Site-directed mutagenesis and chemical modification of cysteine residues of rat glutathione *S*-transferase 3–3

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Rat liver glutathione *S*-transferase (GST) 3–3 is composed of two identical subunits, each containing three cysteine residues, Cys-86, Cys-114 and Cys-173. We have shown previously that Cys-86 is not involved in the enzymic activity of GST 3–3 [Hsieh, Huang, Chen, Lai & Tam (1991) *Biochem. J.* 278, 293–297]. At 50 °C, iodoacetamide can inactivate the enzyme by modifying Cys-86 and Cys-114. Cys-114 can be protected against iodoacetamide inhibition by *S*-(dinitrophenyl)glutathione. Site-directed mutagenesis was used to construct mutants in which serine replaced one (C114S and C173S) or all three (C3S) cysteine residues. These mutants were over-expressed in *Spodoptera frugiperda* cells in a baculovirus system and were found to be fully active. Replacing Cys-86 or Cys-114 with alanine (C86A and C114A) does not diminish the activity of the protein. The results suggest that cysteines are not involved in the enzymic mechanism, and Cys-114 is possibly located at the active site of GST 3–3.

INTRODUCTION

Glutathione *S*-transferases (GSTs; EC 2.5.1.18) are a group of enzymes that catalyse the conjugation of glutathione with a large number of electrophilic alkylating compounds, thereby protecting cells against their potential toxicity [1]. They are also involved in the reduction of organic hydroperoxides [2], the isomerization of prostaglandins [3] and the binding of non-substrate hydrophobic ligands such as bile acids, bilirubin, a number of drugs and thyroid hormones [1,4].

Cytosolic GSTs are dimeric proteins composed of identical or non-identical subunits. For each GST subunit, there is a binding site for GSH (G-site) and a separate binding site for the electrophilic substrate (H-site). Twelve distinct subunits have been identified in rat tissues, and combinations of these subunits result in at least 15 homo- or hetero-dimeric GSTs [5,6]. These subunits are distinguished by their molecular masses, isoelectric points and other properties, and have been classified into four non-homologous multigene families, namely Alpha, Mu, Pi and Theta [6,7].

The primary structure of a large number of GSTs has been determined by protein and DNA sequence analysis [1]. Preliminary X-ray-diffraction studies have also been reported for representatives of mammalian GSTs of classes Alpha, Mu and Pi [8–12]. Recently, the three-dimensional structure of class-Pi GST has been solved [13]. Nonetheless, the structures of class-Alpha and Mu GSTs and the amino acid residues located in the H-site of all three classes of GSTs still remain obscure.

Chemical-modification studies have been carried out in an effort to identify the amino acid residues located at the active site of GSTs. Thiol [14,15], guanidino [16] and imidazole [17] groups have been suggested to be important for catalytic activity. Particular attention has been paid to the possibility of the thiol group(s) being involved in the catalytic function of GSTs. It has been established that a thiol group is located in the proximity of the catalytic site of class-Pi GSTs [18–21]. Interestingly, site-directed mutagenesis of cysteine residues on GST-P demonstrated that none of the four cysteine residues is essential for enzymic

activity [22]. Results from studies on class-Alpha and Mu GSTs are less conclusive. Van Ommen *et al.* [14,23] and Ploemen *et al.* [24] modified and inactivated class-Alpha and -Mu GSTs with tetrachloro-1,4-benzoquinone and its glutathione conjugate, and suggested that GSTs possess one cysteine residue in or near the vicinity of the active site. However, partial thiol modification of class-Alpha (ligandins) and -Mu GSTs without loss of catalytic activity has also been reported [18,19,25–27].

In order to establish the involvements of cysteine(s) in the catalytic function of GSTs, we modified the enzyme with iodoacetamide and replaced the cysteine residues with serine or alanine. In this report, we present conclusive evidence that cysteines are not needed for the enzymic activity of class-Mu GSTs.

MATERIALS AND METHODS

Materials

Restriction enzymes and DNA-modification enzymes were obtained from New England Biolabs (Beverly, MA, U.S.A.). The oligonucleotide-directed '*in vitro*' mutagenesis kit was purchased from Amersham International (Amersham, Bucks., U.K.). The Sequenase kit was obtained from U.S. Biochemical Corp. (Cleveland, OH, U.S.A.). [α - 32 S]dATP for sequencing and iodo[14 C]acetamide (6.25 mM solution; sp. radioactivity 0.1 mCi/ml) for protein modification were products of New England Nuclear (Wilmington, DE, U.S.A.). *Achromobacter* proteinase I was from Wako (Osaka, Japan). *Staphylococcus aureus* V8 proteinase was from Boehringer-Mannheim (Mannheim, Germany). Substrates for enzymic assays were from Merck (Darmstadt, Germany) or Sigma (St. Louis, MO, U.S.A.). Epoxy-activated Sepharose 6B was from Pharmacia Fine Chemicals (Uppsala, Sweden). *S*-(Dinitrophenyl)glutathione (GS-Dnp) was prepared by the method of Lindwall & Boyer [28]. Chemicals used in sequencing were obtained from Applied Biosystems Inc. (Foster City, CA, U.S.A.). All other chemicals used were reagent grade or better.

Abbreviations used: GSTs, glutathione *S*-transferases; Cm, *S*-carbamimethylated; GS-Dnp, *S*-(dinitrophenyl)glutathione; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene.

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Construction and expression of GST 3 mutants

Oligonucleotides 5'-CACCACCTGAGTGGAGAGACA-3' (Cys-86→Ser), 5'-ATCATGCTTAGTTACAACCCC-3' (Cys-114→Ser), 5'-GAGCCCAAGAGCCTGGACGCC-3' (Cys-173→Ser), 5'-CACCACCTGGCTGGAGAGACA-3' (Cys-86→Ala) and 5'-ATCATGCTTGCTTACAACCCC-3' (Cys-114→Ala) were synthesized for site-directed-mutagenesis experiments. Mutagenesis was performed as described by Taylor *et al.* [29]. Construction of single-stranded DNA template for mutagenesis, selection of mutants and expression of mutants in SF9 cells by using a baculovirus system have been described in detail [27]. To ascertain that only the desired mutation had occurred during the manipulations, the fragment encoding GST 3 on the M13mp18 vector for each mutant was sequenced in its entirety.

Purification of enzymes

The expressed wild-type or mutant proteins were purified by affinity chromatography by the method of Mannervik & Guthenberg [30]. GSTs from the affinity column were passed through a preparative Superose 12 column (1.6 cm × 50 cm) with 10 mM-potassium phosphate (pH 7.0)/100 mM-KCl as eluent. The purity of the enzymes was analysed by SDS/PAGE [31]; they were then stored at -70 °C.

Enzyme assay

GST activity was assayed by published methods [32–34] at 25 °C. Protein concentration was determined from the A_{280} . Protein solution with a concentration of 0.56 mg/ml has 1 A_{280} unit [35]. This value was used for both wild-type and mutant recombinant proteins.

Protein modification

GST 3–3 (0.75 nmol) was modified in the dark (24 h at 25 °C, or 3 h at 50 °C) in a solution (100 μ l) containing Tris/HCl buffer (0.06 M, pH 7.9), 100 mM-KCl and 1 mM-iodo[14 C]acetamide (1.6 μ Ci). The reaction was stopped by passing the solution through an Aquapore RP-300 (C_8 ; 0.46 cm × 22 cm) reversed-phase column. For enzymic assay; GST 3–3 was modified with unlabelled iodoacetamide under identical conditions and used without purification. The final concentration of iodoacetamide in the assay solution was less than 0.01 mM. The exact concentration is dependent on the amount of enzyme required in the assay mixture.

Endopeptidase digestion and peptide purification

Modified proteins eluted from the reversed-phase column were dried in a Speed Vac concentrator. Digestions with *Achromobacter* proteinase I and *Staphylococcus aureus* V8 proteinase were carried out as described by Hsieh *et al.* [27]. The digests were separated on an Aquapore RP-300 reversed-phase column, which was eluted at a flow rate of 1 ml/min with a linear gradient of 1%/min of 0.08% trifluoroacetic acid in acetonitrile (buffer B).

Protein sequencing and amino acid analysis

Automated cycles of Edman degradation were performed with an ABI gas/liquid-phase model 470A/900A sequencer equipped with an on-line model 120A phenylthiohydantoin-amino acid analyser [36]. Samples for amino acid analysis were hydrolysed in the gas phase (24 h at 110 °C) with 6 M-HCl containing 1% (w/v) phenol. The hydrolysates were analysed in a Waters PicoTag amino acid analysis system according to the manufac-

turer's instructions. The number of Cm-cysteine residues was calculated as described by Hsieh *et al.* [27].

Determination of kinetic constants

K_m and V_{max} were determined under first-order conditions at low substrate concentration with respect to the varied substrate: for GSH with a fixed concentration of 1 mM-1-chloro-2,4-dinitrobenzene (CDNB), and for CDNB with a fixed concentration of 1 mM-GSH. The catalytic rate constant, k_{cat} , is expressed as maximum velocity per mol of catalytic site or GST 3 subunit [37].

RESULTS AND DISCUSSION

Chemical modification of GST 3–3 with iodoacetamide

GST 3–3 has three cysteine residues (Cys-86, Cys-114 and Cys-173) on each subunit. Incubation with iodoacetamide at 25 °C modifies only Cys-86 and does not affect the enzymic activity of GST 3–3 [27]. When the modification is carried out at 50 °C and the modified protein purified and hydrolysed with 6 M-HCl for quantitative amino acid analysis, 2.0 ± 0.1 Cm-cysteine residues were found per GST 3 subunit. The elevated temperature probably partially unfolded the protein and rendered the cysteine residues more accessible to iodoacetamide.

GST 3–3 was incubated with 1 mM-iodoacetamide at 50 °C. Samples were removed at different time intervals and allowed to cool slowly to room temperature before being assayed for activity. A duplicate sample was purified on a reversed-phase column and the elution profile was monitored at 220 nm. The radioactivity and peak area corresponding to the GST 3 of each sample were determined and expressed as a ratio. Iodoacetamide progressively abolishes the conjugating activity of GST 3–3. The extent of modification is proportional to the disappearance of enzyme activity. On the basis of the specific radioactivity of iodo[14 C]acetamide, we estimated that the enzyme lost 50% of its activity after 60 min of incubation when approx. 1.5 cysteine residues were modified per subunit. At the end of the incubation period (3 h), approx. 2.1 cysteine residues were modified per GST 3–3 subunit. Since we have shown previously [27] that

Fig. 1. (a) H.p.l.c. profile of *Achromobacter* proteinase I digests of iodo[14 C]acetamide-modified GST 3 subunits and (b) radioactivity of the fractions obtained in (a) (measured by liquid-scintillation counting).

Fig. 2. (a) H.p.l.c. profile of *Staphylococcus aureus* V8 proteinase digests of fractions 56–80 from Fig. 1(a), (b) radioactivity in the fractions obtained in (a), (c) radioactivity in the h.p.l.c. fractions of proteinase digests of GST 3–3 labelled with iodo[14 C]acetamide in the presence of *S*-hexylglutathione and (d) *S*-(dinitrophenyl)glutathione

modification of Cys-86 does not affect the enzymic activity of GST 3–3, the inhibition observed probably resulted from the modification of Cys-114 and/or Cys-173.

Identification of Cm-cysteine on GST 3–3

The identity of the modified residues was established by amino acid sequence analysis. GST 3–3 modified at 50 °C was first digested with *Achromobacter* proteinase I. Peptides were separ-

ated on a reversed-phase column (Fig. 1a), and fractions 56–80 (Fig. 1b), which represent 70 % of the total radioactivity, were identified by liquid-scintillation counting, collected and digested with *Staphylococcus aureus* V8 proteinase. Digests were purified as above (Fig. 2a) and 0.5 ml fractions were collected. Liquid-scintillation counting of the fractions revealed three major radioactive peaks (Fig. 2b). Peaks I (fractions 33–37), II (fractions 58–61) and III (fractions 62–65) represent approx. 23 %, 20 % and 21 % of the total radioactivity (c.p.m.) eluted from the column.

Results from peptide-sequencing analysis indicated that peak I is a mixture of three peptides, with sequence: HHLCGETEEE (residues 83–92), YTDSSYEK (residues 22–30) and NQVMDN (residues 101–106). A 20 μ l sample of phenylthiohydantoin-amino acid derivative was removed from each sequencing cycle for liquid-scintillation counting. Cys-86 was the only radioactive amino acid residue identified.

Peak II is a mixture of three peptides including residues 110–121 (LIMLCYNPDFEK), residues 173–181 (CLDAFPNLK) and residues 182–188 (DFLARFE). Cys-114 and Cys-173 are the only labelled residues. Radioactivities recovered from the first (Cys-173) and the (fifth) (Cys-114) regular cycle of the sequencer are in the ratio 1:10. Therefore Cys-173 is only a minor target for iodoacetamide modification.

Peak III has the amino acid sequence NRMQLIMLCYNPDFEK, which represents residues 106–121 of the GST 3 subunit. The phenylthiohydantoin derivative of radioactive Cm-cysteine was also recovered from the sequencer.

Thus we identified by amino acid sequencing that Cys-86 and Cys-114 are the major targets for iodoacetamide modification at 50 °C.

Protection experiments

Protection experiments were carried out to probe the possible location of the reactive thiol group(s). *S*-Methylglutathione can bind reversibly to the G-site of GSTs [21,38]. *S*-Hexylglutathione is a competitive inhibitor for both rat [39] and human [40] GSTs, and GS-Dnp can protect GST from modification with *S*-(*p*-azidophenacyl)glutathione [41]. These compounds are expected to interact with the G-site or both the G- and H-site of GSTs. Modification of GST 3–3 by iodoacetamide was carried out at 50 °C for 3 h in the presence of these compounds. The mixtures after incubation were separated on a reversed-phase column. The radioactivity/peak area ratio of GST 3 was determined and the number of *S*-carbaminoethylated residues calculated. *S*-Methylglutathione is not effective as a protecting reagent. Incubation in the presence of *S*-methylglutathione resulted in 1.9 Cm-cysteine residues per GST 3 subunit. Under these conditions, the enzyme retains 22 % of its original activity. Incubation in the presence of *S*-hexylglutathione and GS-Dnp resulted in the modification of approx. 1.6 and 1.1 cysteine

Table 1. Amino acid sequences of proteinase digests of Cm-GST 3–3

Peak	Iodoacetamide	Iodoacetamide + <i>S</i> -hexylglutathione	Iodoacetamide + GS-Dnp
I	HHLCGETEE (83–92) YTDSSYEK (22–30) NQVMDN (101–107)	HHLCGETEE YTDSSYEK NQVMDN	HHLCGETEE YTDSSYEK NQVMDN
II	LIMLCYNPDFEK (110–121) CLDAFPNLK (173–181) DFLARFE (182–188)	LIMLCYNPDFEK DFLARFE	
III	NRMQLIMLCYNPDFEK (106–121)	NRMQLIMLCYNPDFEK	
IV		NQVMDNRMQLIMLCYNPDFE (101–121)	

Table 2. Enzymic activity of Cm-GST 3-3 and mutants

Activity is expressed as the ratio of the activity of the modified enzyme, v , to that of unmodified control, v_c , $\times 100$.

	Temperature (°C)	Cm-Cys (mol/mol of subunit)	Remaining activity (%)	
			CDNB	DCNB
GST 3-3*	25	0.8 $\pm$ 0.1	94	106
GST 3-3	50	2.1 $\pm$ 0.2	17 $\pm$ 3	4 $\pm$ 2
C86S	50	1.2 $\pm$ 0.2	31 $\pm$ 4	22 $\pm$ 3
C114S	50	1.4 $\pm$ 0.2	105 $\pm$ 14	97 $\pm$ 13
C173S	50	2.0 $\pm$ 0.3	30 $\pm$ 3	21 $\pm$ 1
CallS	50	0.0	86 $\pm$ 9	92 $\pm$ 9

* Data from Hsieh *et al.* [27].

residues per subunit, and the recovery of 64% and 93% of the original enzymic activity respectively. GSH derivatives with a bulky substitution group at the thiol position therefore appear to be better protecting reagents.

In a separate set of experiments, GST 3-3 was preincubated at 50 °C for 5 min before the addition of inhibitors (*S*-hexylglutathione or GS-Dnp) and iodoacetamide. Identical results were obtained from the two sets of experiments. Therefore the inhibitors do not appear to block the modification step by stabilizing the enzyme against thermal unfolding.

The proteinase-digestion and amino acid-sequencing experiments were repeated on samples that had been modified at 50 °C in the presence of inhibitors. Figs. 2(c) and 2(d) show the label recovered in various fractions after proteinase digestion of GST 3-3 labelled with iodo[¹⁴C]acetamide in the presence of *S*-hexylglutathione and GS-Dnp respectively. The peptide sequences recovered from peaks I-IV are shown in Table 1. The data confirm that GS-Dnp almost completely blocked the labelling of Cys-114 with iodoacetamide. Peaks II, III and IV of Fig. 2(c) represent 29% of the total radioactivity eluted from the column. Similar fractions from the sample labelled in the absence of *S*-hexylglutathione (peaks II and III of Fig. 2b) accounted for 40% of the radioactivity eluted from the column. Therefore *S*-hexylglutathione is less effective as a blocking reagent, but still causes a 38% decrease in ¹⁴C uptake for Cys-114. In addition, Cys-173 was not labelled in the presence of *S*-hexylglutathione or GS-Dnp. Therefore *S*-hexylglutathione can only partially block the labelling of Cys-114, whereas GS-Dnp can completely block the labelling.

Construction and expression of GST 3 mutants

Of the three cysteine residues on GST 3-3, only Cys-86 and Cys-114 can be modified with iodoacetamide. The inactivation that results from the modification of these residues could be caused by either the removal of an essential functional group or introduction of steric hindrance. The involvement of Cys-173 cannot be assessed, owing to the inertness of this residue towards iodoacetamide modification. In order to evaluate the involvement of cysteines in the enzymic mechanism of GST 3-3, we resorted to site-directed mutagenesis to replace the cysteines on GST 3-3 individually or altogether with serines. Such substitutions do not appreciably alter the bulk of the side chain [42]. In addition, we replaced the two iodoacetamide-sensitive residues (Cys-86 and Cys-114) individually with alanine to ensure that the less nucleophilic serine oxygen atom would not function in place of the sulphur atom.

A total of five oligonucleotides were used in generating these mutants, which were over-expressed in SF9 cells by using a

baculovirus system [34]. Each mutant protein was purified on a separate newly prepared *S*-hexylglutathione column. The recoveries of the mutant proteins were comparable with the wild-type GST expressed in SF9 cells (1.4-1.6 mg of purified protein/40 ml of cell culture). The mutant proteins were judged to be pure by SDS/PAGE (results not shown).

Enzymic activity and modification of mutant GSTs

In order to confirm the identity of the modified cysteine and correlate the loss of enzyme activity with chemical modification, the wild-type and mutant GST 3-3s were incubated with iodoacetamide at 50 °C. The number of Cm-cysteines and the enzyme activities towards CDBN and 1,2-dichloro-4-nitrobenzene (DCNB) were determined and listed in Table 2. Data from modification of GST 3-3 with iodoacetamide at 25 °C [27] are also listed for comparison. Substitution of serine for cysteine at position 86 or 114 (C86S and C114S) markedly decreases the resulting number of Cm-cysteines. Conversely, replacement of Cys-173 with serine (C173S) does not affect the number of modified thiol groups. After iodoacetamide modification, a decrease in activity was observed for the wild-type enzyme and the C86S and C173S mutants. The activity of mutants devoid of Cys-114 (C114S and CallS) is not significantly affected by the iodoacetamide treatment. These results imply that Cys-86 and Cys-114 are the major targets for iodoacetamide labelling. Since the modification of Cys-86 does not affect enzyme activity, the decreased activity of GST 3-3 and the C86S and C173S mutants could only result from the modification of Cys-114.

Enzyme activity and kinetic data

The wild-type *S*-carbaminoethylated and mutant GSTs were assayed for enzymic activity. Wild-type GST 3-3 was incubated overnight at 25 °C or for 3 h at 50 °C in the absence of iodoacetamide as controls. The results summarized in Table 3 are averages of at least three experiments on duplicate samples and clearly show that the mutants have the same enzyme activity as the control, irrespective of the type of substitution (cysteine $\rightarrow$ serine or cysteine $\rightarrow$ alanine) for all the substrates examined. GST 3-3 with a Cm-Cys-86 (sample modified overnight at 25 °C) is functionally active, whereas a dramatic decrease in activity was observed for the protein (modified at 50 °C for 3 h) with both Cys-86 and Cys-114 *S*-carbaminoethylated.

K_m , V_{max} , and calculated k_{cat} , and k_{cat}/K_m values for the wild-type and mutant GSTs are listed in Table 4. The iodoacetamide-modified enzyme appears as a broad asymmetrical peak on a reversed-phase column, indicative of a mixed population (results not shown). The kinetic parameters for the chemically modified enzyme were not determined. With CDBN as the variable substrate, the K_m and V_{max} values obtained for the mutants and the wild-type enzyme are approximately the same. Replacement of Cys-86 or Cys-114 by either serine or alanine appears to cause a slight but noticeable decrease in affinity for GSH compared with the wild-type enzyme. This conclusion is based on a 24-65% increase in K_m and a 6-40% decrease in k_{cat}/K_m . The K_m for GSH is insensitive to structural alteration at position 173.

The involvement of cysteine in the function of GSTs has been a matter of debate. Using various thiol-modification reagents, Del Boccio *et al.* [21], Lo Bello *et al.* [43] and Tamai *et al.* [18] suggested that Cys-47 is located at the catalytic site of class-Pi GSTs. Results from a crystallographic study [13] indicate that Lys-42, Gln-49 and Pro-51 are located at the G-site of a class-Pi GST from pig lung. Therefore there is conclusive evidence that Cys-47, which is two residues upstream from Gln-49, is actually located at or near the vicinity of the active site of class-Pi GSTs. Nonetheless, using site-directed mutagenesis, Tamai *et al.* [22]

Table 3. Substrate specificities of GST 3–3Results are the means $\pm$ S.E.M. of duplicates of at least three experiments. N.D., not determined.

	Specific activity ($\mu\text{mol}/\text{min}$ per mg)								
	Cm-GST 3–3			C86S*	C114S	C173S	C86A	C114A	CallS
	GST 3–3	25 °C*	50 °C						
CDNB	50.0 $\pm$ 0.3	50.0 $\pm$ 0.3	10.0 $\pm$ 3.0	54.0 $\pm$ 1.0	56.0 $\pm$ 2.0	58.0 $\pm$ 4.0	49.2 $\pm$ 3.0	49.2 $\pm$ 3.0	60.0 $\pm$ 5.0
DCNB	5.0 $\pm$ 0.2	4.7 $\pm$ 0.1	0.1 $\pm$ 0.05	4.9 $\pm$ 0.3	4.7 $\pm$ 0.4	5.1 $\pm$ 0.6	4.6 $\pm$ 0.2	4.6 $\pm$ 0.4	4.8 $\pm$ 0.4
1,2-Epoxy-3-(<i>p</i> -nitro-phenoxy)propane	0.53 $\pm$ 0.02	0.47 $\pm$ 0.04	N.D.	0.58 $\pm$ 0.05	0.56 $\pm$ 0.03	0.54 $\pm$ 0.05	0.50 $\pm$ 0.01	0.51 $\pm$ 0.01	0.50 $\pm$ 0.01
Cumene hydroperoxide	0.29 $\pm$ 0.04	0.29 $\pm$ 0.02	N.D.	0.25 $\pm$ 0.04	0.24 $\pm$ 0.03	0.26 $\pm$ 0.02	0.31 $\pm$ 0.02	0.30 $\pm$ 0.02	0.28 $\pm$ 0.03
Ethacrynic acid	0.14 $\pm$ 0.02	0.14 $\pm$ 0.03	N.D.	0.18 $\pm$ 0.03	0.16 $\pm$ 0.02	0.14 $\pm$ 0.03	0.13 $\pm$ 0.01	0.12 $\pm$ 0.01	0.15 $\pm$ 0.02

* Data from Hsieh *et al.* [27].**Table 4. Kinetic parameters of GST 3–3 and mutants**

For GSH, data were measured with 1 mM-CDNB as co-substrate, and for CDNB, data were measured with 1 mM-GSH as co-substrate.

	GSH				CDNB			
	K_m (μM)	V_{max} ($\mu\text{mol}/\text{min}$ per mg)	k_{cat} (min^{-1})	k_{cat}/K_m ($\text{min}^{-1} \cdot \mu\text{M}^{-1}$)	K_m (μM)	V_{max} ($\mu\text{mol}/\text{min}$ per mg)	k_{cat} (min^{-1})	k_{cat}/K_m ($\text{min}^{-1} \cdot \mu\text{M}^{-1}$)
GST 3–3	83 $\pm$ 5	54 $\pm$ 3	1400	17	32 $\pm$ 2	54 $\pm$ 2	1400	44
C86S	113 $\pm$ 2	55 $\pm$ 2	1430	12	35 $\pm$ 1	52 $\pm$ 1	1350	40
C86A	117 $\pm$ 8	58 $\pm$ 5	1500	13	33 $\pm$ 6	52 $\pm$ 3	1350	42
C114S	103 $\pm$ 3	60 $\pm$ 2	1560	16	36 $\pm$ 4	61 $\pm$ 2	1590	44
C114A	137 $\pm$ 9	52 $\pm$ 4	1350	10	27 $\pm$ 5	49 $\pm$ 7	1270	48
C173S	65 $\pm$ 6	50 $\pm$ 3	1300	20	25 $\pm$ 1	48 $\pm$ 3	1250	48
CallS	106 $\pm$ 7	46 $\pm$ 3	1200	11	33 $\pm$ 3	50 $\pm$ 5	1300	39

concluded that none of the cysteine residues is involved directly in the catalytic activity of rat GST 7–7.

Results from earlier studies on class-Alpha and -Mu GSTs are contradictory. Complete labelling of the cysteine residues of ligandin (1–1 and 1–2) by iodoacetamide under denaturing conditions (6 M-urea) did not affect the catalytic activity on renaturation [24]. Carne *et al.* [26] modified ligandin (a mixture of isoenzyme 1–1 and 1–2) with various thiol-blocking reagents and showed that four thiol groups could be labelled and that the loss of catalytic activity was only observed after the third group was modified. Rat GST 4–4 appeared to be completely inhibited after modification of one of the four cysteine residues with chlorinated 1,4-benzoquinones [14]. With the same compound, modification of one of the two cysteine residues of GST 1–1 also has a major impact on the enzyme activity [22]. Presumably, the modified cysteine is located at or near the active site of GSTs. In these studies, the modified cysteine residue has not been identified. Recently, results from site-directed mutagenesis experiments on a human class-Mu GST [44] and a chick liver class-Alpha GST [45] demonstrated that cysteine residues are not needed for the activity of the enzyme. We report here a similar conclusion for a rat class-Mu GST. Therefore the inhibition observed is most likely the result of steric hindrance.

Reinemer *et al.* [13] solved the three-dimensional structure of a class-Pi GST at the 0.23 nm (2.3 Å) level and proposed that Tyr-106 probably lines the H-site. Hoesch & Boyer [41] identified a similar region on a class-Alpha GST as a possible electrophilic substrate-binding site by using a photoaffinity probe. By aligning

the amino acid sequence of class-Alpha, -Mu and -Pi GSTs (Fig. 3), we can distinguish Cys-114 of GST 3–3 as the residue occupying a position adjacent to Tyr-106 of class-Pi GSTs.

In an earlier report [27], we eliminated the possibility of Cys-86 being located in the active site or contributing to the enzymic activity of GST 3–3. In the present study, we identify Cys-114 as the additional residue modified with iodoacetamide at elevated temperature. This chemical modification of Cys-114 at elevated temperature inactivates the enzyme. The loss of activity is probably the result of steric hindrance or interference by the *S*-carbaminoethyl group on Cys-114 with protein refolding on cooling from 50 °C to room temperature. On the basis of protection experiments, this residue is probably located at or near the active site. This observation is in close agreement with that by Van Ommen *et al.* [14].

The three nucleophilic sulphur atoms on GST 3–3 were individually or entirely replaced by the less nucleophilic serine oxygen with no loss of enzymic activity. Zhang *et al.* [46] observed that His-14, His-83, His-84 and His-167 are all not essential for the activity of the same enzyme. Therefore both the thiol and imidazole groups have been eliminated from involvement in the enzymic mechanism of GST 3–3.

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Mu	GST 3	92	ERIRADIVENQVMDNRMQLIMLCYNP	*
	mGTmu1		ERIRADIVENQVMDTRMQLIMLCYNP	
	GST 4		ERIRVDVLENQAMDTRLQLAMVCYSP	
	Hb		EKIRVDVLENQMTDNHMQLMICYNP	
Pi	Pig GST	83	EAALVDMVNDGVEDIRCKYATLIYTN	∇ ∇ ∇
	GST P		EAALVDMVNDGVEDIRCKYISLIYTN	
	GST 7		EAALVDMVNDGVEDIRCKYGTLIYTN	
Alpha	GST 2	88	ERALIDMYAEGVADLDEIVLHYPYIP	
	Ha		ERALIDMYIEGIADLGEMILLVPCP	
	GST 1		ERALIDMYTEGILDTEMIMQLVICP	
	Mouse Ya		ERALIDMYSEGILDTEMIGQLVLCP	

Fig. 3. Alignment of class-Mu, -Pi and -Alpha GSTs

The amino acid sequence is designated by the universal single-letter code. The number in front of the sequence indicates the position of the first residue. Identical and chemically similar residues (ILMV, KHR, DENQ, ST, AG and FWY) [47] among the three classes are represented in outlined form when aligned. Cys-114 is indicated by an asterisk. The proposed residues lining the H-site [13] are denoted by ∇. The active site located by Hoesch & Boyer [41] using a photoaffinity label is underlined. References: GST 3, pGTR200 [48]; mGTmu1, pmGT10 [49]; GST 4, pGTR187 [50]; Hb, pGTH4 [51]; pig GST [12]; GST P [52]; GST 7, pGP5 [53]; GST 2, pGTB 42 [54]; Ha, pGTH1 [55]; GST 1, pGTR261 [56]; mouse Ya, prYa12 [57].

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