Modification of glutathione S-transferase 3-3 mutants with 2-(S-glutathionyl)-3,5,6-trichloro-1,4-benzoquinone

Identification of the C-terminal tryptic fragment as part of the H-site and evidence that 2-(S-glutathionyl)-3,5,6-trichloro-1,4-benzoquinone is not specific for cysteine labelling

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A triple mutant of rat liver glutathione S-transferase 3-3 that has all three cysteine residues replaced with serine (CallS) and a quadruple mutant with a Tyr-115 to phenylalanine substitution on CallS (CallSY115F) were reacted with 2-(S-glutathionyl)-3,5,6-trichloro-1,4-benzoquinone (GS-1,4-TCBQ). The modified proteins were analysed on a triple-quadrupole mass spectrometer equipped with an electrospray ionization source. At an enzyme: GS-1,4-TCBQ ratio of 1:10, the enzymes were modified at multiple sites. Covalent attachment of a single inhibitor on to the protein was achieved by lowering the enzyme: GS-1,4-TCBQ ratio to 1:1. Results from m.s. analyses suggest that the inhibitor on the CallSY115F mutant exists as a glutathionyl dichlorobenzoquinone derivative. The modifiers of the CallS mutants are glutathionyl monochlorobenzoquinone derivatives. There-

fore, GS-1,4-TCBQ reacts at a single site on CallSY115F, but probably cross-links two regions on wild-type and CallS mutant. To confirm our observation, CallS was modified with 1-chloro-2,4-dinitrobenzene, which specifically labels Tyr-115, before reacting with GS-1,4-TCBQ. The inhibitor formed a glutathionyl dichlorobenzoquinone adduct on the dinitrophenyl–CallS mutant. In addition, the benzoquinone derivative on the protein can be partially removed by 1-chloro-2,4-dinitrobenzene. Peptide mapping and sequencing analysis of the GS-1,4-TCBQ-modified CallS mutant revealed that the C-terminal 16-amino-acid fragment is labelled. Molecular modelling suggests the C(5) and C(6) on the benzoquinone ring of the inhibitor interact with the oxygen atoms of Tyr-115 and Ser-209 respectively.

INTRODUCTION

Glutathione S-transferases (GSTs, EC 2.5.1.18) are a large family of multifunctional dimeric enzymes. They can be classified into four non-homologous species-independent multigene families, namely Alpha, Mu, Pi and Theta (Mannervik et al., 1985; Meyer et al., 1991). These enzymes have at least two distinct functions. They catalyse the conjugation of glutathione to a wide variety of reactive compounds, including electrophiles and hydroperoxides (Coles and Ketterer, 1990; Ketterer and Coles, 1991). Many xenobiotics, along with carcinogens and several therapeutic compounds, are among the substrates for GSTs (Tew et al., 1990; Tsuchida and Sato, 1992). In addition, GSTs act as intracellular binding and transport proteins for non-substrate hydrophobic ligands such as bile acids, bilirubin, a number of drugs and thyroid hormones (Mannervik and Danielson, 1988; Ishigaki et al., 1989).

The active sites of GSTs were initially investigated by modifying the enzyme with side-chain-specific reagents. Carne et al. (1979) suggested that the thiol group is important for catalytic activity of GST 1-1. Desideri et al. (1991) and Del Boccio et al. (1991) presented similar conclusions for GSTs from human placenta and horse erythrocytes respectively. Nonetheless, partial thiol modification of class-Alpha GSTs (Tamai et al., 1990; Shen et al., 1991; Chang et al., 1991) and site-directed mutagenesis of cysteine residues on class-Mu (Hsieh et al., 1991; Chen et al., 1992) and class-Pi (Tamai et al., 1991) GSTs demonstrated that cysteine residues are non-essential for enzymic activity.

Van Ommen et al. (1988, 1989) and Ploemen et al. (1991)

modified and inactivated class-Alpha and -Mu GSTs with tetrachloro-1,4-benzoquinone (TCBQ) and its glutathione conjugate. They suggested that GS-1,4-TCBQ is specific for thiol-group modification and that GSTs possess one cysteine residue in or near the vicinity of the active site.

The three-dimensional structure of GST 3-3 complexed with glutathione has been elucidated by Ji et al. (1992). A preliminary report on the structure of the H-site of GST 3-3 has been published by Armstrong et al. (1993). Tyr-115 is located in the H-site (Liu et al., 1993) and is catalytically important (Johnson et al., 1993; Barycki and Colman, 1993). It is conceivable that van Ommen et al. (1988, 1989) abolished the GST activity by modifying the adjacent Cys-114 with GS-1,4-TCBQ. Given the reactive nature of the quinone compound, it probably could interact with other amino acid side-chain(s) in the absence of thiol groups. Its glutathione conjugate is ideal for affinity-labelling of the H-site amino acids.

Over the past decade, m.s. has become an essential tool for protein structure determination. A high-performance quadrupole instrument could be expected to obtain a mass accuracy of 0.01–0.02% (Jardine, 1990). Using this technique, the molecular mass of most GSTs can be determined with an accuracy of approx. ± 3 –5 Da under optimal conditions.

We have shown previously that a GST 3-3 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) are enzymically active (Chen et al., 1992; Liu et al., 1993). We investigated the interaction of these GST 3-3 mutants devoid of sulphhydryl groups with GS-1,4-TCBQ. The

Abbreviations used: GST, glutathione S-transferase; GS-1,4-TCBQ, 2-(S-glutathionyl)-3,5,6-trichloro-1,4-benzoquinone; TCBQ, tetrachloro-1,4-benzoquinone; CDNB, 1-chloro-2,4-dinitrobenzene; l.c.–e.s.m.s., liquid chromatography–electrospray mass spectrometry.

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resulting enzymes were analysed with m.s., peptide mapping and sequencing. We present evidence that Tyr-115 and the C-terminal fragment are part of the H-site.

MATERIALS AND METHODS

Materials

Recombinant wild-type rat liver GST 3-3 and mutants used in this study have been described previously (Liu et al., 1993). Trypsin was obtained from Boehringer-Mannheim (Mannheim, Germany) and repurified according to Lee and Shively (1990) before use. ^3H -labelled glutathione (1 Ci/mmol) was purchased from New England Nuclear (Wilmington, DE, U.S.A.). Substrates for enzymic assay and TCBQ were purchased from Merck (Darmstadt, Germany). Chemicals and reversed-phase columns used in sequencing were obtained from Applied Biosystem (ABI, Foster City, CA, U.S.A.). Mono S and Superose 12 prepacked columns were purchased from Pharmacia (Uppsala, Sweden). Vydac reversed-phase columns were obtained from H-P Separations Group (Hesperia, CA, U.S.A.). All other chemicals used were reagent grade or better.

Enzyme purification and assay

Harvested *Escherichia coli* cells were suspended in 50 mM Tris-HCl, pH 8.0, 1 mM ethylenedinitrilotetra-acetic acid, 15% (w/v) sucrose, 6 mM 2-mercaptoethanol and 0.5 mM phenylmethanesulphonyl fluoride (30 ml/l of cell culture). Lysozyme was added (1 mg/ml), followed by incubation on ice for 20 min. Cells were lysed by sonication and debris was removed by centrifugation at 4 °C in a Beckman Type 70 Ti rotor for an hour at 50000 rev./min. The expressed wild-type or mutant proteins in the supernatant were purified by affinity chromatography according to the methods of Mannervik and Guthenberg (1981). Proteins after affinity-column chromatography were passed through a Superose 12 gel-filtration column that was eluted with 10 mM Tris/ H_2SO_4 , pH 7.0, and 100 mM K_2SO_4 for removal of unbound *S*-hexylglutathione. Proteins were stored at -70 °C at a concentration of 1 mg/ml or higher with 10 mM dithiothreitol. The purity of the enzymes was analysed with SDS/PAGE (Laemmli, 1970) on a 12.5% polyacrylamide gel. The conjugating activity of the enzymes was assayed at 25 °C with 1-chloro-2,4-dinitrobenzene (CDNB) (Habig and Jakoby, 1981). Protein concentration was determined from 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.

Synthesis of 2-(*S*-glutathionyl)-3,5,6-trichloro-1,4-benzoquinone (GS-1,4-TCBQ)

GS-1,4-TCBQ was synthesized by conjugating glutathione with TCBQ according to the method of van Ommen et al. (1988). The reaction mixture was purified on a Vydac C_{18} (4.6 mm $\times$ 250 mm) reversed-phase column that had been equilibrated in 50 mM Tris/phosphate buffer, pH 2.5. The conjugates were eluted from the column (at a flow rate of 0.6 ml/min) with a linear gradient of 1%/min of methanol. The GS-1,4-TCBQ was identified by m.s. (m/z = 518) and passed through a Superose 12 gel-filtration column that was eluted with 20% (v/v) methanol. The sample was stored at -70 °C. The radioactive conjugate was prepared similarly. The GSH used in the conjugation reaction has a radiospecificity of 0.1 Ci/ μmol .

The concentration of the GS-1,4-TCBQ was determined from the A_{340} value; TCBQ and GSH do not have absorbance at

340 nm. Known amounts of GSH were added to TCBQ at a molar ratio of 1:10 and a u.v. absorption spectrum was obtained. Assuming a single product was formed, a molar absorption coefficient of $6.15 \pm 0.09 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ was determined for GS-1,4-TCBQ at 340 nm.

Protein modifications

Enzymes were incubated with 10 mM dithiothreitol for 15 min and purified by passing through a Superose 12 column eluted with 10 mM Mops, pH 6.5, containing 10 mM K_2SO_4 . The enzymes (2 nmol, 100 μg in 4 ml) were modified immediately at 25 °C (10 min) with an enzyme subunit:GS-1,4-TCBQ ratio of either 1:1 or 1:10. Unreacted GS-1,4-TCBQ was removed by passing the reaction mixture through a Mono S cation-exchange column equilibrated in reaction buffer. After washing with the same buffer, the modified protein was eluted from the column with 10 mM Mops, pH 7.0, containing 100 mM K_2SO_4 . Samples were concentrated to 1.0–1.5 mg/ml in a Centrprep-10 Concentrator (Amicon, Beverly, MA, U.S.A.). Reaction of the GS-1,4-TCBQ-modified enzyme with CDNB was carried out according to Liu et al. (1993) at a protein subunit: CDNB ratio of 1:10 for 20 min.

Alternatively, enzymes were modified first with CDNB at a ratio of 1:10 for 20 min (Liu et al., 1993). Excess CDNB was removed by passing through a Superose 12 column eluted with 10 mM Mops, pH 6.5, containing 10 mM K_2SO_4 . The modified enzyme was reacted with GS-1,4-TCBQ as outlined above, and subjected to liquid chromatography-electrospray m.s. (l.c.-e.s.m.s.) analysis without further purification.

M.s. analysis

The e.s.m.s. analyses were performed on a VG Quattro-Bio-Q (Fisons Instruments, VG Biotech, Altrincham, U.K.), a triple quadrupole instrument with a mass range for singly charged ions of 4000. Samples were introduced from an ABI model 140B h.p.l.c. system and monitored with a Model UVIS 20 (Linear Instruments, NA, U.S.A.) detector equipped with a micro flow-cell. The h.p.l.c. system, the detector and the electrospray probe of the mass spectrometer were connected with 50 μm fused silica tubing. The h.p.l.c. pumps delivered solvent at a constant rate of 350 $\mu\text{l}/\text{min}$. A splitter (1:35) was employed in front of a Rheodyne model 8125 injector, so that the solvent was applied to the column at 10 $\mu\text{l}/\text{min}$. Modified proteins from the Mono S column were injected on to a Brownlee Aquapore C_{18} column (1 mm $\times$ 250 mm) and separated with a linear gradient of 0.32%/min of 0.05% trifluoroacetic acid in acetonitrile. The multiply charged ion peaks from myoglobin (16951 kDa) (Zaia et al., 1992) were used as an external reference for mass scale calibration. Scanning was from m/z 700 to 1700 at 10 s/scan. Data were summed according to the u.v. absorption profile of the samples monitored at 220 nm and processed by the MaxEnt Program (Ferrige et al., 1992).

Trypsin digestion and peptide purification

Modified proteins eluted from the Mono S column with 10 mM Mops, pH 7.0, containing 100 mM K_2SO_4 were digested with trypsin at a substrate to enzyme ratio of 10:1. Proteins were digested for 4 h at 37 °C or 16 h at 25 °C. Resulting peptides were separated on a Vydac reversed-phase column (C_{18} ; 2.1 mm $\times$ 250 mm) at a flow rate of 0.2 ml/min, with a linear gradient of 0.4%/min of 0.05% trifluoroacetic acid in acetonitrile. The elution profile from the column was monitored at 220 and 340 nm.

Protein sequencing

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 (Hewick et al., 1981).

Molecular model building

The protein model constructed is based on the 1.9 Å resolution X-ray structure of GST 3-3 co-crystallized with 1-(S-glutathionyl)-2,4,6-trinitrocyclohexadienate (Ji et al., 1993). The configuration of GS-1,4-TCBQ was assembled using the Chem-Note technique on a Silicon Graphics IRIS 4D/25 workstation with the QUANTA program (Polygen Corp., Waltham, MA, U.S.A.). These two models were annealed by the CHARMM energy-minimization process with the CHARMM/QUANTA program package (Brooks et al., 1983). The hydrogen bonds in the S-glutathionyl group and the protein were constrained during the energy-minimization calculation. The hydrogen bonds, ion pairs and hydrophobic interactions of the model were optimized with 200 steps of steepest-descent minimization, followed by 200 cycles of Powell-conjugate-gradient minimization and 300 cycles of conjugate-gradient minimization.

RESULTS

Modification of GST 3-3 mutants with GS-1,4-TCBQ

GST 3-3 mutants were incubated with dithiothreitol and the molecular mass of the enzymes was determined with e.s.m.s. The results are summarized in Table 1. The results obtained from m.s. are in close agreement with those predicted from cDNA sequencing, indicating that the proteins are not modified before GS-1,4-TCBQ or CDNB treatment. GST 3-3 and GST 3-3 mutants can be easily oxidized during purification and storage (results not shown). Autoxidation of GST 3-3 has also been reported by Hayes and Clarkson (1982) during the isolation of this enzyme from rat liver. The dithiothreitol reduction step is essential for the efficient labelling of the proteins with either GS-1,4-TCBQ or CDNB.

Wild-type GST 3-3 and the CallS mutant were incubated with GS-1,4-TCBQ at a 1:10 or 1:1 ratio, and proteins were removed

Figure 1 The time course of inhibition of GST 3-3 and CallS mutant by GS-1,4-TCBQ

Inhibitor and enzyme were mixed at 25 °C. At different time intervals, 0.25 µg of enzyme samples were transferred into cuvettes containing 1 mM glutathione to terminate the reaction. The remaining activity was determined after adding 1 mM CDNB as the second substrate. GST 3-3 inhibitor at 1:10 (○) and 1:1 (●) ratio. CallS mutant/inhibitor at 1:10 (□) and 1:1 (■) ratio.

at different time intervals for enzymic activity assay. The wild-type enzyme and the GST 3-3 mutant were inactivated in a time-dependent manner at either high or low protein to inhibitor ratio (Figure 1). GST 3-3 lost over half (70–85 %) of the activity within 30 s in the presence of an equal molar concentration of GS-1,4-TCBQ. Similar results were obtained for the CallS mutant containing no cysteine residues.

The molecular mass of the modified CallS mutant was analysed by l.c.–e.s.m.s. The results are summarized in Table 1. At a high inhibitor:enzyme ratio (10:1), the protein is modified at multiple sites. The CallS mutant eluted from the reversed-phase column was seen as a single peak by u.v. monitoring. M.s. analyses indicated that the modified CallS enzyme has a molecular mass of $26\,701 \pm 2$ Da, which is 964 Da higher than the native enzyme (Table 1). Over 90 % of the protein existed in this form (Figure 2a).

The glutathione conjugate of TCBQ has a molecular mass of 517 Da. Covalent binding of this inhibitor with GST 3-3 should eliminate a chloride ion and a proton from the GS-1,4-TCBQ and the enzyme respectively. Therefore, an addition of 481 Da is expected upon formation of an enzyme–glutathionyl dichloro-1,4-benzoquinone complex. Based on the e.s.m.s. data, we can conclude that the inhibitor forms two adducts on each protein subunit.

At an enzyme/inhibitor ratio of 1:1, an increase in molecular mass of 446 Da was observed for the CallS mutant (Table 1, Figure 2b). The result obtained is lower than that expected from adding a single glutathionyl dichloro-1,4-benzoquinone moiety. The data could be explained by assuming that a single GS-1,4-TCBQ molecule cross-links two residues on the enzyme. This process forms a glutathionyl chloro-1,4-benzoquinone adduct by eliminating two chlorides and two protons from GS-1,4-TCBQ and enzyme respectively. Theoretically, the protein modified in this fashion is 444 Da larger than the native protein.

We have shown previously that Tyr-115 is located at the H-site of the enzyme and reacts with CDNB (Liu et al., 1993). A CallSY115F mutant is fully active but cannot be labelled with CDNB. Given the 'targeting' nature of the inhibitor, GS-1,4-TCBQ and CDNB should compete for the same site and possibly

Table 1 Molecular mass of GST 3-3 mutants determined by e.s.m.s.

Numbers in parentheses indicate the difference in molecular mass between modified and native protein subunits determined by e.s.m.s.

	Molecular mass	
	CallS	CallSY115F
GST3		
cDNA sequencing	25734.5	25718.6
E.s.m.s.	25737 ± 3	25720 ± 1
GST3 + GS-1,4-TCBQ		
1:10	26701 ± 2 (+964)	26202 ± 3 (+482)
		26684 ± 2 (+964)
1:1	26183 ± 3 (+446)	26202 ± 3 (+482)
GST3–GS-1,4-TCBQ complex + CDNB	25739 ± 2	25723 ± 4
	26180 ± 4 (+443)	26205 ± 3 (+485)
		26393 ± 3 (+673)
GST3 + CDNB	25905 ± 2 (+168)	25722 ± 2
Dnp-GST3 + GS-1,4-TCBQ	26386 ± 2 (+649)	–

Figure 2 The transformed mass spectra of the CallS mutant

CallS mutant was modified with GS-1,4-TCBQ at 1:10 (a) and 1:1 (b) ratio.

interact with the same residue. To test this hypothesis, we incubated the CallSY115F mutant with an equal molar concentration of GS-1,4-TCBQ (Figure 3b). The modified enzyme has a molecular mass of 26202 ± 3 Da, that is consistent with the formation of a glutathionyl dichloro-1,4-benzoquinone adduct. At a high inhibitor/enzyme ratio (10:1), the majority of the modified protein has a molecular mass of 26200 ± 3 Da. We observed also a component with a molecular mass of 26684 Da, which is 964 Da higher than the native enzyme (Figure 3a). The result is similar to that obtained for the CallS mutant, suggesting the addition of two glutathionyl dichloro-1,4-benzoquinone moieties.

Modification of dinitrophenyl-CallS with GS-1,4-TCBQ

Experiments were done to confirm that GS-1,4-TCBQ and CDNB compete for the same site and that GS-1,4-TCBQ cross-links two different regions of the enzyme. The CallS mutant was modified with CDNB. After purification, the dinitrophenyl-GSTs were further incubated with GS-1,4-TCBQ. Samples were analysed with l.c.-e.s.m.s. after each step of enzyme modification.

The CallSY115F mutant could not be labelled with CDNB, even at a protein/CDNB ratio of 1:70 (Table 1). The modified

Figure 3 The transformed mass spectra of the CallSY115F mutant

CallSY115F mutant was modified with GS-1,4-TCBQ at 1:10 (a) and 1:1 (b) ratio. The product in (b) was reacted with a 10-fold molar excess of CDNB for 6 min (c) and 20 min (d).

CallS mutant has a molecular mass of 25905 Da, which is 168 Da higher than the native protein. At least 95% of the CallS mutant exists in this form.

After removing the excess CDNB, the dinitrophenyl-CallS mutant was incubated with GS-1,4-TCBQ. The resulting protein has a molecular mass of 26386 ± 2 Da (Table 1), which indicates the addition of a glutathionyl-dichloro-1,4-benzoquinone group (481 Da) to the dinitrophenyl-CallS mutant.

Reaction of GS-1,4-TCBQ-modified GST 3-3 mutants with CDNB

The GST 3-3 mutants were modified with GS-1,4-TCBQ at an enzyme/inhibitor ratio of 1:1. Under the modification conditions that we used, unmodified proteins were not observed by m.s. (Figures 2b and 3b). The resulting enzymes were purified and reacted with CDNB for 20 min. After incubation, the modified CallS mutant gives two peaks in the transformed mass spectrum. These two peaks are roughly 4:1 in area and have molecular masses of 26180 and 25739 Da respectively (Table 1). Apparently, CDNB removed the glutathionyl-chlorobenzoquinone group from the CallS to generate the 25739 Da peak. The other peak is 443 Da more than the native enzyme, suggesting the presence of a glutathionyl-chloro-1,4-benzoquinone group.

Figure 4 H.p.l.c. profile of tryptic digests of the GS-1,4-TCBQ-modified CallS mutant

Fraction 1 was collected for further amino-acid-sequence analysis. Bottom solid line, A_{220} ; top solid line, A_{340} ; broken line, [buffer B].

Normally, CDNB does not react with the CallSY115F mutant. However, incubation of glutathionyl-dichlorobenzoquinone-modified CallSY115F mutant (Figure 3b) with CDNB under regular reaction conditions (20 min at enzyme/CDNB ratio of 1:10) gives three peaks at 25723, 26205 and 26393 Da on the transformed mass spectrum (Figure 3d). These peaks exist in a 1:2:2.5 ratio. By shortening the incubation time to 6 min, the ratio of the peak area among these peaks changed to 1:5:2 (Figure 3c). The 25723 and 26205 Da peaks probably represent the unmodified and the glutathionyl-dichloro-1,4-benzoquinone-modified enzyme respectively. Apparently, CDNB removed the glutathionyl-benzoquinone derivative from the enzyme and regenerated the unmodified protein. The CDNB/glutathionyl-benzoquinone complex is capable of binding back on to the protein, generating a protein complex of 26393 Da.

Thus, we present evidence that CDNB can partially remove the glutathionyl-benzoquinone derivative from the enzymes.

Identification of modified peptide in the CallS GST 3-3 mutant

The identity of the modified peptide in CallS was established by amino-acid-sequence analysis. The enzyme was modified with an equal molar concentration of GS-1,4-TCBQ. After purification, the modified enzyme was digested with trypsin and the resulting peptides were separated on a reversed-phase C_{18} narrow-bore column. The eluate profile was monitored at 220 and 340 nm and is presented in Figure 4. The tryptic map of the CallS mutant has a single major peak that absorbs light at 340 nm. Upon sequence analysis, this peak contains a distinct peptide covering the C-terminal 16 amino acids of the protein (YLSTPIFSKLAQWSNK).

DISCUSSION

TCBQ and its glutathione conjugate have been utilized by van Ommen et al. (1988, 1989, 1991) for irreversible inhibition of rat liver GSTs. Using a mixture of GSTs from rat liver at a protein/inhibitor ratio of 1:10, van Ommen et al. (1988) reported an 80% inhibition of enzyme activity at 25 °C within 30 s. Each mol of enzyme subunit covalently binds 0.7 mol of inhibitor. The glutathione moiety seems to target the conjugate to the active site

of the enzyme. The corresponding 2-mercaptoethanol conjugate shows a much lower rate of inhibition. At a 5-fold molar excess of reagent, GST 4-4 binds 3.2–3.5 benzoquinone molecules per subunit, and the resulting protein cannot be modified with iodoacetamide (van Ommen et al., 1989). In addition, human GST A2-2 that possesses no cysteine, was not inhibited and only 0.08 nmol of benzoquinone could be bound per nmol of enzyme subunit (Ploemen et al., 1991). In these reports, the enzymes were modified with radioactive inhibitors and the amounts of labels covalently bound on the enzymes were estimated by scintillation counting. Thus benzoquinone and its glutathione derivative were presented as G-site-targeting inhibitors that react only with thiol groups (van Ommen et al., 1991).

In this study, we utilized *E. coli*-expressed rat GST 3-3 mutants that are devoid of cysteine residues as starting materials. These mutants are active in the CDNB conjugation assay. With glutathione and CDNB as substrates, the K_m and k_{cat} values of these mutants are similar to or better than that of the wild-type enzyme (Liu et al., 1993). The kinetic parameters indicate that the gross catalytic properties of these mutants and wild-type GST 3-3 are similar.

The inhibition kinetics of the wild-type GST 3-3 and the CallS mutant towards GS-1,4-TCBQ are similar (Figure 1). Most importantly, at equal molar concentrations, GS-1,4-TCBQ can effectively inactivate either the wild-type or CallS mutant. The results authenticate the 'targeting effect' of the glutathionyl-benzoquinone derivative as reported by van Ommen et al. (1991), but also show that GS-1,4-TCBQ acts on residues other than cysteines.

Inactivation of CallS mutants occurs through the formation of a glutathionyl-chlorobenzoquinone adduct that theoretically should result in addition of 444 Da to the subunit molecular mass. Experimentally, we obtained an addition of 446 Da to the subunit molecular mass of the CallS mutant. Apparently, the generation of the glutathionyl-chlorobenzoquinone adduct on the GST depends on the presence of Tyr-115. In the absence of Tyr-115, a glutathionyl-dichlorobenzoquinone adduct is formed with the CallSY115F mutant, with an increase of 481 Da (Table 1). Conversely, the functional group of Tyr-115 on the CallS mutant can be masked by modification with CDNB. The resulting enzyme could then form a glutathionyl-dichlorobenzoquinone adduct upon incubation with GS-1,4-TCBQ.

Incubating the enzymes with inhibitors at a 1:10 ratio causes labelling at multiple sites. M.s. analyses show clearly that two molecules of inhibitor can bind to each subunit of the CallS mutant. We have also analysed the modified wild-type GST 3-3 with m.s. Due to the presence of thiol groups that can react with the inhibitor, the resulting spectrum indicates there are multiple components in the sample. The transformed mass spectrum is difficult to interpret but suggests that the protein increases in mass by 1000–1600 Da (results not shown). The result implies the wild-type enzyme can bind covalently more than two inhibitors.

The formation of glutathionyl-chlorobenzoquinone or dichlorobenzoquinone adducts to mutant GST 3-3 can be partially reversed by treatment with CDNB. Using m.s. analysis, we observed complete modification of CallS and CallSY115F mutants with GS-1,4-TCBQ (Figures 2b and 3b). Upon adding CDNB, we started to detect the generation of unmodified proteins. The effect is most pronounced with the CallSY115F mutant, which normally is unreactive towards CDNB (Liu et al., 1993). Upon incubation of the CallSY115F-GS-1,4-TCBQ complex with CDNB, the transformed spectrum of the sample has three peaks. Besides the original fraction, we have a component with a molecular mass matching that of the unmodified protein (25723 Da, Figure 3c), which increased with incubation time

Figure 5 Stereoview of a model of GS-1,4-TCBQ interacting with CallS mutant

(Figure 3d). Van Ommen et al. (1991) reported that the maximal inhibition of GSTs with quinones that could be reached was 90%. The regeneration of unmodified enzymes with CDNB probably accounts for part of the residual activity.

M.s. data strongly suggest that GS-1,4-TCBQ interacts with Tyr-115. Nonetheless, a covalent complex between the inhibitor and a peptide containing Tyr-115 has not been identified. Probably, the adduct is unstable under the basic tryptic digestion condition that we are using.

A covalent complex between GS-1,4-TCBQ and the C-terminal 16-amino-acid fragment of GST 3-3 has been isolated. Construction of chimeric isoenzymes has implicated residues in the C-terminal tail as participating in catalysis (Board and Mannervik, 1991; Zhang et al., 1992). Using photoaffinity labelling, Hoesch and Boyer (1989) reached similar conclusions with a class-Alpha GST. Therefore, isolation of GS-1,4-TCBQ complexed with the C-terminal of GST 3-3 can be expected.

M.s. data suggest the formation of a glutathionyl-chlorobenzoquinone adduct with the CallS mutant. The result implies the inhibitor cross-links two segments of the protein. We have identified the C-terminal peptide covalently linked to the inhibitor. Protection experiments with CDNB and e.s.m.s. results with CallSY115F mutant suggest that Tyr-115 is probably the other residue that interacts with the inhibitor. We have used molecular modelling to substantiate this possibility.

The coordinates of GST 3-3 co-crystallized with 1-(S-glutathionyl)-2,4,6-trinitrocyclohexadienyl (Ji et al., 1993) were employed in devising this model. The inhibitor was assembled and annealed to this model and the final configuration was optimized by the energy-minimization process. In this model (Figure 5), the C(5) and C(6) of the benzoquinone ring are 1.27 Å and 1.58 Å from the oxygen atoms of Tyr-115 and Ser-209 respectively.

According to the X-ray-diffraction model of GST 3-3 complexed with GSH, the $\alpha 4/\alpha 5$ helix-turn-helix and the C-terminal tail form a 19 Å-deep channel in the active site. Tyr-115 is located on the C-terminus of the $\alpha 4$ helix. Both Tyr-115 and Ser-209 are

identified as residues with side-chains near (≤ 8.5 Å) the sulphur of GSH (Ji et al., 1992).

Recently, Ji et al. (1994) elucidated the structure of GST 3-3 complexed with each of the diastereomeric addition products with phenanthrene 9,10-oxide. Residues that have van der Waals contacts with the dihydrophenanthrenyl ring system include Tyr-6, Trp-7, Val-9, and Leu-12 in domain I and Iso-111, Tyr-115, Phe-208, and Ser-209 in domain II. In addition, the hydroxyl group of Tyr-115 forms a hydrogen bond with the 10-hydroxyl group of the product. Substitution of this residue with Phe caused a marked decrease in activity towards phenanthrene 9,10-oxide (Johnson et al., 1993) and 4-phenyl-3-buten-2-one (Ji et al., 1994). Therefore, our conclusions based on m.s. analyses and molecular modelling are in close agreement with those obtained from X-ray crystallographic and kinetic studies.

Tyr-115 is also within hydrogen-bonding distance of the side-chain hydroxyl group of Ser-209 and the main-chain amide nitrogen of the same residue (Armstrong et al., 1993). Ser-209 and Tyr-115 are located at the entrance of the active site. Johnson et al. (1993) postulated that Tyr-115 forms a hydrogen bond to the hydroxyl group of Ser-209, which links the $\alpha 4$ helix with the C-terminal tail of the protein. The net result is a decrease in segmental motion of the protein and product release. With this model, breaking the hydrogen-bonding interactions between the hydroxyl groups of Tyr-115 and Ser-209 would cause an increase in enzymic activity. The kinetic parameters of a Tyr-115 $\rightarrow$ Phe mutation validate this suggestion. Nonetheless, a CallSY115A quadruple mutant is only 20% as reactive as the wild-type enzyme (L.-F. Liu and M. F. Tam, unpublished work). GST 4-4 has Ala at position 209 and its CDNB-conjugating activity is much lower than that of GST 3-3 (Mannervik and Danielson, 1988). It has also been proposed that Tyr-115 positions the C(1) of CDNB in proximity to the thiolate of glutathione by providing hydrophobic interactions for the benzene ring of the substrate (Barycki and Colman, 1993). However, the distance between the hydroxyl of Tyr-115 and the thiolate of glutathione is 7.5 Å (Ji et al., 1992), which is too long for this kind of interaction. Therefore,

the role of Tyr-115 in the CDNB and GSH conjugation reaction has to be further investigated.

In summary, GS-1,4-TCBQ can modify CallS and CallSY115F mutants. The inhibitor can be partially removed with CDNB. The C-terminal 16-amino-acid fragment is covalently complexed with GS-1,4-TCBQ. Tyr-115 and Ser-209 are the two residues that possibly interact with GS-1,4-TCBQ.

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