Characterization of bromosulphophthalein binding to human glutathione S-transferase A1-1: thermodynamics and inhibition kinetics

Doris KOLOBE, Yasien SAYED and Heini W. DIRR¹

Protein Structure–Function Research Programme, School of Molecular and Cell Biology, University of the Witwatersrand, Johannesburg 2050, South Africa

In addition to their catalytic functions, GSTs (glutathione S-transferases) bind a wide variety of structurally diverse non-substrate ligands. This ligandin function is known to result in the inhibition of catalytic function. The interaction between hGSTA1-1 (human class Alpha GST with two type 1 subunits) and a non-substrate anionic ligand, BSP (bromosulphophthalein), was studied by isothermal titration calorimetry and inhibition kinetics. The binding isotherm is biphasic, best described by a set of two independent sites: a high-affinity site and a low-affinity site(s). The binding stoichiometries for these sites are 1 and 3 molecules of BSP respectively. BSP binds to the high-affinity site 80 times more tightly ($K_d = 0.12 \mu\text{M}$) than it does to the low-affinity site(s) ($K_d = 9.1 \mu\text{M}$). Binding at these sites is enthalpically and entropically favourable, with no linkage to protonation events. Temperature- and salt-dependent studies indicate the significance of hydrophobic interactions in the binding of BSP, and that the low-affinity site(s) displays low specificity towards the anion. Binding of BSP results in the release of ordered water molecules at these hydro-

phobic sites, which more than offsets unfavourable entropic changes during binding. BSP inhibition studies show that the binding of BSP to its high-affinity site does not inhibit hGSTA1-1. This site, located near Trp-20, may be related to the buffer-binding site observed in GSTP1-1. The low-affinity-binding site(s) for BSP is most probably located at or near the active site of hGSTA1-1. Binding to this site(s) results in non-competitive inhibition with respect to CDNB (1-chloro-2,4-dinitrobenzene) ($K_i^{\text{BSP}} = 16.8 \pm 1.9 \mu\text{M}$). Given the properties of the H site and the relatively small size of the electrophilic substrate CDNB, it is plausible that the active site of the enzyme can simultaneously accommodate both BSP and CDNB. This would explain the non-competitive behaviour of certain inhibitors that bind the active site (e.g. BSP).

Key words: binding energetics, bromosulphophthalein, glutathione S-transferase A1-1, inhibition kinetics, isothermal titration calorimetry, ligand binding.

INTRODUCTION

Cytosolic GSTs (glutathione S-transferases), a superfamily of multifunctional, dimeric enzymes (see [1] for a review), share a common fold and function as obligate dimers. Although their catalytic mechanisms have been investigated extensively (see [2] for a review), relatively little is known about their ligandin functions. This function refers to the ability of cytosolic GSTs to bind a wide variety of structurally diverse non-substrate compounds including bilirubin, haem, steroids, bile salts, carcinogens, dyes and drugs [3]. Many of these hydrophobic–amphipathic compounds inhibit GST catalysis via complex modes of inhibition involving both competitive and non-competitive mechanisms (see [4] and references therein).

Although a large amount of data is available regarding the nature of ligands involved, their affinities for the various GSTs, and the impact that ligand binding has on catalytic function, the locations and properties of binding sites for non-substrate ligands are largely unknown. Furthermore, since indirect methods have been used in most ligand-binding studies, there is uncertainty regarding the number of sites involved for most of these ligands as well as their binding stoichiometries. When the first crystal structure was solved, it was proposed that the open, solvent-exposed cleft between the subunits could serve as a binding site for non-substrate ligands [5,6]. This region was subsequently shown to bind praziquantel and a conjugate of GSH in the crystal structures of a Schistosomal GST [7] and a class Sigma GST [8] respectively. The intersubunit region was also proposed to be an extended

ligandin-binding site in hGSTA1-1 (human class Alpha GST with two type 1 subunits) [9]. Crystallographic studies have also indicated that the H site of class Pi GST can bind various hydrophobic non-substrate ligands, including the amphipathic anion BSP (bromosulphophthalein) [10] (Figure 1A). The H site, together with the adjacent G site, forms the GST active site near the subunit interface [11]. Molecular recognition at the H site is predominantly hydrophobic in nature, consistent with the hydrophobic character of many non-substrate ligands. Another binding site, unrelated to the active site, has been identified for hydrophilic anionic ligands (e.g. sulphonate buffers) in crystal structures of class Pi GSTP1-1 [10,12,13] (Figure 1A).

In spite of the fact that GSTA1-1 is a major ligand-binding protein in the liver, known historically as ligandin [3], very little is known about the location and properties of its non-substrate-binding sites. The structure of hGSTA1-1 with *S*-benzylglutathione bound at its active sites is shown in Figure 1(B). Fluorescence resonance energy transfer studies have suggested a region at or near the subunit interface of hGSTA1-1 in the binding of the non-substrate ligands ANS (8-anilino-1-naphthalene-1-sulphonic acid) [14] and aflatoxin B1 [15]. Affinity-labelling studies have also identified the subunit interface in the binding of steroid sulphates [16]. A recent kinetic inhibition study, however, indicates that lithocholate and oestradiol disulphate act as competitive rather than non-competitive inhibitors, suggesting that these non-substrate anionic ligands bind at or near the H site of hGSTA1-1 [4]. Since the binding of ANS is sensitive to conformational changes in the C-terminal region of hGSTA1-1 [17], and since this

Abbreviations used: AFB₁, aflatoxin B₁; ANS, 8-anilino-1-naphthalene-1-sulphonic acid; BSP, bromosulphophthalein; CDNB, 1-chloro-2,4-dinitrobenzene; GST, glutathione S-transferase; hGSTA1-1, human class Alpha glutathione transferase with two type 1 subunits; ITC, isothermal titration calorimetry.

¹ To whom correspondence should be addressed (email heinid@gecko.biol.wits.ac.za).

Figure 1 Structures of hGSTP1-1, hGSTA1-1 and BSP

Ribbon representations of (A) hGSTP1-1 complexed with BSP (red ball-and-stick) and Mes (yellow ball-and-stick) and (B) hGSTA1-1 complexed with *S*-benzylglutathione (red ball-and-stick). The Figure was prepared using the MolScript program [46]. (C) The chemical structure of BSP.

region forms part of the enzyme's active site [18]. ANS most probably binds at or near the active site, which explains its ability to inhibit the enzyme's activity competitively [19]. ANS binding to hGSTA1-1 is enthalpically driven and is characterized by a large negative heat capacity change, indicative of a significant hydrophobic component at the protein–ligand interface [20].

BSP, an amphipathic anion due to its negatively charged sulfonate and hydrophobic moieties (Figure 1C), has been used quite extensively as a model compound for probing the catalytic properties of GSTs [21]. Although not a substrate for hGSTA1-1, BSP binds multiple sites in GSTs [10,22–24]. This is unlike ANS, which has one site per subunit [20]. In the present study, we have used ITC (isothermal titration calorimetry) to determine the thermodynamics of BSP binding to hGSTA1-1 together with enzyme inhibition studies to identify BSP-binding sites in hGSTA1-1.

EXPERIMENTAL

Materials

hGSTA1-1 was overexpressed using the pKHA1 plasmid (a gift from Professor B. Mannervik, Uppsala University, Uppsala, Sweden [25]) transformed into *Escherichia coli* BL-21 DE3 cells. The protein was purified on a CM-Sepharose cation-exchange

column (pre-equilibrated with 20 mM sodium phosphate buffer, pH 7.5) using a 0–0.3 M NaCl gradient. The protein was buffer-exchanged into 20 mM sodium phosphate buffer (pH 6.5), containing 100 mM NaCl, 1 mM EDTA and 0.02 % sodium azide. Purity of the protein was confirmed using SDS/PAGE [26] and size-exclusion HPLC. Dimeric protein concentration was determined spectrophotometrically at 280 nm using a molar absorption coefficient of $38\,200\text{ M}^{-1}\cdot\text{cm}^{-1}$. All the other reagents were of analytical grade.

ITC

Titration experiments were performed with a VP-ITC calorimeter from Microcal (Northampton, MA, U.S.A.) by injecting $3\text{ }\mu\text{l}$ increments of a BSP stock solution (3 mM) into the sample cell containing $60\text{--}72\text{ }\mu\text{M}$ protein (monomer concentration) in 20 mM sodium phosphate, 150 mM NaCl, 1 mM EDTA and 0.02 % sodium azide (pH 6.5). The total observed heats of binding were corrected for the heats of dilution before data analysis. Raw data were integrated and analysed with the ORIGIN 5 software (Microcal). Non-linear least-squares fitting of the data generated values for the independent variables ΔH , K_a and N (stoichiometry). Values for the change in Gibbs free energy (ΔG) and the change in entropy (ΔS) were calculated using the following

equations: $\Delta G = -RT \ln K_a$ and $\Delta G = \Delta H - T\Delta S$, where T is the absolute temperature in Kelvin.

To investigate whether the binding of BSP to hGSTA1-1 is coupled with any protonation effects, ITC experiments were performed at 25 °C in different buffers with different ionization enthalpies [27,28]. The buffers used were cacodylate ($\Delta H_{\text{ion}} = -1.96$ kJ/mol), Pipes ($\Delta H_{\text{ion}} = 11.45$ kJ/mol), Mes ($\Delta H_{\text{ion}} = 15.53$ kJ/mol), Hepes ($\Delta H_{\text{ion}} = 21.01$ kJ/mol) and imidazole ($\Delta H_{\text{ion}} = 36.59$ kJ/mol).

Enzyme inhibition studies

The activity of hGSTA1-1 in 0.1 M sodium phosphate, 1 mM EDTA and 0.02 % sodium azide (pH 6.5), at 20 °C was determined spectrophotometrically at 340 nm using GSH and CDNB (1-chloro-2,4-dinitrobenzene) as substrates [29]. All reaction rates were corrected for the corresponding non-enzymic reaction rates. The IC_{50} value for BSP was determined at 1 mM GSH and 1 mM CDNB by varying the concentration of BSP (0–60 μ M) and analysing the data by non-linear regression using SigmaPlot. For inhibition studies, the concentration of glutathione was fixed at 5 mM, whereas that of CDNB was varied in the range 0–2 mM. Assays were performed in the absence and presence of BSP at 0.5, 5, 25 and 100 μ M. Results from these assays were analysed by global non-linear regression (Microcal Origin 5) using the rate equation

$$v = \frac{V_{\text{max}}[S]}{K_m \left(1 + \frac{[I]}{K_i}\right) + [S] \left(1 + \frac{[I]}{\alpha K_i}\right)} \quad (1)$$

which is a general expression not dedicated to any specific reversible inhibition mechanism (see [4]). However, the parameter α provides a quantitative measure of the inhibition mechanism ($\alpha = 1$ for pure non-competitive inhibition, $\alpha = \infty$ for pure competitive inhibition, and values of α between 0 and 1 or between 1 and ∞ indicate mixed inhibition). The regression routine was allowed to vary the parameter α to arrive at the best fit that yielded convergence values for α , K_i^{BSP} , K_m^{CDNB} and V_{max} .

RESULTS AND DISCUSSION

Binding stoichiometry and affinity

Figure 2(A) shows a representative ITC titration of hGSTA1-1 with BSP at 20 °C in sodium phosphate/150 mM NaCl (pH 6.5). The interaction between hGSTA1-1 and BSP is exothermic, and a model of two independent sets of binding sites fits best the biphasic-binding isotherm (Figure 2B). This indicates that each subunit of hGSTA1-1 possesses two types of non-co-operative binding sites for BSP: a high-affinity site and a low-affinity site. The ITC-derived stoichiometry for the two sites indicates that 1 and 3 molecules of BSP bind the high- and low-affinity sites respectively. Earlier binding studies with rat class Alpha GSTs have shown the A1 subunit to possess a high-affinity site for BSP, but that weaker sites are also present [23,24]. The A2 subunit, on the other hand, does not possess the high-affinity site for the organic anion, explaining the observed high-affinity stoichiometry of 1 BSP/dimer of GSTA1-2.

Since it was not possible to resolve the binding energetics for the individual BSP molecules bound at the low-affinity site from the calorimetric trace, the existence of three separate low-affinity sites cannot be excluded if one assumes a stoichiometry of 1 BSP molecule/site. This assumption may, however, not be

Figure 2 A representative calorimetric profile for the titration of hGSTA1-1 with BSP

The experiment was performed at 20 °C with 72 μ M protein monomer in 20 mM sodium phosphate buffer containing 150 mM NaCl, 1 mM EDTA and 0.02 % sodium azide (pH 6.5). (A) Raw exothermic heats associated with 3 μ l injections of 3 mM BSP. (B) The binding isotherm (corrected for heats of dilution) for the results in (A). The solid line through the data represents the best non-linear least-squares fit to the experimental data obtained with the MicroCal ORIGIN 5 software. 1 kcal = 4.184 kJ.

Table 1 Thermodynamic parameters for the binding of BSP to hGSTA1-1 at 20 °C

S.E.M. represents the deviation of experimental data from the fit.

	K_d (μ M)	ΔH_{obs} (kJ/mol)	$T\Delta S$ (kJ/mol)	ΔG (kJ/mol)
High-affinity site	0.12	-23.5 ± 0.2	15.5	-38.9
Low-affinity site(s)	9.1	-7.2 ± 0.1	19.7	-28.3

correct given that the active site of hGSTP1-1 (human class Pi GST with two type 1 subunits) can accommodate two molecules of BSP [10]. Unlike BSP, the organic anion ANS has been shown by ITC to bind hGSTA1-1 with a stoichiometry of 1 molecule/subunit [20].

The affinity for BSP at the high-affinity site of hGSTA1-1 is approx. 80-fold higher than that at the low-affinity site(s) (Table 1). The K_d value for the high-affinity site compares well with

Figure 3 Experimental enthalpies determined at different enthalpies of ionization

Dependence of measured enthalpies for the binding of BSP to the high-affinity site (●) and low-affinity (○) sites on ionization enthalpies of different buffers at pH 6.5, at 25 °C. Solid lines represent linear regressions. The slopes of the lines indicate the number of protons involved in binding. The Y-intercepts yield the binding enthalpy corrected for the enthalpy of ionization of buffer.

those determined for rGSTA1-1 by fluorescence methods [30,31] and for rGSTA1-2 determined by dialysis [23]. The K_d value for the low-affinity site(s) in hGSTA1-1 is similar to that obtained previously from fluorescence measurements (14 μ M; [15]). hGSTA1-1 binds BSP more tightly than it does ANS ($K_d = 65 \mu$ M; [20]).

Binding energetics

The experimentally determined enthalpy, ΔH_{obs} , consists of two parts:

$$\Delta H_{\text{obs}} = \Delta H_b + n_H \Delta H_{\text{ion}} \quad (2)$$

where ΔH_b is the enthalpy of binding, n_H is the number of protons involved in the binding process and ΔH_{ion} is the enthalpy of ionization. Complex formation between ligands and proteins can result in enthalpies of linked protonation effects (i.e. $n_H \Delta H_{\text{ion}}$) due to pK_a changes of groups at the binding interface [32]. The use of buffers with different enthalpies of ionization indicated the absence of linked protonation effects during the BSP–hGSTA1-1-binding process; n_H is -0.004 and -0.03 for the high- and low-affinity sites respectively (Figure 3). Therefore the measured enthalpies were considered to be entirely enthalpies of binding (i.e. $\Delta H_{\text{obs}} = \Delta H_b$). GSTs can bind sulphonate buffers, as shown in crystal structures of hGSTP1-1 [10,12,13]. Furthermore, the sulphonate buffer Hepes, at high concentrations, was shown to compete with BSP for binding to hGSTA1-1 [15]. The use of sulphonate buffers at low concentrations (20 mM) in the present study, however, did not significantly affect the binding parameters of BSP (results not shown).

The more favourable binding enthalpy for BSP at the high-affinity site (Table 1) suggests that more interactions are formed between the ligand and protein when compared with that at the low-affinity site. The changes in entropy are also favourable at both sites (Table 1). Although there are no structural details about the BSP-binding sites in hGSTA1-1, the crystal structure of hGSTP1-1 complexed with BSP indicates the ligand to bind in two different positions at the solvent-exposed active site [10]. The temperature factors for BSP are high, and no electron density was visible for the hydroxybenzylsulphonate moieties indicating high

Figure 4 Thermodynamic parameters for BSP binding

Temperature dependence of the thermodynamic parameters for BSP binding to the high-affinity site (A) and the low-affinity site (B) of hGSTA1-1. ●, ΔH ; ○, $T\Delta S$; ▼, ΔG . The solid lines represent linear regressions.

mobility and lack of constraint at the binding site. The dependence of binding enthalpy on temperature is linear for both types of BSP-binding sites (Figure 4), the slopes of which represent the heat capacity change on complex formation (ΔC_p). For the high-affinity site, the heat capacity change is negative ($\Delta C_p = -0.34 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$), whereas that for the low-affinity site(s) is positive ($\Delta C_p = +0.45 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$). The Gibbs free energy change remains constant with temperature due to enthalpy–entropy compensations at both sites [33]. Formation of a bimolecular interface between protein and ligand results in the burial of their surfaces at the interacting interface. Changes in the heat capacity originate primarily from changes in solvation and are related to changes in solvent-accessible surface areas (see e.g. [34] and references therein). The negative ΔC_p value for BSP binding at the high-affinity site indicates the burial of solvent-exposed non-polar surfaces and, thus, a hydrophobic component in the hGSTA1-1–BSP interaction. ΔG is not affected because of the compensation by entropy. The positive ΔC_p value for BSP binding at the low-affinity site(s) suggests the burial of solvent-exposed polar surfaces. However, the positive ΔC_p value is small and does not exclude the burial of non-polar surface when BSP binds the low-affinity site(s). The small positive ΔC_p value most probably reflects low binding specificity [35]. In the presence of salt, the affinity for BSP was enhanced approx. 3–4-fold at the high-affinity site and approx. 2-fold at the low-affinity site(s), as would be expected for hydrophobic interactions. Hydrophobic interactions also play key roles in the binding of BSP to hGSTP1-1 [10] and ANS to hGSTA1-1 [20]. The two negatively charged sulphonate groups of BSP are not the major determinants for binding.

Although the entropic term $T\Delta S$ changes in opposite directions with temperature for the two BSP-binding sites in hGSTA1-1,

Figure 5 Inhibition of hGSTA1-1 by BSP

The inset shows the absence of inhibition at low concentrations of BSP. Concentrations of BSP corresponding to the K_d values for the high- and low-affinity-binding sites are indicated by arrows.

Figure 4 shows that the entropy changes are favourable at both sites. The contributors to the entropy of binding are changes in conformational degrees of freedom (ΔS_{conf}), changes in solvation (ΔS_{solv}), and changes in translational, rotational and vibrational degrees of freedom (ΔS_{mix}) [36]:

$$\Delta S = \Delta S_{\text{solv}} + \Delta S_{\text{conf}} + \Delta S_{\text{mix}} \quad (3)$$

Binding is expected to restrict the degrees of freedom of mobile and flexible groups at the interactive surfaces of BSP and hGSTA1-1, but the favourable entropic changes after BSP binding indicate that the desolvation at both sites more than offsets unfavourable changes in entropy. Since solvent contributions to entropy are primarily a function of changes in solvent-exposed non-polar surfaces [37,38], the favourable entropy most probably results from the burial of hydrophobic groups together with a release of constrained water molecules at the interface of the interacting molecules.

Binding sites/inhibition of hGSTA1-1 by BSP

In the absence of crystal structures of hGSTA1-1 complexed with non-substrate ligands, the location of binding sites for these ligands is unclear. Nevertheless, BSP inhibition kinetics, together with inhibition, binding and structural data from other studies, provide information for locating putative binding sites for BSP.

BSP is a potent inhibitor of GSTs. Figure 5 shows that the organic anion inhibits the activity of hGSTA1-1 at high BSP concentrations ($\text{IC}_{50} = 7 \mu\text{M}$), but not at low BSP concentrations (see inset to Figure 5). The IC_{50} value is in agreement with the published value of $11 \mu\text{M}$ [39]. Therefore the binding of BSP at its high-affinity site ($K_d = 0.12 \mu\text{M}$) does not affect the enzyme's activity. A previous inhibition study with hGSTA1-1 indicated that BSP is a non-competitive inhibitor with respect to the H site substrate CDNB [15]. This was confirmed in the present study. The global non-linear regression analysis of the inhibition

data obtained by varying the concentration of CDNB at different fixed concentrations of BSP resulted in convergence values of $16.8 \pm 1.9 \mu\text{M}$ and 4 for K_i^{BSP} and α respectively. The values for K_m^{CDNB} ($0.46 \pm 0.06 \text{ mM}$) and V_{max} ($0.021 \pm 0.001 \mu\text{mol/min}$) were similar to those obtained from fitting the kinetic data for the uninhibited enzyme to the Michaelis–Menten equation ($K_m^{\text{CDNB}} = 0.48 \pm 0.03 \text{ mM}$; $V_{\text{max}} = 0.025 \pm 0.001 \mu\text{mol/min}$). The value for the parameter α is indicative of an inhibitor possessing substantial non-competitive character. Fitting of the inhibition data to the equations for pure competitive ($\alpha = \infty$) and pure non-competitive ($\alpha = 1$) inhibition did not yield the best fits (results not shown). In a recent inhibition study with hGSTA1-1, the global non-linear regression analysis of the data also indicated haematin to exhibit non-competitive inhibition with respect to CDNB ($\alpha = 3.4$), whereas both lithocholic acid and oestradiol 3,17-disulphate were competitive inhibitors ($\alpha = \infty$) [4].

Each subunit of hGSTA1-1 has a single ANS-binding site to which the C-terminal region of the protein contributes structurally [17,20]. Given that the C-terminal region also forms an integral part of the H site [18] and that ANS competitively inhibits class Alpha GSTA1-2 with respect to CDNB [19], ANS most probably binds at or near the H site of hGSTA1-1. Furthermore, AFB₁ (aflatoxin B₁), a potent hepatocarcinogen, competes with ANS for the same binding site in hGSTA1-1 [15]. The exo-8,9-epoxide of AFB₁ binds the H site of class Alpha GSTs, where it is conjugated with GSH [40–42]. The binding of BSP to hGSTA1-1 is, however, non-competitive with respect to ANS [15]. Although not a substrate for hGSTA1-1, BSP binds the H site of GSTs [10,22]. The crystal structure of hGSTP1-1 complexed with BSP indicates that each active site of the enzyme can accommodate two molecules of the anionic ligand [10], consistent with a binding stoichiometry of three BSP molecules per site for hGSTA1-1 (the present study). However, BSP inhibits GSTP1-1 non-competitively with respect to CDNB [43].

The active site of GSTs consists of two adjacent regions: a highly specific and predominantly polar G site for the binding of GSH, and a large non-specific H site that is capable of binding a variety of small and large hydrophobic substrates (see [11]). Therefore in view of the properties of the H site and the relatively small size of the electrophilic substrate CDNB, it is plausible that the active site of GSTs can simultaneously accommodate both inhibitor and CDNB. This would explain the non-competitive behaviour of certain inhibitors that bind the active site (e.g. BSP). Further studies will, however, be required to establish the structural basis by which these non-competitive inhibitors function. It should also be noted that each active site of a GST from *Arabidopsis thaliana* has been shown to accommodate two molecules of a GSH conjugate [44].

Since the binding of BSP to its high-affinity site does not inhibit hGSTA1-1, this site is not related to the active site (see above). The high-affinity binding site for BSP appears to be situated near the lone Trp-20 in helix 1 of hGSTA1-1. BSP is highly efficient in quenching the fluorescence of Trp-20. BSP binding diminishes the accessibility of Trp-20 to the solvent, and replacing Trp-20 with phenylalanine reduces the affinity for BSP [15]. 2-Hydroxy-5-nitrobenzyl alcohol has also been shown to bind at or near Trp-20 in rat GSTA1-1 and A2-2, and this site is separate from the ANS-binding site [47]. However, unlike BSP, 2-hydroxy-5-nitrobenzyl alcohol did not inhibit their enzymic activity. Furthermore, affinity-labelling studies also indicated the presence of Trp-20 in the BSP-binding-site peptide of the A1 subunit [45]. The sulphonate buffer Hepes, at high concentrations, has been found to compete with BSP for binding to hGSTA1-1 and does not inhibit enzyme activity [15]. In this regard, crystal structures of hGSTP1-1 indicate the presence of a potential

non-substrate binding site between helix 1, strand 2 and helix 8 in each subunit, to which sulphonate buffer anions bind (see Figure 1) [10,12,13]. The sequence of the Trp-20-containing BSP-binding-site peptide of the A1 subunit corresponds to this region [45]. Mes buffer has an impact on the binding of anionic ligands to hGSTP1-1, but does not inhibit the enzyme's activity [13]. It is noteworthy that the buffer-binding site in hGSTP1-1 is involved in crystal contacts [10,12,13], and, therefore, this might explain why the larger BSP molecule does not bind there in the crystallized protein. In view of the high mobility of BSP bound at the active site of hGSTP1-1 [10], it is possible that the active site represents the low-affinity rather than the high-affinity site for BSP. Inhibition studies with GSTP1-1 indicate that the H site is not the primary site for BSP, but that inhibition occurs only when the anion binds its secondary site located at or near the active site [31].

This work was supported by the University of Witwatersrand, the Andrew Mellon Foundation, the South African National Research Foundation (grant no. 205359) and the Wellcome Trust (grant no. 060799).

REFERENCES

- Sheehan, D., Meade, G., Foley, V. M. and Dowd, C. A. (2001) Structure, function and evolution of glutathione transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily. *Biochem. J.* **360**, 1–16
- Armstrong, R. N. (1998) Mechanistic imperatives for the evolution of glutathione transferases. *Curr. Opin. Chem. Biol.* **2**, 618–623
- Litwack, G., Ketterer, B. and Arias, I. M. (1971) Ligandin: a hepatic protein which binds steroids, bilirubin, carcinogens and a number of exogenous organic anions. *Nature (London)* **234**, 466–467
- Lyon, R. P. and Atkins, W. M. (2002) Kinetic characterisation of native and cysteine 112-modified glutathione S-transferase A1-1: reassessment of nonsubstrate ligand binding. *Biochemistry* **41**, 10920–10927
- Reinemer, P., Dirr, H. W., Ladenstein, R., Schaffer, J., Gallay, O. and Huber, R. (1991) The three-dimensional structure of class Pi glutathione S-transferase in complex with glutathione sulfonate at 2.3 Å resolution. *EMBO J.* **10**, 1997–2005
- Dirr, H., Reinemer, P. and Huber, R. (1994) Refined crystal structure of porcine class Pi glutathione S-transferase (pGST P1-1) at 2.1 Å resolution. *J. Mol. Biol.* **243**, 72–92
- McTigue, M. A., Williams, D. R. and Tainer, J. A. (1995) Crystal structures of a schistosomal drug and vaccine target: glutathione S-transferase from *Schistosoma japonica* and its complex with the leading antischistosomal drug praziquantel. *J. Mol. Biol.* **246**, 21–27
- Ji, X., von Rosenvinge, E. C., Johnson, W. W., Armstrong, R. N. and Gilliland, G. L. (1996) Location of a potential transport binding site in a sigma class glutathione transferase by X-ray crystallography. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 8208–8213
- Le Trong, I., Stenkamp, R. E., Ibarra, C., Atkins, W. M. and Adman, E. T. (2002) 1.3-Å resolution structure of human glutathione S-transferase with S-hexyl glutathione bound reveals possible extended ligandin binding site. *Proteins* **48**, 618–627
- Oakley, A. J., Lo, B. M., Nuccetelli, M., Mazzetti, A. P. and Parker, M. W. (1999) The ligandin (non-substrate) binding site of human Pi class glutathione transferase is located in the electrophile binding site (H site). *J. Mol. Biol.* **291**, 913–926
- Dirr, H., Reinemer, P. and Huber, R. (1994) X-ray crystal structures of cytosolic glutathione S-transferases. Implications for protein architecture, substrate recognition and catalytic function. *Eur. J. Biochem.* **220**, 645–661
- Ji, X., Tordova, M., O'Donnell, R., Parsons, J. F., Hayden, J. B., Gilliland, G. L. and Zimniak, P. (1997) Structure and function of the xenobiotic substrate-binding site and location of a potential non-substrate-binding site in a class Pi glutathione S-transferase. *Biochemistry* **36**, 9690–9702
- Prade, L., Huber, R., Manoharan, T. H., Fahl, W. E. and Reuter, W. (1997) Structures of class Pi glutathione S-transferase from human placenta in complex with substrate, transition-state analogue and inhibitor. *Structure* **5**, 1287–1295
- Sluis-Cremer, N., Naidoo, N. N., Kaplan, W. H., Manoharan, T. H., Fahl, W. E. and Dirr, H. W. (1996) Determination of a binding site for a non-substrate ligand in mammalian cytosolic glutathione S-transferases by means of fluorescence-resonance energy transfer. *Eur. J. Biochem.* **241**, 484–488
- Sluis-Cremer, N., Wallace, L., Burke, J., Stevens, J. and Dirr, H. (1998) Aflatoxin B1 and sulphobromophthalein binding to the dimeric human glutathione S-transferase A1-1: a fluorescence spectroscopic analysis. *Eur. J. Biochem.* **257**, 434–442
- Vargo, M. A. and Colman, R. F. (2001) Affinity labeling of rat glutathione S-transferase isozyme 1-1 by 17β-iodoacetoxy-estradiol-3-sulfate. *J. Biol. Chem.* **276**, 2031–2036
- Dirr, H. W. and Wallace, L. A. (1999) Role of the C-terminal helix 9 in the stability and ligandin function of class alpha glutathione transferase A1-1. *Biochemistry* **38**, 15631–15640
- Sinning, I., Kleywegt, G. J., Cowan, S. W., Reinemer, P., Dirr, H. W., Huber, R., Gilliland, G. L., Armstrong, R. N., Ji, X., Board, P. G. et al. (1993) Structure determination and refinement of human α class glutathione transferase A1-1, and a comparison with the Mu and Pi class enzymes. *J. Mol. Biol.* **232**, 192–212
- Ketley, J. N., Habig, W. H. and Jakoby, W. B. (1975) Binding of nonsubstrate ligands to the glutathione S-transferases. *J. Biol. Chem.* **250**, 8670–8673
- Sayed, Y., Hornby, J. A., Lopez, M. and Dirr, H. (2002) Thermodynamics of the ligandin function of human class Alpha glutathione transferase A1-1: energetics of organic anion ligand binding. *Biochem. J.* **363**, 341–346
- Mannervik, B. and Danielson, U. H. (1988) Glutathione transferases: structure and catalytic activity. *CRC Crit. Rev. Biochem.* **23**, 283–337
- Jakobson, I., Warholm, M. and Mannervik, B. (1979) The binding of substrates and a product of the enzymatic reaction to glutathione S-transferase A. *J. Biol. Chem.* **254**, 7085–7089
- Tipping, E., Ketterer, B., Christodoulides, L. and Enderby, G. (1976) The non-covalent binding of small molecules by ligandin. *Eur. J. Biochem.* **67**, 583–590
- Bhargava, M. M., Ohmi, N., Listowsky, I. and Arias, I. M. (1980) Structural, catalytic, binding, and immunological properties associated with each of the two subunits of rat liver ligandin. *J. Biol. Chem.* **255**, 718–723
- Stenberg, G., Bjørnstedt, R. and Mannervik, B. (1992) Heterologous expression of recombinant human glutathione transferase A1-1 from a hepatoma cell line. *Protein Expr. Purif.* **3**, 80–84
- Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680–685
- Baker, B. M. and Murphy, K. P. (1996) Evaluation of linked protonation effects in protein binding reactions using isothermal titration calorimetry. *Biophys. J.* **71**, 2049–2055
- Fukada, H. and Takahashi, K. (1998) Enthalpy and heat capacity changes for the proton dissociation of various buffer components in 0.1 M potassium chloride. *Proteins* **33**, 159–166
- Habig, W. H. and Jakoby, W. B. (1981) Assays for differentiation of glutathione S-transferases. *Methods Enzymol.* **77**, 398–405
- Sugiyama, Y., Sugimoto, M., Stolz, A. and Kaplowitz, N. (1984) Comparison of the binding affinities of five forms of rat glutathione S-transferases for bilirubin, sulfbromophthalein and hematin. *Biochem. Pharmacol.* **33**, 3511–3513
- Sato, K., Hatayama, I., Tsuchida, S. and Sato, K. (1991) Biochemical characteristics of a preneoplastic marker enzyme glutathione S-transferase P-form (7-7). *Arch. Biochem. Biophys.* **285**, 312–316
- Baker, B. M. and Murphy, K. P. (1996) Evaluation of linked protonation effects in protein binding reactions using isothermal titration calorimetry. *Biophys. J.* **71**, 2049–2055
- Lumry, R. and Rajender, S. (1970) Enthalpy–entropy compensation phenomena in water solutions of proteins and small molecules: a ubiquitous property of water. *Biopolymers* **9**, 1125–1227
- Brokx, R. D., Lopez, M. M., Vogel, H. J. and Makhatazde, G. I. (2001) Energetics of target peptide binding by calmodulin reveals different modes of binding. *J. Biol. Chem.* **276**, 14083–14091
- Ladbury, J. E., Wright, J. G., Sturtevant, J. M. and Sigler, P. B. (1994) A thermodynamic study of the *trp* repressor–operator interaction. *J. Mol. Biol.* **238**, 669–681
- Murphy, K. P. (1999) Predicting binding energetics from structure: looking beyond ΔG° . *Med. Res. Rev.* **19**, 333–339
- Ross, P. D. and Subramanian, S. (1981) Thermodynamics of protein association reactions: forces contributing to stability. *Biochemistry* **20**, 3096–3102
- Sturtevant, J. M. (1977) Heat capacity and entropy changes in processes involving proteins. *Proc. Natl. Acad. Sci. U.S.A.* **74**, 2236–2240
- Björnstedt, R., Stenberg, G., Widersten, M., Board, P. G., Sinning, I., Jones, T. A. and Mannervik, B. (1995) Functional significance of arginine 15 in the active site of human class α glutathione transferase A1-1. *J. Mol. Biol.* **247**, 765–773
- Buetler, T. M., Slone, D. and Eaton, D. L. (1992) Comparison of the aflatoxin B₁-8,9-epoxide conjugating activities of two bacterially expressed α class glutathione S-transferase isozymes from mouse and rat. *Biochem. Biophys. Res. Commun.* **188**, 597–603
- Hayes, J. D., Judah, D. J., Neal, G. E. and Nguyen, T. (1992) Molecular cloning and heterologous expression of a cDNA encoding a mouse glutathione S-transferase Yc subunit possessing high catalytic activity for aflatoxin B₁-8,9-epoxide. *Biochem. J.* **285**, 173–180
- Johnson, W. W., Ueng, Y. F., Widersten, M., Mannervik, B., Hayes, J. D., Sherratt, P. J., Ketterer, B. and Guengerich, F. P. (1997) Conjugation of highly reactive aflatoxin B₁ exo-8,9-epoxide catalyzed by rat and human glutathione transferases: estimation of kinetic parameters. *Biochemistry* **36**, 3056–3060

-
- 43 Bico, P., Erhardt, J., Kaplan, W. and Dirr, H. (1995) Porcine class Pi glutathione S-transferase: anionic ligand binding and conformational analysis. *Biochim. Biophys. Acta* **1247**, 225–230
- 44 Reinemer, P., Prade, L., Hof, P., Neufeld, T., Huber, R., Zettl, R., Palme, K., Schell, J., Koelln, I., Bartunik, H. D. et al. (1996) Three-dimensional structure of glutathione S-transferase from *Arabidopsis thaliana* at 2.2 Å resolution: structural characterization of herbicide-conjugating plant glutathione S-transferases and a novel active site architecture. *J. Mol. Biol.* **255**, 289–309
- 45 Bhargava, M. M. and Dasgupta, A. (1988) Binding of sulfobromophthalein to rat and human ligandins: characterization of a binding-site peptide. *Biochim. Biophys. Acta* **955**, 296–300
- 46 Kraulis, P. J. (1991) MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. *J. Appl. Crystallogr.* **24**, 946–950
- 47 McCarthy, R. M., Farmer, P. and Sheehan, D. (1996) Binding of 2-hydroxy-5-nitrobenzyl alcohol to rat α class glutathione S-transferases; evidence for binding at tryptophan 21. *Biochim. Biophys. Acta* **1293**, 185–190

Received 8 January 2004/12 May 2004; accepted 18 May 2004

Published as BJ Immediate Publication 18 May 2004, DOI 10.1042/BJ20040056