSH analogue and for CDNB, the concentrations required to obtain half the maximal rate of reaction were

Table 4 Kinetic constants for the conjugation of small thiols with CDNB catalysed by GST A1-1 in the presence of GSH analogues

Thiol substrate	GSH analogue 'activator'	K_m (mM) (CDNB)	$s_{0.5}$ (mM) (GSH analogue)	$s_{0.5}$ (mM) (thiol substrate)	k_{cat} (s^{-1})
2-Mercaptoethanol	SMeG	0.34 ± 0.02	0.67 ± 0.18	54 ± 1.9	0.31 ± 0.03
DTT	SMeG	0.34 ± 0.02	0.75 ± 0.04	56 ± 0.8	0.33 ± 0.03
DTT	γGlu-Ala-Gly	0.25 ± 0.01	0.41 ± 0.07	54 ± 1.2	0.26 ± 0.06

comparable with the K_m values for GSH and CDNB in the normal conjugation reaction; the maximal rate of conjugation of CDNB to 2-mercaptoethanol was less than 1% of the maximal rate of its conjugation to GSH, though comparable with the rate of conjugation of poor substrates such as ethacrynic acid (Table 2). By contrast, the binding of the small thiols is clearly very weak, with half-maximal rates being obtained at > 50 mM. The absolute requirement for the presence of a GSH analogue if catalysis of CDNB conjugation to DTT or 2-mercaptoethanol is to be observed shows that the small thiols cannot be binding to the enzyme at the G-site.

It was noted above that the effect of GSH analogues in increasing the affinity of the enzyme for CDNB suggests that binding of ligands to the G-site induces a conformational change which affects the H-site. If this conformational change not only increases the affinity for CDNB but also has the effect of increasing its reactivity towards thiols, this would provide a possible explanation for the requirement for G-site ligands in the small-thiol conjugation reaction. Support for the idea that the same conformational change underlies both effects comes from studies of the C-terminally truncated enzyme GST ΔCT. Both effects of G-site ligands, on CDNB binding (Table 3) and on promoting the small-thiol conjugation reaction (results not shown), are abolished, suggesting that both depend on changes in the C-terminal 12 residues of the protein.

Conclusions

Discussions of the mechanism of GST-catalysed conjugation have centred on the activation of the thiol of GSH (by a decrease in its pK_a), in which the hydroxy functional group of a residue close to the N-terminus of the protein (Tyr^9 in GST A1-1) is believed to play a key role [17–19]. The present work shows that this residue is not uniformly important for all substrates: substitution of this tyrosine residue by a phenylalanine residue has a marked effect on the rate of conjugation of the good substrate CDNB, but very little effect on the rate of conjugation of the poor substrate ethacrynic acid. This could be explained if product dissociation were rate-limiting for ethacrynic acid conjugation, as shown very recently for wild-type rat GST A1-1 [36], but not for CDNB conjugation by human GST A1-1; the data would require that for ethacrynic acid product dissociation be rate-limiting both in the wild-type enzyme and in the Y9F mutant, where, judging by the results with CDNB, the chemical step appears to be at least 170-fold slower.

There is also evidence that the C-terminal residues (211–222) of the protein, which form a helix in the structure of the *S*-benzylGSH complex [8], are important for catalysis [20], and they have very recently been shown to determine the dissociation of the GSH–ethacrynic acid conjugate from rat GST A1-1 [36]. From the studies reported here of ligand binding and catalysis by the wild-type and ΔCT enzymes, we come to the following conclusions.

(i) The binding of GSH to the G-site produces a conformational change in the protein which is transmitted to the H-site, involving the C-terminal residues of the protein. Although the evidence presented here for the involvement of residues 211–222 is indirect, based as it is on studies of GST ΔCT, recent X-ray-crystallographic comparisons of the enzyme alone and its GSH complex have shown that GSH binding does indeed affect the conformation of these C-terminal residues (C. S. Allardyce, L.-Y. Lian, G. C. K. Roberts and P. C. E. Moody, unpublished work). Crystal structures of GST A1-1 show that the C-terminal 12 residues are flexible in the free protein, but, in the presence of ligands, form an amphipathic helix which completes the H-site ([8,12]; C. S. Allardyce, L.-Y. Lian, G. C. K. Roberts and P. C. E. Moody, unpublished work). It has been suggested that the helical conformation of the C-terminus is favoured by a hydrogen bond between the side chain of Arg^{221} (the penultimate residue) and the side chain of Asp^{42}, and that GSH binding may favour this indirectly by the interaction of Arg^{45} with the glycyl carboxy group of GSH [38]. Alternatively, or additionally, it is likely that Phe^{220} plays an important role, since this residue in the C-terminal helix contacts the glutathionyl moiety of bound *S*-benzylglutathione.

(ii) The effect of this change in the H-site is to increase its affinity for the substrate CDNB and to increase the rate of reaction of CDNB with thiol compounds. The mechanism by which this apparent activation takes place cannot be deduced from the present experiments. However, NMR studies of selectively labelled GST A1-1 suggest that CDNB binds in several orientations within the H-site in the absence of a GSH analogue, but in a single orientation when the G-site is occupied ([39]; L.-Y. Lian, C. S. Allardyce and G. C. K. Roberts, unpublished work). The apparent 'activation' of CDNB may thus arise from an increase in the proportion of the substrate which binds in a productive mode. Analogous increases in substrate affinity and/or turnover seen in other GSTs which lack the C-terminal helix appear to be mediated in other ways. For example, in GST P1-1 effects appear to be transmitted between the G- and H-sites through helix 2 [34,35], while in rat GST M4-4 the hydroxy group of Tyr^{115}, a residue not conserved in alpha class GSTs, appears to be important [40].

This GSH-induced conformational change also provides a possible explanation for some aspects of the specificity of the enzyme. Among GSTs, enzymes of the Alpha class exhibit the most stringent specificity for GSH, although GSH is bound in a similar extended conformation to all GSTs. The GSH 'fragments' γGlu-Cys and Cys-Gly bind to the enzyme with affinities similar to that of GSH, but are poor substrates. This could be explained if these GSH fragments (unlike γGlu-Ala-Gly) were unable to produce the conformational change. By the same token, the very low catalytic-centre activity observed for ethacrynic acid, a substrate which binds well to the enzyme and which is intrinsically more reactive than CDNB, suggests that there must be a mechanistic difference between the conjugation of CDNB and ethacrynic acid. As noted above, one possibility is that product dissociation is determined by the C-terminal residues of the enzyme [36] and is rate-limiting for ethacrynic acid but not for CDNB conjugation. If the C-terminal residues are important both for the conformation change leading to substrate 'activation', and for product dissociation, the effect of their removal will depend on a balance between these two effects. Thus, in the case of CDNB conjugation by human GST A1-1, C-terminal truncation decreases k_{cat} by a factor of 149, suggesting that any effect in speeding up product dissociation (which is probably not rate-limiting in this case) is outweighed by the unfavourable effect in decreasing the proportion of CDNB bound in a productive orientation. By contrast, the 50-fold increase in k_{cat} seen with ethacrynic acid suggests that the postulated effect on product dissociation dominates. Interestingly, in ethacrynic acid conjugation by rat GST A1-1, where rate-limitation by product dissociation and the involvement of the C-terminal residues has been clearly demonstrated [36], C-terminal truncation leads to a modest (3–6-fold) decrease in k_{cat}, suggesting that in this case the two effects may be of similar magnitude. Crystallographic and NMR studies of a number of complexes of the enzyme which are currently in progress (C. S. Allardyce, L.-Y. Lian G. C. K. Roberts and P. C. E. Moody, unpublished work) will provide tests of these hypotheses.

C.S.A. was supported by a Prize Studentship and Prize Fellowship from the Wellcome Trust, and P.D.McD. by a studentship from the Biotechnology and Biological Sciences Research Council. We are also grateful for financial support from the Medical Research Council.

REFERENCES

1 Mannervik, B. (1985) Adv. Enzymol. Relat. Areas Mol. Biol. **57**, 357–391
2 Hayes, J. D. and Pulford, D. J. (1995) Crit. Rev. Biochem. Mol. Biol. **30**, 445–600
3 Mannervik, B., Awasthi, Y. C., Board, P. G., Hayes, J. D., Di Ilio, C., Ketterer, B., Listowsky, I., Morgenstern, R., Muramatsu, M., Pearson, W. R. et al. (1992) Biochem. J. **282**, 305–306
4 Wilce, M. C. J. and Parker, M. W. (1994) Biochim. Biophys. Acta **1205**, 1–18
5 Reinemer, P., Dirr, H. W., Ladenstein, R., Schaffer, J., Gallay, O. and Huber, R. (1991) EMBO J. **10**, 1997–2005
6 Ji, X., Zhang, P., Armstrong, R. N. and Gilliland, G. L. (1992) Biochemistry **31**, 10169–10184
7 Ji, X. H., von Rosenvinge, E. C., Johnson, W. W., Tomarev, S. I., Piatigorsky, J., Armstrong, R. N. and Gilliland, G. L. (1995) Biochemistry **34**, 5317–5328
8 Sinning, I., Kleywegt, G. J., Cowan, S. W., Reinemer, P., Dirr, H. W., Huber, R., Gilliland, G. Armstrong, R. N., Ji, X., Board, P. G. et al. (1993) J. Mol. Biol. **232**, 192–212
9 Dirr, H., Reinemer, P. and Huber, R. (1994) J. Mol. Biol. **243**, 72–92
10 Garcia-Saez, I., Parraga, A., Phillips, M. F., Mantle, T. J. and Coll, M. (1994) J. Mol. Biol. **237**, 298–314
11 Raghunathan, S., Chandross, R. J., Kretsinger, R. H., Allison, T. J., Penington, C. J. and Rule, G. S. (1994) J. Mol. Biol. **238**, 815–832
12 Cameron, A. D., Sinning, I., L'Hermite, G., Olin, B., Board, P. G., Mannervik, B. and Jones, T. A. (1995) Structure **3**, 717–720
13 Wilce, M. C. J., Board, P. G., Feil, S. C. and Parker, M. W. (1995) EMBO J. **14**, 2133–2143
14 Bruns, C. M., Hubatsch, I., Ridderstrm, M., Mannervik, B. and Tainer, J. A. (1999) J. Mol. Biol. **288**, 427–439
15 Board, P. G. and Mannervik, B. (1991) Biochem. J. **275**, 171–174
16 Armstrong, R. N. (1994) Adv. Enzymol. Relat. Areas Mol. Biol. **69**, 1–44
17 Dirr, H., Reinemer, P. and Huber, R. (1994) Eur. J. Biochem. **220**, 645–661
18 Stenberg, G., Board, P. G. and Mannervik, B. (1991) FEBS Letts. **293**, 153–155
19 Liu, S., Zhang, P., Ji, X., Johnson, W. W., Gilliland, G. L. and Armstrong, R. N. (1992) J. Biol. Chem. **267**, 4296
20 Kolm, R. H., Sroga, G. E. and Mannervik, B. (1992) Biochem. J. **285**, 537–540
21 McDonagh, P. D. (1998) Ph.D. Thesis, University of Leicester
22 Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 488–492
23 McDonagh, P. D., Judah, D. J., Hayes, J. D., Lian, L.-Y., Neal, G. D., Wolf, C. R. and Roberts, G. C. K (1999) Biochem. J. **339**, 95–101
24 Simula, T. P., Glancey, M. J. and Wolf, C. R. (1993) Carcinogenesis **14**, 1371–1376
25 Habig, W. H., Pabst, M. J. and Jakoby, W. B. (1974) J. Biol. Chem. **249**, 7130–7139
26 Allardyce, C. S. (1996) Ph.D. Thesis, University of Leicester
27 Gill, S. C. and von Hippel, P. H. (1989) Anal. Biochem. **182**, 319–326
28 Briggs, G. E. and Haldane, J. B. S. (1925) Biochem. J. **19**, 338–339
29 Adang, A. E. P., Brussee, J., Meyer, D. J., Coles, B., Ketterer, B., van der Gen, A. and Mulder, G. J. (1988) Biochem. J. **255**, 721–724
30 Atkins, W. M., Wang, R. W., Bird, A. W., Newton, D. J. and Lu, A. Y. H. (1993) J. Biol. Chem. **268**, 19188–19191
31 McTigue, M. A., Williams, D. R. and Tainer, J. A. (1995) J. Mol. Biol. **246**, 21–27
32 Sluis-Cremer, N., Naidoo, N. N., Kaplan, W. H., Manoharan, T. H., Fahl, W. E. and Dirr, H. W. (1996) Eur. J. Biochem. **242**, 484–488
33 Barycki, J. J. and Colman, R. F. (1997) Arch. Biochem. Biophys. **345**, 16–31
34 Caccuri, A. M., Ascenzi, P., Antonini, G., Parker, M. W., Oakley, A. J., Ciessi, E., Nuccetelli, M., Battistoni, A., Bellizia, A. and Ricci, G. (1996) J. Biol. Chem. **271**, 16193–16198
35 Ricci, G., Caccuri, A. M., Lo Bello, M., Rosato, N., Mei, G., Nicotra, M., Chiessi, E., Mazzetti, A. P. and Federici, G. (1996) J. Biol. Chem. **271**, 16187–16192
36 Nieslanik, B. S., Dabrowski, M. J., Lyon, R. P. and Atkins, W. M. (1999) Biochemistry **38**, 6971–6980
37 Principato, G. B., Danielson, U. H. and Mannervik, B. (1988) FEBS Lett. **231**, 155–158
38 Widerstern, M., Bjornestedt, R. and Mannervik, B. (1996) Biochemistry **35**, 7731–7742
39 Lian, L.-Y. (1998) Cell. Mol. Life Sci. **54**, 359–362
40 Barycki, J. J. and Colman, R. F. (1993) Biochemistry **32**, 13002–13011

Received 25 May 1999/29 July 1999; accepted 20 August 1999