Characterization of the ligandin site of maize glutathione S-transferase I

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INTRODUCTION

GSTs (glutathione S-transferases; EC 2.5.1.18) are dimeric detoxification enzymes that catalyse a wide variety of conjugations of GSH to hydrophobic electrophilic compounds [1,2]. Each GST monomer has two domains, an α/β domain that includes helices α1–α3 and a largely α-helical domain comprising helices α4–α9. The former contains a glutathione-binding site (denoted as G-site) on top of the α-domain. A hydrophobic pocket (known as the H-site) lies largely between the domains in which a generally hydrophobic substrate binds and reacts with the glutathione to form a conjugate product [3,4].

GSTs were originally termed ligandins because, in addition to their enzymic roles, they bind to large lipophilic molecules (> 400 Da), such as bile acids, fatty acids, bilirubin, haem and certain drugs, leading to the suggestion that GSTs are involved in the storage and rapid transport of these molecules in the aqueous phase of the cell [5–10]. The precise functions of GST binding to non-substrate ligands remain unclear [10–13]. The existence of a distinct ligandin-binding site (L-site) has been confirmed by the findings that ligands can bind in the dimer interface of GSTs from Schistosoma japonica [14] and squid [15]. In the case of Arabidopsis enzyme, the L-site, as identified by crystallography, is located next to the G-site between the side-chains of helices α3′/α3‴ and α5′ [16]. A different picture has emerged from the case of the human GST P1-1 isozyme where the L-site is located in the electrophile substrate binding site [17].

Triazine dyes have proven to be useful and versatile tools for probing structure–function relationships and ligandin function in a variety of proteins, including GSTs [17–20]. CB3GA (Cibacron Blue 3GA) has been used as a probe to classify mouse GST isoenzymes [3] and also as the probe to characterize mouse GST I in their metabolism or transport to target sites. The present results provide evidence for the binding of plant hormones and flavonoids to GST I, which probably indicates the involvement of GST I in their metabolism or transport to target sites.

MATERIALS AND METHODS

Materials
dNTPs and restriction enzymes were obtained from Roche. GSH, CDNB (1-chloro-2,4-dinitrobenzene), gibberellic acid,
2-naphthoxyacetic acid, indole-3-butryic acid, kinetin and CB3GA were obtained from Sigma–Aldrich Co. XL1-Blue Escherichia coli cells and Pfu DNA polymerase were from Stratagene. Quercetin, cyanidin, luteolin and naringenin were purchased from Fluka. VBAR {Vilmax Blue A–R; 1-amino-4-[3-(4,6-dichlorotiazin-2-ylamino)-4-sulphophenylamino]anthraquinone-2-sulphonic acid} was from Vilmax S.A., Buenos Aires, Argentina. The same dye is also available from Fluka under the commercial name Procion Blue MX-R®.

Cloning, expression and purification of maize GST I and other GSTs

The cloning of maize GST I into a pQE70 expression vector to yield the pQEGST expression plasmid was described in [26]. Expression and purification of GST I were performed as described previously [26]. Human spleen haematoipoietic prostatic gland D synthase, was purified as described in [27], whereas soybean GST4 was cloned, expressed and purified as described in [28]. Recombinant human GST A1-1 was expressed in E. coli and purified as described previously [29]. The expression vector for human GST A1-1 was a much appreciated gift from Professor W. M. Atkins (Department of Medicinal Chemistry, University of Washington, Seattle, WA, U.S.A.).

Dye purification

CB3GA and VBAR were purified to > 95 % purity on lipophilic Sephadex LH 20 as described previously [18,19].

Site-directed mutagenesis

Site-directed mutagenesis was performed as described previously [30]. The pairs of oligonucleotide primers used in the PCR reactions were as follows: for the Ile118 → Phe mutation, 5'-CCCATCAACCTCGCACC GCCC-3' and 5'-CAGCATCTC-GTATCGGAGCC-3'; for the Trp12 → Leu mutation, 5'-ATGTCGAATCGGAGTTGC-3'; and 5'-CACC CGCCCG-TACGCTTTATCGG-3'; for the Phe15 → Leu mutation, 5'-ATCACCTCGCACC GCCACACG CACG-3' and 5'-GGGCACGATCTGATCTGGAGCC-3'. Sites of mutation are indicated in italics. The expression construct pQEGST encoding the wild-type maize GST I was used as template DNA in all mutagenesis reactions. The Asn49 → Ala, Gln53 → Ala and Ser57 → Ala mutants were described in [25]. All mutations were verified by DNA sequencing on an Applied Biosystems Sequencer 373A with the DyeDeoxy Terminator Cycle sequencing kit.

Assay of enzyme activity and protein

Enzyme assays for the CDNB conjugation reactions were performed at 30 °C according to the published methods [25,29,32]. Observed reaction velocities were corrected for spontaneous reaction rates when necessary. All initial velocities were determined in triplicate in buffers equilibrated at 30 °C. Catalytic-centre activities (turnover numbers) were calculated on the basis of one active site per subunit. Protein concentration was determined by the method of Bradford [31] using BSA (fraction V) as standard.

Enzyme-inactivation studies

Inactivation of GST I was performed at 25 °C in an incubation mixture containing, in a total volume of 1 ml, 50 μmol of potassium phosphate buffer, pH 7.0, 0–20 nmol of VBAR and 0.1 unit of enzyme (GST assay at 30 °C). The rate of inactivation was monitored by periodically removing samples for assay of enzymic activity. Initial rates of inactivation (kobs, min⁻¹) were deduced from plots of log (percentage of activity remaining) against time (min) for several dye concentrations, and the slopes were calculated by unweighted linear-regression analysis. Kd determinations were performed by non-linear-regression analysis from the plot depicting the apparent rate constants of GST I inactivation against VBAR concentration as described in [18,19]. Inactivation studies of GST I by VBAR in the presence of S-nitrobenzyglutathione were performed in a total volume of 1 ml (25 °C) and the reaction mixture contained 50 μmol of potassium phosphate buffer, pH 7.0, 4.1 μM VBAR, 1 μmol of S-nitrobenzyglutathione and 0.2 unit of GST.

In order to calculate the pKd of the reactive side chain of the amino acid residue involved in the nucleophilic modification of GST I by VBAR, enzyme-inactivation experiments were performed at various pH values (6.5–8.5) in 50 mM potassium phosphate buffer, containing 20 μmol of VBAR. The pKd value was obtained from the plot of the apparent rate constant of inactivation (kobs, min⁻¹) against pH [18] and fitting the kobs to the equation: kobs = kint/(1 + 10pKn-pH), where kint is the intrinsic pH-independent rate constant. Analysis was performed with the Graft program (Erithacus Software Ltd.).

For the studies of inactivation of GST I by VBAR in the presence of competitive compounds (CB3GA, 2,4-dichlorophenoxyacetic acid, kinetin, gibberellic acid, indole-3-butryic acid, indole-3-acetic acid, 2-naphthoxyacetic acid, quercetin, cyanidin, luteolin and naringenin), the reaction mixture contained in a total volume of 1 ml at 25 °C: 50 μmol of potassium phosphate buffer, pH 7.0, 0–50.1 μM VBAR, 2 units of GST I and the competitively binding compound, typically 0.01–0.5 μmol. Initial rates of inactivation were deduced from plots of log (% of activity remaining) against time (min) for several VBAR concentrations, and the slopes were calculated by unweighted linear-regression analysis. For the determination of dissociation constants of competitive compounds, the initial rates of inactivation were analysed by the equation:

\[ \frac{1}{k_{obs}} = 1/k_1 + K_d/(k_3[\text{VBAR}]/(1 + ([I]/K_i)) \]

where kobs is the observed rate of enzyme inactivation for a given VBAR concentration, k1 is the maximal rate of inactivation (min⁻¹), I is the competitive inhibitor, and Ki is its apparent dissociation constant [19,39]. The apparent dissociation constants of competitive compounds were calculated from double-reciprocal plots depicting the apparent rate constants of GST I inactivation against VBAR concentration in the presence of a constant concentration of the competitive compound, as described in [19].

Inactivation of other GST isoenzymes (human GST A1-1, soybean GST4 and human haematoipoietic prostatic gland D synthase) was performed at 25 °C in 1 ml of incubation mixture containing 50 μmol of potassium phosphate buffer, pH 7.0, 2 μM VBAR and 2 units of enzyme.

Stoichiometry of VBAR binding to GST I

GST (115 μg) in 50 mM potassium phosphate buffer, pH 7.0, was inactivated with 10 nmol of VBAR at 25 °C until the remaining activity was less than 5 %. The dye-inactivated enzyme was separated from the free dye by ultrafiltration (in an Amicon stirred cell 8050 carrying a Diaflo YM10 ultrafiltration membrane; cut-off, 10 kDa) after extensive washing with distilled water. Further separation was achieved by gel-filtration chromatography by applying the inactivating dye–enzyme complex to a Sephadex G-25 column (9 cm × 1.6 cm) equilibrated with water, and collecting fractions (0.5 ml) at a flow rate of 10 ml/h. The solution with dye-inactivated GST was then freeze-dried and stored at −20 °C.
The freeze-dried VBAR–GST I covalent complex was diluted 5-fold with 8 M urea, and the absorbance was determined spectrophotometrically at 620 nm using a molar absorption coefficient of 11.6 litre · mmol⁻¹ · cm⁻¹ (8 M urea) [18]. The protein concentration was determined by the method of Lowry [33]; no dye interference was observed in protein determinations [18].

Chymotryptic digestion of VBAR–GST I and peptide purification using HPLC

In order to covalently block the free thiol groups before peptide purification, freeze-dried VBAR–GST I covalent complex (200 µg) was dissolved in 0.1 M Hepes/NaOH buffer, pH 7.0, and was denatured by the addition of solid urea to a final concentration of 6 M. N-Ethylmaleimide was added to the denatured enzyme to a final concentration of 10 mM, and modification was effected during 30 min incubation at 25 °C. The enzyme was then dialyzed against 0.1 M ammonium bicarbonate buffer, pH 7.8, and digested by the addition of 15 µg of chymotrypsin. The digestion was allowed to continue for 10 h at 25 °C before the mixture was freeze-dried and stored dry at −20 °C. Separation of the resulting peptides was achieved on a C₄ reverse-phase 55 ODS2 Spherisorb silica column (250 mm × 4.6 mm internal diameter). Analysis was effected with a water/acetonitrile linear gradient containing 0.1% trifluoracetic acid (0–80% acetonitrile in 80 min) at a flow rate of 0.5 ml/min. Fractions of 0.5 ml were collected. The effluents were monitored at both 220 nm and 620 nm.

N-terminal amino acid sequence analysis

N-terminal amino acid sequence of VBAR-labelled peptide was carried out on an Applied 542 Biosystems Protein sequencer, model 470A, equipped with an on-line phenylthiohydantoin analyser, model 120A.

Difference spectroscopy

Difference spectral titrations were performed in a PerkinElmer Lambda16 double-beam double monochromator UV/Vis spectrophotometer at 30 °C. Enzyme solution [1 ml; 0.5 mg of wild-type GST I or mutant enzyme (Trp1² → Leu, Phe⁸⁵ → Leu, Lys⁸⁶ → Ala, Asn⁹⁰ → Ala, Gin⁹¹ → Ala, Ser¹⁰⁷ → Ala or Ile¹⁰⁸ → Phe) in 20 mM potassium phosphate, pH 6.5] and enzyme solvent (1 ml; 20 mM potassium phosphate, pH 6.5) were placed in the sample and reference black-wall silica cuvettes (10 mm pathlength) respectively, and the baseline difference spectrum was recorded in the range 700–400 nm. Identical volumes (2–5 µl) of CB3GA solution (0.5 mM) were added to both cuvettes and the difference spectra were recorded after each addition. The difference absorption at 670 nm was measured relative to a zero-absorbance reference area at 750 nm. The data were analysed according to [19,34], using the equation:

\[ \Delta A = \frac{\Delta A_{\text{max}}[\text{dye}]}{K_d + [\text{dye}]} \]  

where \( \Delta A \) is the difference absorption at 670 nm after each addition of dye, and \( \Delta A_{\text{max}} \) is the maximum difference absorption at 670 nm at saturated concentration of CB3GA.

Kinetic inhibition studies with CB3GA

Initial velocities for the GST-catalysed reaction with GSH as variable substrate were measured at 30 °C in a total volume of 1 ml of total mixture containing 100 mM potassium phosphate buffer, pH 6.0, 1 mM CDNB and 0.05–1 mM GSH in the presence of 2.2, 5.5 or 10.0 µM CB3GA dye, or in its absence. With CDNB as variable substrate, the reaction cuvette contained in a total volume of 1 ml: 100 mM potassium phosphate buffer, pH 7.5, 1 mM GSH and 0.05–0.5 mM CDNB, again in the presence of 2.2, 5.5 or 10.0 µM CB3GA dye, or in its absence. The kinetic and inhibition constants were deduced from Lineweaver–Burk plots.

Docking of CB3GA and VBAR into the GST I binding site

Structural superpositions of GSTs were carried out with LSQMAN [35]. The program O [36] was used for manual manipulations, modelling of mutations and structural visualization. CNS [37] was used for energy minimization of predicted structures and PyMOL (http://www.pymol.org) for preparation of structure figures.

RESULTS AND DISCUSSION

Kinetic inactivation studies

When maize GST I was incubated with VBAR (Figure 1A) at pH 7 and 25 °C, the enzyme was progressively inactivated (Figure 1B), whereas in the absence of VBAR, virtually no change in activity was observed under identical conditions. The observed rate of inactivation was dependent upon VBAR concentration as illustrated in Figure 1(C). This indicated that the reaction obeyed pseudo-first-order saturation kinetics, and was consistent with a Kitz and Wilson model [39] for reversible binding of reagent before covalent modification according to the equation:

\[ E + \text{VBAR} \overset{k_1}{\rightarrow} E:VBAR \overset{k_2}{\rightarrow} E-\text{VBAR} \]  

where \( E \) represents the free enzyme; \( E:VBAR \) is the reversible complex and \( E-\text{VBAR} \) is the covalent product [18,40,41]. The steady-state rate equation for the interaction is:

\[ k_{\text{obs}} = k_1[VBAR]/(K_d + [VBAR]) \]  

From the data shown in Figure 1(C), a \( K_d \) value of 35.5 ± 2.2 µM and an apparent maximal rate constant \( k_1 \) of 0.47 min⁻¹ were determined. These results are in agreement with the view that VBAR forms a Michaelis-type reversible complex, GST:VBAR, and that formation of a covalent complex is rate-limiting [18,39–41]. The inactivation of GST I by VBAR is irreversible, and enzyme activity cannot be recovered by extensive dialysis nor after gel-filtration chromatography on Sephadex G-25 columns in the presence or absence of 6 M urea.

The ability of specific ligands (e.g. substrates and inhibitors) to prevent enzyme inactivation by an irreversible inhibitor is the usual criterion used in arguing for binding site-directed modification [40,41]. The inactivation of GST I was reduced significantly by the presence of its ligands. In the absence of protecting ligands, GST I retained about 13.2% activity after incubation with 4.1 µM VBAR in the first 8 min. In the presence of 1 mM \( S \)-nitrobenzylglutathione, the enzyme retained about 75.8% of its activity.

The specificity of the VBAR interaction with GST I is demonstrated further from the stoichiometry of incorporation and kinetic inhibition studies. Completely inactivated GST I binds 2.1 mol of dye/mol of dimeric enzyme. Kinetic inhibition studies (results not shown) have shown that CB3GA behaved as a competitive inhibitor towards CDNB and GSH with \( K_i \) values of 8.2 ± 0.4 µM and 11.3 ± 0.5 µM respectively. The above findings suggest that the VBAR-binding site probably overlaps both the H-site and G-site.
peptide) emerged from the column. The blue peak was subjected to N-terminal amino acid sequencing. Automated Edman sequence analysis of the labelled peptide gave the sequence ATAE-HXSPEHLVRNP, where X indicates that no phenylthiohydantoin derivative was detected in the cycle. By comparison with the amino acid sequence of GST I, the X in the peptide was identified as Lys41, indicating that the ε-amino group of Lys41 is the reactive group that is responsible for the nucleophile attack to the dichlorotriazine ring of the dye. Lys41 is located in the β-short helical segment of α-helix 2 and is involved in direct hydrogen bond interactions with the Gly- and γ-Glu-carboxylate moieties of GSH. A multifunctional role has been attributed to this residue based on steady-state kinetics, difference spectroscopy and limited proteolysis studies [25,26,32]. For example, this residue contributes directly to the electrostatic field of the active site, influences the ionization of the -SH group of bound glutathione and exhibits high enthalpy of ionization with ΔH° of 35.3 ± 3 kJ/mol [25,29]. Based on limited proteolysis experiments, it has been shown that this residue is a sole point of attack by trypsin and contributes indirectly to the dynamics of the short 3₁₀-helical segment of α-helix 2 (residues 35–46) [25,43].

The effect of pH on enzyme inactivation by VBAR was measured to obtain more information about the chemical mechanism of Lys41 labelling. The pseudo-first-order rate constant of inactivation of GST I by VBAR was increased as the pH was raised from 6.5 to 8.5 (Figure 2), suggesting that the reaction takes place with the deprotonated form of the ε-amino group of Lys41 and depends strongly on its nucleophilicity. This indicates that the reaction proceeds via a nucleophile-substitution reaction on the electrophile carbon of the dichlorotriazine group of the VBAR. The sigmoid-shaped pH profile of Lys 41 alkylation by VBAR yields an estimated pKₐ value of 7.8 ± 0.2 (Figure 2). The low pKₐ may explain the strong selectivity of Lys41 for alkylation.

The interaction of CB3GA with GST I

The monochlorotriazine dye CB3GA is markedly less reactive than the corresponding dichloro forms (e.g. VBAR) and was therefore unable to inactivate GST I, instead behaving as a competitive inhibitor against VBAR. Figure 3 shows GST I inactivation (20 μM VBAR) in the presence of CB3GA (5–20 μM).
Figure 3. Effects of competing CB3GA dye on the time course for the inactivation of GST I by VBAR at pH 7.0 and 25 °C. All incubations contained 20 µM VBAR, in the absence (*) or in the presence of CB3GA: 5.1 µM (○) or 20.1 µM (△). Incubation was also performed in the presence of 50.6 µM CB3GA (□), without VBAR.

It is evident that CB3GA prevents enzyme inactivation by VBAR. This indicates that CB3GA competes with VBAR for GST I binding, suggesting that these compounds bind GST I at the same site. The reduced reactivity of CB3GA and of other monochlorotriazine dye with target enzymes has been observed before [19,21,22].

CB3GA exhibited marked spectral changes upon interacting with the binding site of GST I. In the presence of GST I, the absorption spectrum of CB3GA undergoes a red shift, producing difference spectra consisting of a positive maximum in the 670–680 nm region. Figure 4(A) depicts original difference spectra. CB3GA displays two broad peaks (a 670 nm positive and a 555 nm negative) and an isosbestic point at 610 nm, following a shift from its original absorbance maximum (614 nm). The shape and the wavelengths that corresponded to maximum and minimum of the dye spectrum remained unchanged during titration experiments. Furthermore, no time-dependent changes of absorbance, which are indicative of the formation of one type of complex [19], were observed. The increase in the absorption at positive maximum after each addition of the CB3GA exhibits a hyperbolic dependence on the concentration of dye, indicating the formation of CB3GA–GST I complex [19,34,42]. The intercept on the abscissa corresponds to the dissociation constant of the CB3GA–GST I complex, $K_d = 12.1 \pm 0.5$ µM.

Subramanian [44] has shown that the shape of the spectrum describing the dye–enzyme complex is characteristic of the nature of interaction. The difference spectra of CB3GA, in a high-salt environment, is characterized by positive maximum (690 nm) and negative double minima (630 and 585 nm), whereas the difference spectrum of the dye in binary aqueous solvents displayed a positive peak and a shoulder at approx. 655 and 610 nm respectively, with a small negative contribution below 550 nm. From Figure 4(A), it therefore appears that the anthraquinone chromogen of CB3GA is probably located in a rather hydrophobic environment. This observation is supported further by the molecular-modelling studies (see below).

Assessment of binding characteristics of selected mutants of GST I by difference spectroscopy

The maize GST I exhibits unique characteristics with respect to H-site size, shape and flexibility [24]. The size and shape of the H-site is determined mainly by the hydrophobic residues Trp12, Phe35 and Ile118 [24]. Previous data, from this laboratory, based on dynamic simulations and limited proteolysis studies of GST I, have shown that Asn49 and Gln53 modulate indirectly the flexibility of the H-site [25,26]. Asn49 influences the mobility of the short 310-helical segment of $\alpha$-helix 2, which is related to the modulation of the affinity of H-site for the electrophile substrate. The other segment of protein with high mobility, which is modulated by Gln53, is the $\alpha$-helix 3′′ that contains conserved key residues of the H-site (e.g. Ile118).

To characterize further and locate precisely the dye-binding site of GST I, site-directed mutagenesis of selected H-site residues (Ile118, Trp12 and Phe35; Figure 5) and G-site (Asn49, Lys51, Gln53 and Ser67) was performed and the binding characteristics of the mutated enzymes for CB3GA were determined using difference spectroscopy. The results are summarized in Table 1. All but one mutant tested exhibited differences in binding affinity for CB3GA. Phe35 → Leu, Asn49 → Ala, and Gln53 → Ala mutants had reduced binding affinity for CB3GA, whereas Ile118 → Phe and
Trp → Leu mutants showed increased affinity compared with the wild-type enzyme. The mutant Ile → Phe exhibited the lowest $K_d$ (3.6 µM; Table 1). On the other hand, the mutations of Ser → Ala and Lys → Ala did not seem to affect the affinity of the enzyme for CB3GA. Each of the residues whose mutation leads to altered CB3GA binding could either interact directly with the dye molecule or influence affinity indirectly through changes in overall enzyme structure. The second possibility may be ruled out since far-UV spectra of mutant and wild-type GSTs are indistinguishable [25,26].

### Molecular modelling

Molecular-modelling studies were employed to provide a detailed picture of CB3GA interaction with GST I. The only available structures of GST I are those in complex with glutathione conjugates: the atrazine conjugate [43] and the lactoylglutathione complex [24]. Neither of these ligands bears any strong structural resemblance to CB3GA. We therefore examined whether the structure of CB3GA in complex with human GST P1-1 [17] would provide useful information to aid docking of CB3GA to GST I. Although CB3GA in its entirety was present in the crystallization, electron density was only present for the anthraquinone chromophore portion. GST I and GST P1-1 share only around 24% sequence identity. Nevertheless, examination of the superimposed structures revealed broadly similar H-sites. Although the human H-site may be described as deeper and slightly narrower, there are clear similarities in the nature of the amino acids lining the H-site in the region into which CB3GA binds in the human GST P1-1 structure. For example, Phe, a CB3GA-ligating residue in the human GST complex is replaced by an alanine residue in GST I, but in a structural superposition, GST I residue Phe occupies the same position, replacing a valine residue in the human enzyme. Similarly, on the opposite side of the CB3GA, the hydrophobic binding surface consisting of Tyr and Gly in the human enzyme is replaced by an equally hydrophobic surface formed by the side chains of Phe and Ile. Based on these considerations, we initially positioned CB3GA into the GST I–atrazine conjugate structure (after removal of the original ligand) based on the superposition of that structure and the human GST complex with CB3GA. Reassuringly, this produced no serious steric clashes between CB3GA and GST I. Minor steric clashes with Met and Phe could be manually removed through a tilt of around 30° of the anthraquinone group around an axis defined by the two carbonyl groups on its central ring. After this rotation, the anthraquinone ring occupied more of the same space occupied by the atrazine ring in the structure of GST I in complex with the atrazine conjugate and made stronger hydrophobic interactions with residues Phe and Ile. After computational refinement by energy minimization with the program CNS [37], the final model of dye binding to GST I was obtained. As mentioned previously, only the anthraquinone chromophore portion of CB3GA was defined by electron density in the complex with human GST and the anthraquinone sulphonate is responsible for the relatively high affinity interaction between CB3GA and GST P1-1 [17]. As shown in Figure 5, the remainder of the dye molecule attaches to the anthraquinone ring at a highly solvent exposed position. Thus the prediction for the complex with GST I is that, as for human GST, only the anthraquinone portion binds specifically to the enzyme. No modelling of the complete structures of CB3GA or VBAR was therefore attempted.

The predicted mode of interaction of the anthraquinone chromophore portion of VBAR and CB3GA with GST I is shown in Figure 5. Consistent with the shape of the spectrum describing the dye–enzyme complex (Figure 4A) [44], the bulk of the interactions with the enzyme are hydrophobic and involve residues Met, Trp, Phe, and Met (Table 1). In addition, three hydrogen bonds may be formed, two by Asn and one by Gln (Figure 5). The model explains well the data presented in Table 1. The weaker binding by the Phe → Leu mutant reflects the significant hydrophobic interactions present in the model between Phe and the ligand. Similarly, the hydrogen bond made by Gln to the anthraquinone group is likely to be responsible for the reduced affinity of the Gln → Ala mutant. The greatest loss of affinity is seen for the Asn → Ala mutant, an initially surprising result given its distance from the ligand (Figure 1). However, earlier work has shown that Asn is a key residue, its mutation leading to effects that can be explained by the perturbation of the structure of a β-turn adjacent to Gln [26]. These multiple effects, along with the unexpectedly induced cooperativity for substrate binding [26] can readily explain the loss of dye affinity by indirect mechanisms. The lack of effect on dye
binding of mutation at Ser147 is explained readily by its distance from the anthraquinone ligand (Figure 5). Two mutations enhance affinity for dye, Ile113 → Phe and, to a lesser extent, Trp12 → Leu. Figure 3 illustrates how the former mutation may improve binding affinity. A phenylalanine residue at position 118, in a favourable rotameric conformation, can lie on top of the first anthraquinone ring, leading to more extensive hydrophobic interactions. The origin of the lesser enhancement of binding affinity observed for the Trp12 → Leu mutant may be due to the reorientation of Phe114 to occupy the space released by the presence of a smaller amino acid at position 12 and thereby be able to form additional hydrophobic interactions with ligand. The lack of strong effect on dye binding on mutation of Lys12, the amino acid residue target of covalent modification, support further the idea, discussed earlier, that only the anthraquinone portion of the dye binds specifically to the enzyme. It seems that the chlorotriazine ring does not contribute to the binding affinity. Nevertheless, VBAR bound through its anthraquinone moiety to the enzyme, as in Figure 5, could simultaneously react with Lys12; the separation of the NB atom (marked with an asterisk in Figure 5) and the reactive position of the dye would be up to around 10 Å, while the NB atom and the side chain amino group of Lys12 are separated by 8.5 Å in the model. Clearly, sufficient flexibility exists between the anthraquinone ring and the chemically reactive centre of VBAR to allow the latter’s reaction with the former bound as in Figure 5.

Comparison of the CB3GA–GST I model (Figure 5) with the crystallographic resolved structure of CB3GA–GST P1-1 [17] show a similar overall pattern of hydrophobic interactions in the H-site combined with hydrogen bonds to the sulphonic acid group. In the CB3GA–GST P1-1 complex, the anthraquinone chromophore makes hydrophobic contacts with Phe8, Val10, Ile113, Tyr118 and Gly205. There are possible hydrogen-bonding interactions between the hydroxy group of Tyr7 and the anthraquinone ring carbonyl group of CB3GA, and a salt bridge between the sulphonic acid group of CB3GA and the side chain of Arg111. No basic residues are suitably positioned to make salt bridges with the ligand in the GST I structure.

Reaction of VBAR with other GST isoenzymes

To demonstrate the wider applicability of VBAR as an affinity label for other GST isoenzymes, such as human GST A1-1, soybean GST4, human prostaglandin D synthase, inactivation studies were carried out. Table 2 summarizes the observed pseudo-first-order rates of inactivation at 10 μM VBAR, in the presence or absence of 1 mM S-nitrobenzylglutathione. All enzymes were inactivated at pH 7.0 and 25 °C. The protection effect of S-nitrobenzylglutathione indicates the specificity of the reaction. The inactivation of the enzymes by VBAR was irreversible and enzyme activity could not be recovered by extensive dialysis against 1000 vol. of potassium phosphate buffer, pH 8, containing 1 M KCl, or after gel-filtration chromatography on a Sephadex G-25 column in the presence or absence of 6 M urea.

It is interesting to note that Lys41, the target of VBAR for GST I, is conserved in soybean GST4 (Lys40) and human prostaglandin D synthase (Lys43), and an equivalent lysine residue is found in GST A1-1 (Lys41). It is therefore likely that this lysine residue is also the target of VBAR in the other enzymes. However, we cannot rule out a completely different binding mode of VBAR to these enzymes.

Testing of plant hormones and flavonoids for binding to GST I

The ability of VBAR to specifically inactivate the GST I was explored to demonstrate the binding of several plant hormones and flavonoids. The ability of these ligands to alter the kobs for enzymic activity.

Table 2  Observed rates of inactivation (k obs) of GST isoenzymes by VBAR in the presence or absence of 1 mM S-nitrobenzylglutathione

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>kobs × 10⁻¹ (min⁻¹)</th>
<th>Protection from inactivation (%)</th>
</tr>
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<tbody>
<tr>
<td>Human GST A1-1</td>
<td>23.2</td>
<td>84.3</td>
</tr>
<tr>
<td>Soybean GST 4</td>
<td>18.6</td>
<td>89.8</td>
</tr>
<tr>
<td>Human haematopoietic prostaglandin</td>
<td>1.94</td>
<td>90.2</td>
</tr>
</tbody>
</table>

Figure 6  Binding of plant hormones to GST I at pH 7.0 and 25 °C

Inactivation of GST I by VBAR (10 μM) was carried out in the presence (■), or in the presence of competing ligands (0.01 μmol): gibberellic acid ([□]), 2-naphthoacetic acid ([●]), indole-3-butyric acid ([○]). At the times indicated, aliquots were withdrawn and assayed for enzymic activity.

Testing of plant hormones and flavonoids for binding to GST I

The ability of VBAR to specifically inactivate the GST I was explored to demonstrate the binding of several plant hormones and flavonoids. The ability of these ligands to alter the kobs for enzymic activity.

Figure 6 shows GST I inactivation in the presence of non-substrate ligands. Inactivation of GST I by VBAR was inhibited by luteolin and quercetin, whereas naringenin and cyanidin had no effect. Naringenin is a colourless flavonoid that has unconjugated carbon bonds in the C-ring, resulting in a non-planar structure; all other flavonoids examined are planar molecules. Indole-3-acetic acid and p-coumaric acid did not inhibit GST I inactivation by VBAR. The apparent dissociation constants of ligands (Table 3) were calculated from double-reciprocal plots of the apparent rate constants of GST I inactivation against different VBAR concentrations, in the presence of a constant concentration of non-substrate ligand. The apparent dissociation constants determined by this method are generally in good agreement with that determined by direct binding or kinetic studies [18,19,39,45]. Of all the compounds tested, gibberellic acid exhibited the highest affinity (Kd, 5.2 μM; Table 3) for GST I, indicating that the enzyme might have a novel functional role in the metabolism or transport of this compound to its target site. It seems reasonable to suppose that gibberellic acid binds in the H-site of GST I, but its generalized hydrophobic nature,
the absence of crystallographic structures for similar molecules and the magnitude of the structural differences observed between different GST I complex structures mean that modelling of its mode of interaction is not feasible.

Several GST isoenzymes are able to interact with plant hormones and flavonoids with distinct binding specificities [45–48]. For example, a monomeric GST isoenzyme from maize with molecular mass of 30 kDa was isolated and found to bind to several coumaric acid isomers and other phenylpropanoids such as trans-cinnamic acid, ferulic acid, and coniferyl alcohol [46]. The recombinant GSTF2 from Arabidopsis thaliana binds indole-3-acetic acid and the artificial auxin 1-naphthylphthalamic acid [47]. The GST isoenzymes AN9 from petunia and GST III from maize are inhibited by several flavonoids, whereas horse liver GST is not [45]. The GST isoenzymes GST1-1 and GST2-1 from Nicotiana tabacum could be inhibited by 2,4-dichlorophenoxyacetic acid, but only the GST1-1 isoenzyme is inhibited by the structurally related compound 2,4-dichlorobenzoic acid [48]. Interestingly, in the present work, indole-3-acetic acid and p-coumaric acid did not seem to bind GST I. Variation in binding specificity between different GST isoenzymes is a well-known feature of GSTs and is believed to be relevant to their specific in vivo roles in the metabolism or transport of certain plant hormones and flavonoids into the plant cell [45].

The precise functions of ligandin GST binding to non-substrate ligands remain unclear. One possibility is that binding of non-substrate ligands to GST I prevents oxidation of the molecules in vivo (e.g. flavonoids) [45]. Another possibility is that GST I prevents cellular damage from cytotoxic and genotoxic compounds that can oxidize protein and intercalate into DNA [45]. The third possibility is that GST I facilitates delivery of the ligands to specific receptors or cellular compartments. Future work that will elucidate the actual role of giberelleic acid binding is underway in this laboratory.

Conclusions

From both the biochemical studies and the molecular modelling approach described here, it is evident that the L-site of GST I is located mainly in the H-site. The results of the present study have practical significance, since they provide the basis for the rational design of new engineered GSTs with altered ligandin properties. In addition, the mutagenesis data could be used as an initial point for analysing the specificity of other plant GSTs of unknown structure. The usefulness of CB3GA and VBAR and the experimental approach described in the present study may be explored further for probing the ligandin function of other GST isoenzymes. Our data draw attention to the possibility that GST I may be involved in metabolism of the plant hormone giberelleic acid.

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