Reversible modification of rat liver glutathione S-transferase 3–3 with 1-chloro-2,4-dinitrobenzene: specific labelling of Tyr-115

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Rat liver glutathione S-transferase 3–3 (GST, EC 2.5.1.18), a triple mutant with all three cysteine residues replaced with serine (CallS) and a quadruple mutant with a Tyr-115 to phenylalanine substitution on CallS (CallSY115F) were overexpressed in *Escherichia coli* under the control of a *phoA* promoter. Using this system, we obtained over 35 mg of fully active pure protein/litre of cell medium. GST 3–3 and CallS mutant were modified with 1-chloro-2,4-dinitrobenzene (CDNB), a model substrate for the enzyme, in the absence of GSH. Dinitrophenol, but not S-

methylglutathione, inhibits this process. The dinitrophenyl groups are readily removed from the enzyme with GSH, but much more slowly with dithiothreitol. Results from peptide mapping and amino acid sequence analyses indicate that CDNB modifies the cysteine residues and Tyr-115 on wild-type GST 3–3, but only Tyr-115 on CallS. In addition, CDNB cannot modify the CallSY115F mutant. We propose that Tyr-115 is located at or near the H-site of GST 3–3.

INTRODUCTION

Glutathione S-transferases (GSTs, EC 2.5.1.18) are a group of multifunctional dimeric proteins which catalyse the conjugation of GSH to a wide variety of electrophilic alkylating compounds, thereby protecting cells against their potential toxicity [for reviews, see Mannervik and Danielson (1988) and Coles and Ketterer (1990)]. They are also involved in the reduction of organic hydroperoxides (Tan et al., 1986), the isomerization of prostaglandins (Christ-Hazelhof and Nutgeren, 1979) and the binding of non-substrate hydrophobic ligands such as bile acids, bilirubin, a number of drugs and thyroid hormones (Mannervik and Danielson, 1988; Ishigaki et al., 1989).

Cytosolic GSTs from mammalian sources have been well characterized. Twelve distinct subunits have been identified in rat tissues, and combinations of these subunits result in at least 15 homo- or hetero-dimeric GSTs. These isoenzymes are classified into four non-homologous species-independent multigene families, namely, Alpha, Mu, Pi and Theta (Mannervik et al., 1985; Meyer et al., 1991).

The complexity of the GST isoenzyme system confers a special challenge for isolating pure GSTs for structural and functional studies. Apparently homogeneous GST preparations isolated from animals may contain more than one structurally related isoform. Therefore expression of GST clones in eukaryotic or prokaryotic systems for large-scale production of functionally active homogeneous enzyme without contamination with closely related isoenzyme(s) becomes an attractive option. We have previously successfully expressed a GST 3 cDNA clone in *Spodoptera frugiperda* (SF9) cells with the use of a baculovirus system (Hsieh et al., 1989). However, the processes of screening for recombinant virus and maintaining the SF9 cell cultures are time consuming. Therefore an *Escherichia coli* expression system may be an attractive alternative for efficient production of wild-type and mutant GSTs.

For each GST subunit, there is a binding site for GSH (G-site) and a separate binding site for the electrophilic substrate (H-

site). The primary structure of a large number of GSTs has been determined by protein- and DNA-sequence analysis (Mannervik and Danielson, 1988). Furthermore, Reinemer et al. (1991) have solved the three-dimensional structure of a class-Pi GST co-crystallized with a GSH derivative. Mutational substitution of residues implicated by crystal structure has indicated the interaction of tyrosine, arginine, glutamine and aspartic acids with the substrate GSH (Manoharan et al., 1992). The interaction of Tyr-6 of a class-Mu enzyme with GSH has also been confirmed by X-ray crystallography and site-specific mutagenesis (Liu et al., 1992). However, the structures of class-Alpha and -Theta GSTs and the amino acid residues located in the H-site of all four classes of GSTs still remain obscure.

Inactivation of GSTs by the electrophilic co-substrate 1-chloro-2,4-dinitrobenzene (CDNB), in the absence of GSH, has been well documented (Pabst et al., 1974; Corrigan et al., 1989; Adams and Sikakana, 1990). A similar compound, fluoro-2,4-dinitrobenzene, was employed by Sanger (1959) for peptide sequence analysis. Jackson and Young (1987) showed that this compound is reactive towards histidine, tyrosine, cysteine and lysine residues. As CDNB is a co-substrate of GST, inactivation by this compound is probably a result of alkylation or modification of residues located at the active site of the enzyme. We report here the heterologous expression of a class-Mu enzyme, GST 3–3, in *E. coli* and the utilization of CDNB as an affinity probe for locating residues at the H-site. We identified Tyr-115 on GST 3–3 as the residue that can be reversibly labelled with CDNB.

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 Corp. (Amersham, Bucks., U.K.). The Sequenase kit was obtained from US Biochemical Corp.

Abbreviations used: GST, glutathione S-transferase; CDNB, 1-chloro-2,4-dinitrobenzene; Dnp, dinitrophenyl.

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(Cleveland, OH, U.S.A.). [α - ^{35}S]Thio]dATP for sequencing was purchased from New England Nuclear (Wilmington, DE, U.S.A.). Trypsin was obtained from Boehringer-Mannheim (Mannheim, Germany) and repurified as described by Lee and Shively (1990) before use. Substrates for enzymic assay were from Merck (Darmstadt, Germany) or Sigma (St. Louis, MO, U.S.A.). Chemicals used in sequencing were obtained from Applied Biosystems (Foster City, CA, U.S.A.). All other chemicals used were reagent grade or better.

Construction and expression of wild-type and GST 3 mutants in *E. coli*

The Bluescript phagemids (pKs⁺/Yb₁ and pKs⁺/CallS), containing the coding region of the wild-type GST 3 subunit and a GST 3 mutant in which all three original cysteines were replaced by serines, have been described previously (Hsieh et al., 1989; Chen et al., 1992). Oligonucleotides 5'-ATGCTTAGTTTCAACCCCGAC-3' and 5'-AGCGCCAGACATATGCCTATG-3' were synthesized in order to mutate the Tyr-115 to phenylalanine and introduce a unique *Nde*I restriction endonuclease site at the location of the ATG start codon for the GST 3 gene on both phagemids. Site-directed mutagenesis was performed as described by Taylor et al. (1985). Construction of single-stranded DNA templates for mutagenesis has been described in detail (Hsieh et al., 1991). The fragment encoding GST 3 on the M13mp18 vector for each mutant was sequenced in its entirety to confirm that only the desired mutations had occurred during the manipulations. The *Nde*I-*Bam*HI fragment (702 bp) of each plasmid (pNde/Yb₁, pNde/CallS and pNde/CallSY115F), containing the GST 3 coding region, was inserted into the *Nde*I-*Pst*I sites of a pBac expression vector using a *Bam*HI-*Pst*I linker. The recombinant plasmid was transformed into *E. coli* TG 1 cells for protein expression by the method of Craig et al. (1991). Bacteria transformed with expression plasmids were grown in Luria broth to exponential phase before dilution (10000-fold) into the induction medium (Craig et al., 1991). Cells were allowed to grow at 30 °C until the culture reached an absorbance at 600 nm of 1.2 (about 18 h) for maximum induction of recombinant protein expression. Cultivation of cells at higher temperature (37 °C) produces insoluble proteins with unprocessed N-termini and should be avoided.

Enzyme purification and assay

Harvested cells were suspended in 50 mM Tris/HCl, pH 8.0, containing 1 mM ethylenedinitrilotetra-acetic acid, 15% (w/v) sucrose, 6 mM 2-mercaptoethanol and 0.5 mM phenylmethane-sulphonyl fluoride (100 ml/l of cell culture). Lysozyme was added (0.1 mg/ml), and the mixture was incubated on ice for 20 min. Cells were lysed by sonication, and debris was removed by centrifugation in a Beckman 45 Ti rotor for 1 h at 205600 *g* (42000 rev./min). The expressed wild-type or mutant proteins in the supernatant were purified by affinity chromatography as described by Mannervik and Guthenberg (1981). Proteins after affinity chromatography were passed through a preparative Superose 12 gel-filtration column (Pharmacia, Uppsala, Sweden) which was eluted with 100 mM potassium phosphate, pH 7.0, for removal of *S*-hexylglutathione. Proteins were stored at -70 °C at a concentration of 1 mg/ml or higher. The purity of the enzymes was analysed by SDS/PAGE (Laemmli, 1970) and isoelectric focusing (Hsieh et al., 1988).

GST-conjugating activity, peroxidase activity and isomerase activity were determined at 25 °C with CDNB, cumene hydroperoxide and Δ^5 -androstene-3,17-dione as the respective substrates

by the respective methods (Habig et al., 1974; Lawrence and Burke, 1976; Habig and Jakoby, 1981). The rates of all the enzymic reactions in this study were obtained by subtracting the non-enzymic rate from the observed reaction rates. Protein concentration was determined with the A_{280} . Protein solution with a concentration of 0.56 mg/ml has 1 A_{280} unit (Hsieh et al., 1991). This value was used for both wild-type and mutant recombinant proteins. Concentrations of CDNB-modified GST samples were determined by the dye-binding method of Bradford (1976), using unmodified GST as protein standard.

Protein modification

GST 3-3 (40 nmol) was modified at 25 °C (20 and 30 min for wild-type and mutants respectively) in a solution (400 μ l) containing potassium phosphate buffer (0.1 M, pH 7.0) and 1 mM CDNB. For enzymic assay, the reaction was stopped and free CDNB was removed by passing the solution through an analytical Superose 12-gel filtration column eluted with a potassium phosphate buffer (0.02 M, pH 7.0) at a flow rate of 0.6 ml/min. The protein and the unchanged CDNB were eluted from the column at 23 and 97 min respectively. By calculation, the protein and excess CDNB should be resolved within 90 s.

Trypsin digestion and peptide purification

For trypsin digestion, the modified protein was purified with a Vydac reversed-phase column (C18; 0.46 cm $\times$ 25 cm) equilibrated in 0.08% trifluoroacetic acid/water. Protein was eluted from the column (flow rate 0.6 ml/min) with a linear gradient of 0.32%/min of 0.05% trifluoroacetic acid in acetonitrile (buffer B). Eluate from the column was detected with a diode array detector (model 1000S; Applied Biosystems). Modified protein was dried down in a Speed Vac concentrator, then redissolved in 100 μ l of 0.1 M NH_4HCO_3 , pH 8.0. Digestion with trypsin (16 h at 37 °C) was carried out at a substrate/enzyme ratio of 20:1 (w/w). Resulting peptides were separated on the same Vydac column, which was eluted at a flow rate of 0.3 ml/min with the same gradient and buffer conditions as mentioned above.

Protein sequencing

Automated cycles of Edman degradation were performed with an Applied Biosystems gas/liquid-phase model 470A/900A sequencer equipped with an on-line model 120A phenylthiohydantoin-amino acid analyser (Hewick et al., 1981).

Determination of kinetic constants

K_m and V_{max} were determined as outlined by Pabst et al. (1974). Kinetic constants were determined with GSH (0.1–1.0 mM) and CDNB (0.01–0.1 mM) as the variable substrates, and CDNB and GSH concentration constant at 1 mM respectively. The catalytic rate constant, k_{cat} , is expressed as maximum velocity/mol of catalytic site or GST 3 subunit (Segel, 1975).

RESULTS AND DISCUSSION

Expression of recombinant GST 3 in *E. coli*

Wild-type GST 3-3 and mutants were overexpressed in *E. coli*. After induction and harvest, cells were lysed with SDS sample buffer and analysed by SDS/PAGE on a 15% (w/v) polyacrylamide gel. The expressed wild-type and mutant enzymes have an apparent molecular mass of 26.5 ± 1.2 kDa (data not

Table 1 Substrate specificities and kinetic parameters of recombinant GST 3-3 and mutants expressed in *E. coli*

Isomerase and peroxidase activity were determined with Δ^5 -androstene-3,17-dione and cumene hydroperoxide as second substrates respectively. Results are means $\pm$ S.E.M. of duplicates of at least three experiments. N.D., not detected.

	Wild-type	CallS	CallSY115F
CDNB-conjugating activity ($\mu\text{mol}/\text{min}$ per mg)	48.4 $\pm$ 0.7	72.2 $\pm$ 0.8	113.7 $\pm$ 2.3
Isomerase activity ($\mu\text{mol}/\text{min}$ per mg)	0.026 $\pm$ 0.002	0.027 $\pm$ 0.002	N.D.
Peroxidase activity ($\mu\text{mol}/\text{min}$ per mg)	0.14 $\pm$ 0.01	0.14 $\pm$ 0.01	0.10 $\pm$ 0.01
CDNB			
K_m (μM)	25.1 $\pm$ 3.0	39.1 $\pm$ 2.1	52.7 $\pm$ 3.7
k_{cat} (s^{-1})	24.3 $\pm$ 0.8	36.6 $\pm$ 1.5	78.8 $\pm$ 1.9
k_{cat}/K_m ($\mu\text{M}^{-1} \cdot \text{s}^{-1}$)	0.97	0.94	1.50
GSH			
K_m (μM)	75.9 $\pm$ 5.8	126.7 $\pm$ 17.2	120.9 $\pm$ 12.4
k_{cat} (s^{-1})	23.1 $\pm$ 2.4	34.3 $\pm$ 2.1	50.7 $\pm$ 1.2
k_{cat}/K_m ($\mu\text{M}^{-1} \cdot \text{s}^{-1}$)	0.30	0.27	0.42

shown). Based on densitometric scanning of the SDS/polyacrylamide gel, the protein bands depicting wild-type GST 3, CallS and CallSY115F represent 13.4, 17.8 and 16.3 % of the total soluble proteins respectively. The recombinant enzymes were purified on an *S*-hexylglutathione-Sepharose 6B column. We obtained 39 $\pm$ 1.5 mg, 35 $\pm$ 0.8 mg and 36 $\pm$ 2 mg of purified wild-type GST 3, CallS and CallSY115F respectively from 1 litre of cell culture.

Class-Alpha (Wang et al., 1989; Stenberg et al., 1992; Hayes et al., 1992), -Mu (Lai et al., 1989; Zhang and Armstrong, 1990; Vorachek et al., 1991) and -Pi GSTs (Tamai et al., 1991; Kong et al., 1991) have been expressed in *E. coli*. Expression of recombinant human GST A1-1, a class-Alpha enzyme, was induced by adding isopropyl β -D-thiogalactopyranoside; approx. 55 mg of purified GSTs was obtained from 1 litre of culture medium (Stenberg et al., 1992). Using a similar isopropyl β -D-thiogalactopyranoside induction system, low-level expression of GST 3-3 has been reported by Lai et al. (1989). In addition, the N-terminal proline residue of the recombinant GST 3-3 is omitted from the mature protein.

Overexpression of class-Mu GSTs in *E. coli* has been achieved with a heat-induction system. Rat liver GST 3 (Zhang and Armstrong, 1990) and human muscle GST 4 (Vorachek et al., 1991) genes were inserted into an expression vector, pMG27NS, under the control of the P_T promoter of λ phage. Approx. 20 mg of purified GST 3 and 50 mg of GST 4 in the crude lysate were obtained on heat induction. In the present phosphate-starvation system, we obtained over 35 mg of purified GST 3 protein/l of cell medium. This value is at the upper end of the range previously reported for overexpression of class-Mu GSTs.

Characterization of recombinant GST 3 in *E. coli*

Wild-type and GST 3 mutant enzymes were subjected to N-terminal sequence analysis. The N-terminal methionine residue had been removed from the mature recombinant proteins. The first 25 amino acid residues were identical with the sequence reported for GST 3 subunit. The C-terminal sequence was confirmed by digesting the proteins with Lys-C protease and isolating the C-terminal fragment for peptide-sequence analysis. The heptapeptide had a sequence of LAQWSNK. We conclude therefore that we had expressed a full-length GST 3 protein.

The molecular mass of the native protein was determined by gel-filtration chromatography using a Pharmacia Superose 12 analytical column. BSA (66 kDa), chicken egg ovalbumin

(44 kDa), bovine milk β -lactoglobulin (35 kDa), human carbonic anhydrase B (28 kDa) and soya-bean trypsin inhibitor (20.1 kDa) were used as standards for the calibration curve. The column was eluted with 50 mM Tris/ H_2SO_4 , pH 7.6, and 100 mM K_2SO_4 . Molecular masses of 50.1, 50.1 and 49.0 kDa were obtained for wild-type GST 3, CallS and CallSY115F respectively, therefore the expressed proteins must have existed in a dimeric form.

The native molecular mass of GST 3-3 expressed in SF9 cells has been determined previously to be 42 kDa by gel-filtration chromatography (Hsieh et al., 1989). A system containing 100 mM potassium phosphate, pH 7.0, and 100 mM KCl was used as the elution buffer in that study. Using the same elution buffer, GST 3 expressed in *E. coli* has a molecular mass of 40.1 $\pm$ 0.5 kDa. In addition, Zhang and Armstrong (1990) reported that GST 3 expressed in *E. coli* has a molecular mass of 46 kDa by gel-filtration chromatography in a sodium phosphate/NaCl buffer system. Therefore the native molecular mass of GST 3 as determined by gel-filtration chromatography is highly dependent on the buffer.

The conjugating activity, peroxidase activity, isomerase activity and kinetic constants of the expressed proteins determined with GSH and CDBN are listed in Table 1. The CDBN-conjugating activity and the K_m^{CDBN} values of the wild-type GST 3 in this study (48 $\mu\text{mol}/\text{min}$ per mg and 25 μM) are comparable with that reported by Hsieh et al. (1991) for the same enzyme expressed in SF9 cells (50 $\mu\text{mol}/\text{min}$ per mg and 32 μM) and by Lai et al. (1989) for the variant GST 3 expressed in *E. coli* (45 $\mu\text{mol}/\text{min}$ per mg and 36 μM). Nonetheless, the K_m^{GSH} value reported in the present study (76 μM) is significantly lower than that reported for the GST 3 variant (202 μM) of Lai et al. (1989) or the GST 3 expressed in *E. coli* under the control of the λ_{lac} promoter (112 μM) (Zhang et al., 1991).

A significant increase in the CDBN-conjugating activity can be observed for the CallSY115F mutant when compared with the wild-type GST 3-3. The upsurge in activity is caused by an increase in k_{cat} , rather than a decrease in K_m of the mutants for the substrates. The calculated k_{cat}/K_m value for the CallSY115F mutant is 1.5 times higher than that of the wild-type enzyme.

Conversely, the isomerase activities of the mutants are lower than that of the wild-type enzyme. We could not detect any isomerase activity for the CallSY115F mutant when Δ^5 -androstene-3,17-dione was used as substrate. We have also used 1,2-epoxy-3-(*p*-nitrophenoxy)propane to measure the isomerase activities of the enzymes, obtaining specific activities of 0.14, 0.12 and 0.05 $\mu\text{mol}/\text{min}$ per mg for the wild-type

Figure 1 Protein difference spectra for modification of wild-type GST 3-3 (a) and CallS mutant (b)

Enzymes were treated with 1 mM CDNB in 100 mM potassium phosphate, pH 7.0 (25 °C), and difference spectra were recorded with a double-beam spectrophotometer from 1 to 100 min as indicated.

enzyme, CallS and CallSY115F respectively. Therefore the isomerase activities of our expressed enzymes are lower than that reported for the GST 3-3 expressed in SF9 cells (Hsieh et al., 1991) or the GST 3-3 variant (Lai et al., 1989).

The peroxidase activities, with cumene hydroperoxide as substrate, were determined for the expressed proteins (Table 1). The values obtained are similar among the wild-type and mutants, and slightly higher than that reported for the variant GST 3-3 (0.10 $\mu\text{mol}/\text{min}$ per mg) (Lai et al., 1989). Nonetheless, this value is still substantially lower than that observed for the wild-type GST 3-3 expressed in SF9 cells (0.29 $\mu\text{mol}/\text{min}$ per mg) (Hsieh et al., 1991).

Thus we present here an alternative method for overexpressing GST in *E. coli*. The resulting wild-type and mutated GST 3-3 enzymes exhibit CDNB-conjugating, isomerase and peroxidase activities. Most importantly, they all show good CDNB-binding activity as indicated by the low K_m values.

Modification of GST 3-3 with CDNB

GST 3-3 was incubated with 1 mM CDNB in the absence of GSH in a quartz cuvette (light-path 1 cm) and the modification reaction was monitored spectrophotometrically. Enzyme subunit/CDNB ratio was maintained at 1:10. GST 3-3 and CDNB at twice the concentration as the reaction mixture were placed separately into a double-sector quartz cuvette (light-path 0.5 cm/sector) and used as a blank. The difference spectra clearly illustrate an increase in absorbance at 330 nm as a function of time (Figure 1a). Under similar reaction conditions, the CallS mutant shows an increase in absorbance at 305 nm (Figure 1b).

The data presented in Figure 1 can be plotted as absorbance versus incubation time. The resulting graphs are asymptotic in shape (results not shown) and demonstrate clearly that the rate of absorbance increase decreases noticeably after 20 and 30 min of incubation for the wild-type GST 3-3 and CallS mutant respectively.

Wild-type GST 3-3 and CallS mutant were allowed to react with CDNB for 20 and 30 min respectively, and the reactions were stopped by passing the mixtures through a gel-filtration column. Protein fractions were collected and further separated on a C18 reversed-phase column. GST 3-3 and CallS mutant were resolved into two components (Figures 2a and 2d). The slower-moving constituent (peak II) of both samples could be detected by monitoring the elution profile at 340 nm, suggesting that it carries dinitrophenyl (Dnp) derivative(s). To confirm our conclusion, near-u.v. spectra for the components, as they were eluted from the column, were recorded with a diode array detector. The results are presented in Figures 2(b) and 2(c) and 2(e) and 2(f) for wild-type GST 3-3 and CallS mutant respectively. The spectrum for peak I of each sample (Figures 2b and 2e) is identical with a control not incubated with CDNB (results not shown). The increase in near-u.v. absorbance between 300 and 350 nm (Figures 2c and 2f) clearly indicates that peak II represents proteins that have been covalently modified with CDNB.

On prolonged incubation (120 min) of the enzymes with CDNB, peak I of both samples disappears. The wild-type GST 3-3 sample gives a broad peak on reversed-phase chromatographic analysis, with retention time similar to that of peak II of Figure 2(a). With the same analysis, the CallS sample gives a single sharp peak with a retention time identical with that of peak II in Figure 2(b) (results not shown).

We incubated bovine erythrocyte carbonic anhydrase, soya-bean trypsin inhibitor, bovine brain hsc70, glutathione reductase and denatured GST 3-3 with CDNB. The resulting mixtures were analysed by the method outlined above. None of these proteins can be modified by CDNB, including GST 3-3 that has been denatured by passage through a reversed-phase column. The results suggest that the modification reaction is specific and native protein conformation is required.

Covalent binding of GST isoenzymes by electrophilic co-substrate CDNB, in the absence of GSH, is well documented. Pabst et al. (1974) reported a binding of 0.4 mol of CDNB/mol of GST A. CDNB inactivates the enzyme with time. Human GST p (Adams and Sikakana, 1990) and human lung acidic GST (Corrigall et al., 1989) are also irreversibly inactivated by CDNB. Human lung acidic GST can incorporate 3 mol of CDNB/mol of enzyme. In contrast, basic and near-natural human lung GSTs are still active after binding of 1.7 and 4.0 mol of CDNB/mol of enzyme respectively. In the present study, we produced a mixture of modified and unmodified GST 3-3 or CallS mutant on incubation with CDNB. Under regular assay conditions, these mixtures of modified and unmodified enzymes have the same kinetic parameters as proteins not subjected to CDNB treatment (results not shown).

Removal of the Dnp group from the modified enzymes

GST 3-3 and CallS mutant were modified with CDNB for 20 and 30 min respectively, and excess reagents were removed by gel-filtration chromatography. The samples eluted from the column were mixtures of modified (Dnp-GST or Dnp-CallS) and unmodified proteins. The Dnp-GST/GST 3 and Dnp-CallS/CallS ratios were approx. 1:2 and 2:3 respectively. These mixtures were incubated with GSH, dithiothreitol or *S*-

Figure 2 H.p.l.c. profile of GST 3-3 (a), CalIS (d) and CalISY115F mutant (g) after modification with CDNB

The photodiode spectra of peaks I and II separated in (a) and (d) are presented in (b), (c) and (e), (f) respectively. The photodiode spectrum of CalISY115F mutant after reaction with CDNB is presented in (h). In (a), (d) and (g): bottom solid line, A_{220} ; top solid line, A_{340} ; ----, [buffer B].

methylglutathione, and the constituents of the samples were separated on a C18-reversed-phase column. The relative amounts of modified and unmodified proteins were determined by integrating the area of the protein peaks eluted from the column, assuming similar absorption coefficients at 220 nm. The results were normalized to the control (modified sample not treated with GSH or dithiothreitol) (Table 2).

With a 30 s incubation period, GSH (1 mM final concentration) can remove over 95% of the Dnp groups from Dnp-

GST 3-3. By prolonging the incubation period to 2 min, we observed a 99% removal. Dithiothreitol is less successful in removing the Dnp moiety. After a 30 min incubation with 5 mM dithiothreitol, over 20% of the Dnp groups were retained on the enzyme. By increasing the dithiothreitol concentration to 10 mM, we could improve the removal rate to 93% over the same period. S-Methylglutathione is ineffective at eliminating the Dnp group. Approx. 98% of the Dnp groups were retained on Dnp-GST after a 30 min incubation with 1 mM S-methylglutathione.

Table 2 Removal of dinitrophenyl group from modified wild-type GST 3-3 and CallS mutant

Addition to modified enzyme	Relative amount of Dnp-GST	
	GST 3-3	CallS mutant
None	100	100
GSH (1 mM)		
30 s	4.4	1.5
2 min	0.9	0
Dithiothreitol (5 mM)		
15 min	45.4	30.6
30 min	22.7	25.4
Dithiothreitol (10 mM)		
15 min	24.2	22.7
30 min	6.4	5.5
S-Methylglutathione (1 mM)		
30 min	97.9	95.4

Table 3 Effect of inhibitors on CDNB modification of wild-type GST 3-3 and CallS mutant

	Relative amount of modified enzyme	
	GST 3-3	CallS mutant
Dinitrophenol (mM)		
0	100	100
1	87.2	60.7
2	72.2	45.1
3	58.8	43.5
4	56.4	26.9
S-Methylglutathione (mM)		
0	100	100
1	99.8	119.4
2	106.7	143.4
3	106.8	140.2
4	101.6	140.2

Compared with wild-type GST 3-3, the Dnp group on Dnp-CallS is easier to remove. Over 98 % was removed by 1 mM GSH within 30 s (Table 2). After a 2 min incubation period, no Dnp derivatives on Dnp-CallS could be detected. Dithiothreitol can remove 70-94 % of the Dnp groups from Dnp-CallS, depending on the incubation time and concentration of dithiothreitol used. Again *S*-methylglutathione is ineffective at eliminating the Dnp group from the enzyme, strongly suggesting that a thiol group is needed for the process.

In a previous report by Pabst et al. (1974), incubation of CDNB-modified GST A with 1 mM GSH and 1 mM dithiothreitol for 5 min removed only 65 and 45 % of the label respectively. The authors suggested that the Dnp group that cannot be removed by GSH may be attacked by non-specific alkylation, whereas the Dnp group that can be removed may be bound as substrate.

Protection experiments

Protection experiments were carried out to discover the location of the side chain(s) reactive towards CDNB. Dinitrophenol, which is structurally similar to CDNB except for the substitution

of a hydroxy for a chloro group, should compete for the H-site of GSTs. *S*-Methylglutathione can bind reversibly to the G-site (Principato et al., 1988; Del Boccio et al., 1991). Modification of GST 3-3 and CallS mutant was carried out with 1 mM CDNB in the presence of increasing amounts of these compounds. The mixtures were separated on a reversed-phase column after incubation, and the relative amount of Dnp-GST formed in each mixture was determined as described above and is given in Table 3.

Dinitrophenol shows a moderate degree of protection of GST 3-3 against CDNB modification. At a dinitrophenol/CDNB ratio of 2:1, Dnp-GST formation is decreased by 28 %. The amount of protection can be increased to 45 % by raising the dinitrophenol/CDNB ratio to 4:1. Dinitrophenol is more effective at protecting CallS mutant against CDNB modification. With equal amounts of dinitrophenol and CDNB, the formation of Dnp-CallS is decreased by 40 %. By increasing the concentration of dinitrophenol to 4 mM, we observed a 73 % decrease in the formation of Dnp-CallS (Table 3).

S-Methylglutathione is completely ineffective at protecting GST 3-3 or CallS mutant against CDNB modification. It has been suggested that binding of GSH to GST is accompanied by a conformational change. On binding of *S*-methylglutathione, GST 4-4 can catalyse the conjugation of 2-mercaptoethanol to CDNB; the activating effect of *S*-methylglutathione is concentration-dependent, having a maximum in a narrow concentration interval between 2 and 3 mM (Principato et al., 1988). Presumably the peptide part of *S*-methylglutathione induces a conformation change in the protein that is necessary for catalysis to occur. After assuming the active conformation, the enzyme can then accept thiol compounds such as 2-mercaptoethanol as substrates for catalytic reaction. We noticed a slight increase in modification (6 %) of wild-type GST 3-3 by CDNB in the presence of 2-3 mM *S*-methylglutathione (Table 3). A more marked increase (40 %) was observed for the CallS mutant over the same concentration range of *S*-methylglutathione (Table 3). Results from peptide mapping and amino-acid-sequence analyses indicate that CDNB labels the same residue on CallS mutant in the presence or absence of *S*-methylglutathione. We speculate that the CallS mutant is not as rigid as wild-type GST 3-3, and assumes a more open structure on *S*-methylglutathione binding.

Identification of Dnp amino acid on GST 3-3 and CallS

The identify of the modified residues was established by amino-acid-sequence analysis. The modified GST 3-3 and CallS mutant were purified on a reversed-phase column, and the fractions containing Dnp-GST and Dnp-CallS were collected for trypsin digestion. The resulting peptides were separated on a reversed-phase C18 regular-bore column, and the elution profiles were monitored at 220 and 340 nm. The tryptic maps for CallS, Dnp-GST and Dnp-CallS are presented in Figures 3(a), 3(b) and 3(c) respectively. The tryptic map for CallS does not contain peaks absorbing at 340 nm. Conversely, there are eight peaks exhibiting 340 nm absorbance generated from trypsin digestion of Dnp-GST. The amino acid sequences of peptides from these peaks are summarized in Table 4.

With the exception of peak 1, which is the major fraction absorbing at 340 nm, all peaks contain two or three peptides each. The sequence for each peptide was inferred from that of the total protein. The sequences of 16 peptides were deduced and they cover seven regions (residues 1-10, 32-47, 52-67, 82-95, 108-123, 144-172 and 173-186) for a total of 114 residues. Of these 16 peptides, 11 carry a cysteine residue, and they are distributed among peaks 1-7. There are four, five and two

Figure 3 H.p.l.c. profile of *Achromobacter* proteinase 1 digests of Callis mutant (a), Dnp-GST 3-3 (b) and Dnp-Callis (c)

Fractions 1-8 from (b) and 1 and 2 from (c) were collected for further amino acid sequence analysis. Bottom solid line, A_{220} ; top solid line, A_{340} ; ----, [buffer B].

Table 4 Amino acid sequence of trypsin digests of CDNB-modified GST 3-3 and CallS mutant

	Wild-type	CallS
Peak 1	KHHLGGETEER (82-93)	SYNPDFEK (114-121)
Peak 2	YAMGDAPDYDRSQWLN (32-47)	MLIMLSYNPDFEK (108-121)
	HHLCGETEER (83-93)	
Peak 3	PMILGYWNR (1-10)	
	CYNPDFEK (114-121)	
Peak 4	KHHLGGETEERIR (82-95)	
	MLIMLCYNPDFEK (108-121)	
	CLDAFPNLKDFLAR (173-186)	
Peak 5	KHHLGGETEER (82-93)	
	MLIMLCYNPDFEKQK (108-123)	
Peak 6	LGLDFNLPYLIDGSR (52-67)	
	MLIMLCY (108-115)	
Peak 7	MLIMLCYNPDFEK (108-121)	
	CLDAFPNLK (173-181)	
Peak 8	RPWFAGDKVTYVDLAYDILDQYHIFEPK (144-172)	
	VTYVDLAYDILDQYHIFEPK (152-172)	

peptides containing Cys-86, Cys-114 and Cys-173 respectively. The phenylthiohydantoin derivative of dinitrophenyl-cysteine, if it exists, is probably unstable in the sequencer and evades detection in the phenylthiohydantoin analyser. The recovery of the phenylthiohydantoin-tyrosine from the 14-residue peptide of peak 7 (MLIMLCYNPDFEK) is extremely low. This residue is probably modified. Peak 8 has a high A_{220}/A_{340} ratio, and we believe that the concentrations of the phenylthiohydantoin-amino acids generated from this peptide that absorb at A_{340} are too low for detection in the sequencer.

We have proved previously that cysteines are not needed for the enzymic activity of GST 3-3, and the mutant with cysteine to serine substitutions (CallS) is fully active with all the substrates tested (Chen et al., 1992). Jackson and Young (1987) pointed out that a Dnp group can be added to the side chain of the cysteine residue. Therefore we incubated CallS with CDNB, and the tryptic map of the resulting DNP-CallS is presented in Figure 3(c). Two peaks absorbing at 340 nm were obtained with sequences of SYNPDFEK (residues 114-121) and MLIMLSYNPDFEK (residues 108-121). The recoveries of phenylthiohydantoin-tyrosine (residue 115) from the sequencer for both peptides are drastically decreased (results not shown), clearly confirming the fact that Tyr-115 is the modified residue.

To confirm the sequencing data, the CallSY115F mutant was incubated with CDNB for 30 min, then analysed on a reversed-phase column after excess CDNB had been removed by gel-filtration chromatography. A single peak not absorbing at 340 nm was eluted from the column (Figure 2g), and the near-u.v. spectrum for this peak (Figure 2h) was identical with that of a GST 3-3 sample not treated with CDNB.

In its reaction with rat liver GST 3-3, CDNB demonstrates the characteristics of an affinity label. A limited number of sites on the enzyme are covalently modified. For the wild-type enzyme, reaction is limited mostly to peptides containing cysteine residues. Incidentally, Tyr-115 is located right next to a cysteine residue, and we have to resort to modification of the CallS mutant to confirm that cysteine residues on GST 3-3 are labelled non-specifically. The inability of CDNB to modify the CallSY115F quadruple mutant demonstrates the specific interaction between Tyr-115 and CDNB.

Results from protection experiments and the fact that Dnp groups can be removed from the modified enzymes with GSH

and dithiothreitol support our conclusions. The wild-type enzyme has Dnp groups on cysteine as well as tyrosine residues. The removal of the Dnp group with GSH from the tyrosine residue located in the H-site is a rapid process, whereas the thiolysis reaction involved in the removal of the Dnp groups from the cysteines is comparatively slow. Therefore the removal of Dnp label from the CallS mutant is much faster than that from the wild-type enzyme. With the same analogy, dinitrophenol protects the H-site of the enzymes from CDNB labelling, but is ineffective in preventing the cysteine residues located outside the active site of GST 3-3 from being modified. Consequently, dinitrophenol is a better protecting reagent for CallS than for the wild-type enzyme.

The involvement of a tyrosine in the G-site of GST has been demonstrated repeatedly by several groups (Stenberg et al., 1991; Liu et al., 1992; Kong et al., 1992; Manoharan et al., 1992; Wang et al., 1992). Using n.m.r. spectroscopy, Penington and Rule (1992) suggested that Tyr-115 of GST 4-4 is part of the hydrophobic binding site. Hoesch and Boyer (1989) identified a similar region on a class-Alpha GST as a possible electrophilic substrate-binding site by using a photoaffinity probe.

More concrete evidence for the localization of Tyr-115 in the hydrophobic binding site of GST is provided by Colman and co-workers. These authors modified GST 3-3 (Katusz et al., 1992) and 4-4 (Katusz and Colman, 1991) with *S*-(4-bromo-2,3-dioxobutyl)glutathione, a reactive analogue of GSH. Using kinetic analysis, tryptic mapping and amino-acid-sequencing analysis, they proposed that Tyr-115 is located at the H-site of the enzyme. Their approach and ours are similar, except that they used a GSH analogue with a reactive group that possibly attaches to the H-site of the enzyme, whereas we used a substrate that is known to bind specifically to the H-site. The conclusions from their studies and ours are in complete agreement.

The crystal structure of GST 3-3 has been elucidated by Ji et al. (1992). A portion of the hydrophobic face of the $\alpha 4$ helix (residues 104-115) has been proposed to be part of the H-site. The side chain of Tyr-115 is located within 0.85 nm (8.5 Å) of the sulphur atom of GSH. We present evidence here to support the notion that Tyr-115 is located in the H-site. However, this residue is probably only involved in the binding of xenobiotic substrates, as elimination of the hydroxy group on Tyr-115 (CallSY115F) has no effect on the CDNB-conjugating and peroxidase activity of the enzyme (Table 1).

In conclusion, we present here an alternative method for overexpressing GST in *E. coli*. We modified GST 3-3 and mutants with CDNB in the absence of GSH, and demonstrated the specific labelling of Tyr-115 with this compound. We propose that Tyr-115 is located in the H-site of GST 3-3.

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