Implications of the ligandin binding site on the binding of non-substrate ligands to Schistosoma japonicum-glutathione transferase

Zeyad Yassin, Emilia Ortiz-Salmerón, Federico García-Maroto, Carmen Barón, Luis García-Fuentes*

Department of Physical Chemistry, Biochemistry and Inorganic Chemistry, Faculty of Experimental Sciences, University of Almería, La Cañada de San Urbano, Almería, 04120, Spain

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Abstract

The binding interactions between dimeric glutathione transferase from Schistosoma japonicum (Sj26GST) and bromosulfophthalein (BS) or 8-anilino-1-naphthalene sulfonate (ANS) were characterised by fluorescence spectroscopy and isothermal titration calorimetry (ITC). Both ligands inhibit the enzymatic activity of Sj26GST in a non-competitive form. A stoichiometry of 1 molecule of ligand per mole of dimeric enzyme was obtained for the binding of these ligands. The affinity of BS is higher ($K_d = 3.2 \mu M$) than that for ANS ($K_d = 195 \mu M$). The thermodynamic parameters obtained by calorimetric titrations are pH-independent in the range of 5.5 to 7.5. The interaction process is enthalpically driven at all the studied temperatures. This enthalpic contribution is larger for the ANS anion than for BS. The strongly favourable enthalpic contribution for the binding of ANS to Sj26GST is compensated by a negative entropy change, due to enthalpy-entropy compensation. $\Delta G^{\circ}$ remains almost invariant over the temperature range studied. The free energy change for the binding of BS to Sj26GST is also favoured by entropic contributions at temperatures below 32 °C, thus indicating a strong hydrophobic interaction. Heat capacity change obtained for BS ($\Delta C_p = (−580.3 \pm 54.2) \text{cal} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$) is twofold larger (in absolute value) than for ANS ($\Delta C_p = (−294.8 \pm 15.8) \text{cal} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$). Taking together the thermodynamic parameters obtained for these inhibitors, it can be argued that the possible hydrophobic interactions in the binding of these inhibitors to L-site must be accompanied by other interactions whose contribution is enthalpic. Therefore, the non-substrate binding site (designated as ligandin) on Sj26GST may not be fully hydrophobic.

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1. Introduction

The gene family of glutathione S-transferases (GSTs; EC 2.5.1.18) is represented by membrane-bound and soluble enzymes, which function in the detoxification of alkylating agents. The soluble cytosolic GSTs seem to be implicated in the development of tumour resistance towards various alkylating, electrophilic anti-cancer drugs [1–4]. Furthermore, due to their abundance and binding properties, they represent a large reserve of high-capacity binding proteins that can sequester a large variety of structurally diverse nonsubstrate ligands with high-to-moderate affinity [5]. This ligand-binding (ligandin) function has involved GSTs in the intracellular uptake and transport of hydrophobic non-substrate compounds. They may also serve to prevent the accumulation of hydrophobic molecules at lipophilic sites within the cell. The ligandin function has implications in the regulation of GST activity since ligand binding can inhibit catalysis.

Unlike the catalytic properties of GSTs, which have been studied extensively [6], little is known about the structural and thermodynamic basis of their ligandin function [7]. Although much data are available regarding the ligands involved, their affinities for various GSTs, and the impact of non-substrate ligand binding on catalytic function, details about the location and properties of ligand-binding sites are unclear. A dimeric quaternary structure is required for the

* Corresponding author. Tel.: +34-950-015618; fax: +34-950 015008.
E-mail address: l.garcia@ual.es (L. García-Fuentes).
formation of a fully functional active site, which is located near the subunit interface (Fig. 1C). An examination of the structural features of the subunit interfaces for the various gene classes reveals the existence of two major interface types; namely, the alpha/mu/pi/Sj26GST type and the sigma/theta type. In the first type, a hydrophobic lock-and-key intersubunit motif is the major structural feature conserved in the dimeric interface [7]. In the second type the interface is hydrophilic with an extensive network of polar interactions, therefore lacking the hydrophobic lock-and-key motif [8].

Taking into account this observation, it might be expected that hydrophobic contribution would be the major contribution in the binding of non-substrate ligands to Sj26GST. Furthermore, since indirect methods, such as fluorescence spectroscopy, have been used to determine the binding stoichiometry for many of the ligands, there is uncertainty concerning the number of sites involved. Crystallographic studies have indicated one binding site (named as ligandin or L-site) for the drug praziquantel (PZQ) in the Schistosoma GST [9], located along the dimer interface (Fig. 1C). It was also known that ligands such as 8-anilino-1-naphthalene sulfonate (ANS), aflatoxin B1 [10], and steroid sulfates [11] bind at this site. The stoichiometry of non-substrate ligands binding to dimeric GSTs appears to depend on the ligand size and on whether it binds the intersubunit cleft (1:1 or 2:1). Stoichiometry of organic anion binding has also been shown to be dependent on the subunit type; i.e. high-affinity binding occurs on the Alpha 1 but not the Alpha 2 subunit, resulting in stoichiometry ratios of 2:1 for Alpha 1-1 homodimers and 1:1 for Alpha 1-2 heterodimers [12]. It is also known that both ANS and bromosulfophthalein (BS) (Fig. 1A and B) compete for the L-site, and inhibit the catalytic activity of \( \pi \)-class GST in a non-competitive form [13,14]. Alternatively, the binding of non-substrate ligands to L-site of Schistosoma japonicum GST can inhibit or not the enzymatic activity of the protein. Thus, the binding of PZQ does not result in the enzymatic inhibition of GST [15], whereas the binding of either ANS or BS inhibits its activity. On the other hand, Oakley et al. [16] showed by crystallographic studies that the L-site is located in the electrophile binding site (H-site) in hGSTP1-1. Taking together all these findings, it can be argued that binding of non-substrate ligands to the L-site in glutathione transferases appears to depend on the class and type of transferase and non-substrate ligand.

In order to know the nature of the L-site in Sj26GST and its possible implication in the binding of ligands (analogous to GSH substrate) to the G-site of each monomer, a thermodynamic study of the binding of these non-substrate ligands. Fluorescence spectroscopy and isothermal titration calorimetry (ITC) studies have been performed under different experimental conditions (temperature and pH), to obtain the forces and contributions responsible for the binding of these non-substrate ligands.

2. Materials and methods

2.1. Reagents and buffers

All chemicals were of analytical grade and were used without further purification. GSH, S-hexylglutathione, BS and ANS were purchased from Sigma. The ANS concentration was measured spectrophotometrically at 350 nm using as extinction coefficient 5000 M\(^{-1}\)cm\(^{-1}\). DTT and EDTA were from Pharmacia-Biotech and Riedel-Hae\ss n, respectively. Buffer reagents were from Sigma. All solutions were made with distilled and deionized (Milli Q) water.

2.2. Expression and purification

The pGEX-6P-1 plasmid (Amersham Pharmacia Biotech) was used to overexpress the Sj26GST in Escherichia coli strain JM105. A single colony of E. coli cells was incubated overnight at \( 37 ^\circ C \) with 200–250 rpm shaking in Luria–Bertani (LB) culture medium containing 100 \( \mu g/ml \)
of tetracycline. This culture was diluted 10 times in fresh LB medium with 100 µg/ml of tetracycline and grown at 37 °C until the absorbance at 600 nm reached 0.6–1. At this moment, isopropyl-β-D-thiogalactopyranoside was added at a final concentration of 1.0 mM to induce overexpression of Sj26GST. Cultures were incubated for 3–5 h at 37 °C, after which the bacterial cells were harvested by centrifugation at 7000 rpm for 10 min at 4 °C. The bacterial pellet was resuspended in 1/20 volume of phosphate-buffered saline (PBS) and sonicated on ice in short burst. Triton X-100 (1% final concentration) was added and the solution was gently stirred at room temperature for 30 min to facilitate solubilization of proteins.

The supernatant was then collected by centrifugation at 7000 rpm for 10 min at 4 °C, after which it was applied directly to a glutathione affinity column. After the Sj26GST protein was bound to the matrix, it was washed with PBS buffer to remove nonspecifically bound proteins. Sj26GST was eluted with 10 mM of reduced glutathione. The enzyme showed a single-band pattern in SDS polyacrylamide gel electrophoresis. Purified protein was stored at −80 °C. Protein solutions were prepared by dialysis of the enzyme against several changes of buffer solution at 4 °C.

2.3. Enzyme assays and inhibition studies

The GSH transferase activity was measured spectrophotometrically (Beckman DU-7400 spectrophotometer) at 25 °C using 1-chloro-2,4-dinitrobenzene (DTNB) and GSH as substrates. The appearance of S-(2,4-dinitrophenyl)-glutathione was monitored at 340 nm [17]. All enzyme activities were corrected for nonenzyme rates. Protein concentrations were determined from absorbance measurements at 278 nm, using a molar extinction coefficient of 7.01 × 10^4 M⁻¹ cm⁻¹ [18].

ANS and BS solutions were prepared freshly in the same buffer solution (sodium phosphate at pH 6.5) as the protein. Inhibition assays with these ligands were carried out mixing 1.5 µg of Sj26GST and the inhibitor (either ANS or BS). This solution was incubated at 25 °C for 2 min, after which the reaction was initiated by the addition of GSH 1 mM and CDNB 1 mM. Thus, apparent inhibition constants for ANS and BS, using CDNB as co-substrate, were determined at fixed concentrations of GSH and co-substrate and at different concentrations of non-substrate ligand (either ANS or BS).

2.4. Equilibrium dialysis

The equilibrium dialysis experiments with ANS were performed at 25 °C using a Dianorm equilibrium dialysis system with Spectra Por 12–14 kDa molecular mass cut-off membranes and a Beckman DU-7400 spectrophotometer with a cuvette of 0.2-cm pathlength. The enzyme and ANS samples (500 µl each) were added to opposite sides of the dialysis membrane with a Hamilton microsyringe. The dimeric Sj26GST and ANS concentrations were 135.2 µM and 35.3 mM, respectively, both in phosphate buffer at pH 6.5. After 4-h dialysis, the ANS concentration was measured in the compartment without protein. Thus, the concentration of free ligand was calculated. The concentration of bound ANS was determined from the difference between the ANS concentration placed in a chamber (35.3 mM) and two times the ANS concentration measured in the side without protein. Controls, with ligand alone, revealed that dialysis equilibrium had been attained, and with enzyme alone, showed no loss in activity during dialysis. A triplicate of each sample was done, and the final measure is the average.

2.5. Fluorescence measurements

Spectrofluorimetric titrations and folding/unfolding studies were performed at room temperature in a Perkin Elmer LS50B spectrofluorometer interfaced to a computer for data collection and analysis. The study of the folding/unfolding under equilibrium conditions was done as previously described [19]. Equilibrium-binding with ANS or BS was measured by quenching of the intrinsic tryptophan fluorescence of Sj26GST. The excitation wavelength was 295 nm, and fluorescence was monitored at 340 nm. The excitation and emission bandpasses were 4 nm in both cases. A 2.0-ml solution of GST in a 4.0-ml quartz fluorescence cell was stirred after each addition of ligand. The buffer used for fluorescence measurements was 50 mM sodium phosphate, 5 mM NaCl, 1 mM EDTA and 1 mM of DTT at pH 6.5.

Taking into account that for a macromolecule with equal and independent n sites, which have a characteristic microscopic association constant, K, for the ligand, L, the saturation fraction, Y, can be expressed as:

\[
Y = \frac{\Delta F}{\Delta F_{\text{max}}} = \frac{K[L]}{1 + K[L]} \tag{1}
\]

where \(\Delta F\) indicates the fluorescence-quenching change observed at nonsaturating ligand concentrations (e.g. ANS or BS), and \(\Delta F_{\text{max}}\) is the maximum fluorescence-quenching variation detected at saturating ligand concentration. In addition, \([L]\) in Eq. (1) is the free concentration of the non-substrate ligand (e.g. ANS or BS). On the other hand, \([L]\) can be expressed as:

\[
[L] = [L]_0 - nY[Sj26GST], \tag{2}
\]

where \([L]\) and \([Sj26GST]\) are the total non-substrate ligand and protein concentrations, respectively. Using an iterative method, in which Eqs. (1) and (2) were used, the parameters K and n can be calculated. The software used was Micromath (Scientific Software). At this point, it is very convenient to underline that n was obtained using this procedure only for BS, whereas n is fixed at 1 for ANS (in this case n was determined by equilibrium dialysis).
2.6. Isothermal titration microcalorimetry

The isothermal calorimetric experiments were performed on an MCS isothermal titration microcalorimeter (ITC) from Microcal Inc., as described by Ortiz-Salmerón et al. [20,21]. The sample cell was filled with 1.8 ml of the buffer (control) or enzyme solution (effective volume of 1.38 ml). The injector was filled with 250 µl of the ligand. The titration was initiated by the first (preliminary) injection of 1 µl, followed by 25–30 injections (5 µl each) of the ligand. During the experiment, the enzyme solution was stirred at a constant rate of 400 rpm.

Calorimetric titrations of Sj26GST in the presence of non-substrate ligand (either ANS or BS) were carried out mixing together the protein solution with the non-substrate ligand, after which this solution was used to fill the sample cell. This solution was titrated with S-hexylglutathione following a similar procedure at that above indicated.

All the calorimetric data were presented after correction for the background. The background heat was slightly different than that obtained for the dilution of the ligand in the buffer medium, and in those cases, the heat produced at the end of the titration (where the enzyme was saturated by the ligand) was taken as the measure of the background heat.

The data analysis produced three parameters, viz. stoichiometry (n), association constant (K), and the standard enthalpy changes (ΔH°) for the binding of the ligand to Sj26GST. The standard free energy change (ΔG°) for the binding was calculated according to the relationship ΔG° = −RTlnK. Given the magnitudes of ΔG° and ΔH, the standard entropy changes (ΔS°) for the binding process were calculated according to the standard thermodynamic equation ΔG° = ΔH° − TΔS°.

The buffer solutions for pH experiences were as follows: sodium acetate 50 mM for pH 5.5–6, and sodium phosphate 50 mM at pH values from 6.5 to 7.5. All buffer solutions also contained 5 mM NaCl, 1 mM EDTA and 1 mM of DTT.

2.7. Calculation of solvent-accessible surface areas

The value of ΔC_p associated with ligand–protein interactions as well as the ΔH can be approximated from the three-dimensional structure of the protein–ligand complex, as a simple function of the change in the polar (ΔASA_{pol}) and apolar (ΔASA_{ap}) surface areas associated with the binding event [22–24]. According to Freire and coworkers, the relationships between both ΔC_p and ΔH with the solvent accessible surfaces areas are

\[ ΔC_p = 0.45ΔASA_{ap} - 0.26ΔASA_{pol} \quad (3) \]

\[ ΔH_{60} = -8.44ΔASA_{ap} + 31.4ΔASA_{pol} \quad (4) \]

ΔC_p, ΔH_{60}, and ΔASA in cal·K^{-1}·mol^{-1}, cal·mol^{-1}, and Å^2 units, respectively. ΔH_{60} is the enthalpy change at 60 °C. This temperature is taken as a reference because it is the mean value of the denaturation temperatures of the model proteins used in the analysis.

3. Results and discussion

3.1. Equilibrium unfolding studies

Unfolding/folding experiments of this enzyme in the presence of the inhibitors ANS or BS were performed to study their influence on the unfolding parameters. Fig. 2 shows the urea-unfolding curves in the presence of ANS or BS. The stability parameters [ΔG_W^U and m values] were:

- 16.4 kcal/mol; 3.21 kcal·mol^{-1}·M^{-1} and 22.3 kcal mol^{-1}; 4.6 kcal·mol^{-1}·M^{-1}, for the unfolding in the presence of ANS or BS, respectively. The differences between the values calculated for both non-substrate ligands are within the experimental error, and correlate well with those obtained for the enzyme without inhibitor [18.3 kcal/mol and 3.5 kcal·mol^{-1}·M^{-1}]. Therefore, the presence of these inhibitors and possibly any other non-substrate ligand does not alter the dimeric structure of the protein and, consequently, the unfolding process was almost insensitive to the occupancy of the L-site. On the other hand, some authors [25] also argue that ANS in the ANS–hGSTA1-1 complex does not modify the enzyme stability, suggesting that the ligand does not change the protein conformation.

3.2. Fluorescence binding experiments

The binding of ANS and BS to Sj26GST as a function of the ligand concentration was followed by intrinsic fluores-
cence at 25 °C and pH 6.5 (Fig. 3), following the procedure indicated in Materials and methods. The fluorescence emission spectra displayed in Fig. 3 correspond to ANS concentrations between 0 and 450 µM (0, 20, 48, 90, 260 and 450 µM), and BS concentrations between 0 and 18 µM (0, 1.5, 3, 6.5 and 18 µM)—see panels A and B, respectively. The fluorescence emission of ANS bound to the enzyme appears about 470 nm in Fig. 3A.

Fig. 4 depicts the titration of this enzyme with BS as a plot of the binding degree, \(m\), vs. the free inhibitor concentration. Extrapolation of the upper part of the plot leads to a value of 1 mol of inhibitor bound per mole of dimeric enzyme at saturation. Fitting of these experimental data to a non-cooperative model gives a value for the association constant, \(K\), equal to \((3.1 \pm 0.2) \times 10^3 \text{ M}^{-1}\) \((K_d = 3.2 \text{ µM})\). This value is approximately 60-fold higher than that obtained for the binding of ANS \((5.1 \pm 0.2) \times 10^3 \text{ M}^{-1}\) \((K_d = 195 \text{ µM})\).

Therefore, at pH 6.5, BS binds to Sj26GST more tightly than does ANS. Furthermore, the inhibition constants determined by kinetic assays in the presence of these inhibitors (see Materials and methods) were 139.8 ± 8.2 and 2.06 ± 0.16 µM for ANS and BS, respectively. These values are in agreement, within the experimental error, to those obtained by both fluorescence and ITC (see below).

On the other hand, Bico et al. [13] obtained \(K\) values slightly different to those above indicated for the binding of these ligands to the class \(\pi\) GST. In this case, the dissociation constant for BS is only 12-fold higher than that for ANS [13]. In addition, whereas the behaviour found for the binding of BS to Sj26GST is non-cooperative, a positive cooperativity was found in the binding with human glutathione \(S\)-transferase A1-1, hGSTA1-1 [10]. Moreover, a stoichiometry of 2 ANS molecules/dimer was determined by ITC for hGSTA1-1 [7]. All these findings prove a dependence of the binding of non-substrate ligands with the type of transferase.

3.3. Calorimetric study

In order to study the binding of either ANS or BS to S. japonicum-GST, a thermodynamic analysis using isothermal titration microcalorimetry was performed under different experimental conditions. Fig. 5 shows the isothermal microcalorimetric data for the titration of GST from S. japonicum(Sj26GST) with ANS (Fig. 5A) or BS (Fig. 5B) in phosphate buffer at pH 6.5 and 30 °C. The solid smooth line shown in Fig. 5 (bottom panels) is the best fit of the experimental data according to a non-cooperative model providing the thermodynamic parameters shown in Table 1.

As it can be observed in Table 1, at 25 °C, the \(\Delta H\) value obtained for the binding of ANS to Sj26GST was found to be 9.6 kcal/mol more favourable than that for the binding of BS. In contrast, the \(\Delta G^\circ\) value for the binding of ANS was 2.5 kcal/mol less favourable than that for BS. Consequently, the binding of BS to the enzyme was 12 kcal/mol entropically more favourable than that for ANS.

We further investigated the effects of pH on the binding of both ligands a pH values around pH 6.5 (between 5.5 and
7.5). Fig. 6 shows the thermodynamic parameters obtained within this pH range by ITC at 25 °C. As it is shown in this figure, the thermodynamic parameters remain more or less constant within the studied pH range. However, it is possible to obtain some clues about the binding process for these inhibitors in the pH range studied. As it can be observed, in the BS binding to Sj26GST, the entropy change is favourable (positive) (Fig. 6B), whereas it is unfavourable (negative) for ANS (Fig. 6A). In both cases, $D_{H}$, $D_{S}$, and $D_{G}$ remain more or less constant within the pH range. Therefore, there is no enthalpy–entropy compensation with increasing pH value.

On the other hand, we have performed several calorimetric titrations of Sj26GST, in the presence of either ANS (10 mM) or BS (0.1 mM), with S-hexylglutathione (2 mM) providing dissociation constants of 3.8 and 3.4 μM, respectively. In both cases, a stoichiometry of 2 mol of inhibitor/mol of dimeric enzyme was obtained. These dissociation constants are similar to those determined under the same conditions but in the absence of non-substrate ligand (3.6 μM). Overall, the following conclusions can be drawn: (1) the binding site (L-site) appeared to be distinct from the G-site, since both ANS or BS still bound to it in the presence of S-hexylglutathione; (2) the binding of these ligands to the L-site does not seem to produce a profound conformational change in the dimeric structure that could affect the binding of the substrate (GSH) or competitive inhibitors (such as S-hexylglutathione) to the G-sites.

Table 1

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$T$ (K)</th>
<th>Stoichiometry ($n$)</th>
<th>$-\Delta H$ (kcal-mol$^{-1}$)</th>
<th>$T\Delta S$</th>
<th>$-\Delta G^{\circ}$ (kcal-mol$^{-1}$)</th>
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<tr>
<td>ANS</td>
<td>298.4</td>
<td>1$^a$</td>
<td>12.53 ± 0.32</td>
<td>−7.74 ± 0.03</td>
<td>4.78 ± 0.03</td>
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<td>303.2</td>
<td>1</td>
<td>13.66 ± 0.28</td>
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<td>4.76 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>308.2</td>
<td>1</td>
<td>15.48 ± 0.34</td>
<td>−10.71 ± 0.02</td>
<td>4.77 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>313.3</td>
<td>1</td>
<td>16.80 ± 0.52</td>
<td>−11.97 ± 0.03</td>
<td>4.83 ± 0.04</td>
</tr>
<tr>
<td>BS</td>
<td>298.3</td>
<td>0.98 ± 0.01$^b$</td>
<td>2.86 ± 0.29</td>
<td>4.35 ± 0.06</td>
<td>7.21 ± 0.07</td>
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<tr>
<td></td>
<td>303.4</td>
<td>0.93 ± 0.04$^b$</td>
<td>6.95 ± 0.49</td>
<td>0.11 ± 0.04</td>
<td>7.05 ± 0.04</td>
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<tr>
<td></td>
<td>308.4</td>
<td>1.02 ± 0.02$^b$</td>
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<td>−1.93 ± 0.08</td>
<td>7.33 ± 0.07</td>
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<tr>
<td></td>
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<td>1.21 ± 0.03$^b$</td>
<td>11.70 ± 0.31</td>
<td>−3.72 ± 0.07</td>
<td>8.01 ± 0.06</td>
</tr>
</tbody>
</table>

Uncertainties are standard errors in the fitting of the curves.

$^a$ Determined by equilibrium dialysis. In the fitting of the calorimetric data this value was kept fixed to 1.

$^b$ Determined by the fitting of the calorimetric titration data.
3.4. Stoichiometry of the binding process to the L-site

Since the affinity of ANS to Sj26GST is low, the calorimetric titration data did not allow us to determine accurately the stoichiometry of the binding process [26]. It is clear that by using a higher protein concentration (in our case, above 2 mM), the stoichiometry could have been obtained. However, at those concentrations the protein solution aggregates during the calorimetric titration making the instrumental baseline unstable.

An accurate determination of the stoichiometry can only be obtained when the c-value, defined as the product of total macromolecule concentration in the cell and the association constant, ranges from about 1 to 1000 [26]. Sayed et al. [7] obtained a stoichiometry of 2 ANS molecules per dimer of human class alpha GSTA1-1 by ITC. In the experimental data described by Sayed et al. [7] the c-value is lower than unity, and consequently the deduced stoichiometry could not be too realistic.

By inspection of our calorimetric curves with ANS (Fig. 5A), we can estimate the ANS concentration where the enzyme is practically saturated by this inhibitor. Thus, the stoichiometry for ANS was measured by equilibrium dialysis at that total concentration of inhibitor (see Materials and methods). At the equilibrium, the free ligand concentration was 17.6 mM, and 1 mol ANS was bound per mole of dimeric enzyme.

However, for the binding of BS the affinity is high enough and the three parameters can be determined together (stoichiometry, enthalpy change and the association constant), using a lower protein concentration [26]. A stoichiometry close to unity is reached in all titrations with BS (Table 1).

3.5. Temperature dependence

Phosphate buffer was used because it is known to have only a small enthalpy of ionization ($\Delta H_{\text{ion}} \approx 1$ kcal mol$^{-1}$) in conjunction with only a slight pKa change with rising temperature ($\Delta pK_a/dT = -0.0028$ K$^{-1}$). Therefore, the observed enthalpy $\Delta H$ will be practically the binding enthalpy. To determine the heat capacity change associated with the binding processes of the ANS and BS inhibitors to Sj26GST, a series of ITC experiments was performed at different temperatures, ranging from 25 to 41 °C.

Table 1 summarizes the temperature dependence of all the thermodynamic parameters for the binding of either ANS or BS to Sj26GST. It should be pointed out that all calorimetric titration experiments reported in Table 1 have been performed (at least) in duplicate, and the values represent the average of these independent experiments.

The interaction process is enthalpically driven at all the studied temperatures, this enthalpic contribution being larger for ANS than for BS (Table 1). Similarly, the binding of ANS to several proteins has been shown to be enthalpically driven [27,28]. Figs. 7 and 8 show a marked change of $\Delta H$ and $\Delta S^0$ with temperature, whereas $\Delta G^0$ remains almost the same. In Fig. 7 a linear dependence of $\Delta H$ with the temperature was observed and a strong negative change in heat capacity was obtained from its slope. The heat capacity change for ANS ($\Delta C_p = -294.8 \pm 15.8$ cal K$^{-1}$ mol$^{-1}$) is less negative than that for BS ($\Delta C_p = -580.3 \pm 54.2$ cal K$^{-1}$ mol$^{-1}$). Such a difference is quantitatively consistent with a stronger hydrophobic interaction for the binding of BS.

A plot of $\Delta H$ vs. $\Delta S^0$ values for the binding of ANS or BS at different temperatures shows a slope near unity (Fig. 8), and has been described as enthalpy–entropy compensation. Similar behaviours have been reported in a number of previous studies using other protein–ligand complexes [20,29–31]. Therefore, $\Delta G^0$ itself is almost unaffected over the temperature range investigated, and the same is true for the affinity constant that is in a similar order of magnitude than the association constants obtained by kinetic studies and fluorescence, as it was indicated above.

Besides, as it is shown in Table 1, $\Delta S^0$ for BS is positive (favourable entropically) at 25 and 30 °C, changing to a negative value at around 33 °C. Clearly, at the intersection point, $\Delta G^0 = \Delta H$ (32 °C and $-7.35$ kcal mol$^{-1}$), implying that the entropic contributions to BS–Sj26GST binding are equal to zero. Hence, $\Delta G^0$ for BS–Sj26GST binding is exclusively contributed by $\Delta H$ at 32 °C, whereas at temperatures below 32 °C, the free energy change is mainly entropic, indicating a strong hydrophobic contribution. However, for the binding of ANS, $\Delta S^0$ is always negative (entropically unfavourable) in the studied temperature
range. From the analysis of the binding parameters for these two non-substrate ligands, a different binding mode is predicted, and consequently the contributions involved must be also different.

3.6. Molecular interpretation of the thermodynamic parameters

It has been widely recognized that $\Delta C_p^o$, derived from the temperature dependence of enthalpic changes for protein–ligand interactions, is one of the most valuable thermodynamic parameters for inferring structural changes in proteins. From the measured enthalpy change at 25 °C, the corresponding value at 60 °C (calculated using $\Delta H_{60} = \Delta H_{25} + \Delta C_p (60-25)$) would be $-22.84$ and $-23.15$ kcal-mol$^{-1}$ for ANS and BS, respectively. Moreover, if we admit the Eqs. (3) and (4) to be valid for binding processes, we would calculate the changes in accessible surface areas: $\Delta ASA_{ap} = -1280 \, \text{Å}^2$; $\Delta ASA_{pol} = -1070 \, \text{Å}^2$ and $\Delta ASA_{ap} = -2030 \, \text{Å}^2$; $\Delta ASA_{pol} = -1280 \, \text{Å}^2$, for the binding of ANS and BS, respectively. Thus, the $\Delta ASA$s are larger in the binding with BS than those for ANS binding, which could be an indication of both larger conformational change and a larger hydrophobic contribution for BS than for ANS.

On the other hand, we can use Eq. (3) to obtain an estimation of $\Delta C_p$ value by knowing the solvent accessible surfaces area (polar and apolar) upon complex formation with these ligands. This determination requires to know the three-dimensional structure of the complexes. The structures of Sj26GST with either ANS or BS have not been solved. However, the structure of the Sj26GST in a complex with PZQ is known [9]. The structure and size of PZQ are similar to those for ANS and BS. Besides, these three inhibitors have comparable ASAs values ($\sim 504$, $\sim 430$ and $\sim 660 \, \text{Å}^2$ for PZQ, ANS and BS, respectively). Thus, we could use the structural data of this analogous complex to estimate the changes in accessible surface areas for the binding of ANS and BS to Sj26GST. The surface area calculations indicate that a total of $640 \, \text{Å}^2$ of apolar surface area and $140 \, \text{Å}^2$ of polar surface area are buried upon binding ($\Delta ASA_{ap} = -640 \, \text{Å}^2$; $\Delta ASA_{p} = -140 \, \text{Å}^2$). Solvent accessible surface

Fig. 7. Temperature dependence of $\Delta H$ on the interaction of ANS (A) and BS (B) to Sj26GST. In panel A, the solid line is the linear regression analysis of the experimental data for a slope ($\Delta C_p^o$) of $-0.29 \pm 0.02$ kcal-mol$^{-1}$K$^{-1}$ and an intercept (at 0 K) of $75.52 \pm 4.84$ kcal-mol$^{-1}$. In panel B, the solid line is the linear regression analysis of the experimental data for a slope ($\Delta C_p^o$) of $-0.58 \pm 0.05$ kcal-mol$^{-1}$K$^{-1}$ and an intercept (at 0 K) of $169.60 \pm 16.57$ kcal-mol$^{-1}$.

Fig. 8. Enthalpy–entropy compensation plots for the binding of ANS (A) or BS (B) to Sj26GST. In panels A and B, the experimental data for $\Delta G^o$ and $\Delta H$ are displayed by cross lines and open squares, respectively. The linear regression analysis for the data of panel A of $\Delta H$ vs. $T \Delta S^o$ yields magnitudes for the slope and the intercept of $1.01 \pm 0.01$ and $-4.70 \pm 0.08$ kcal-mol$^{-1}$, respectively. In panel B, such an analysis yields magnitudes for the slope and intercept of $1.08 \pm 0.06$ and $-7.38 \pm 0.19$ kcal-mol$^{-1}$, respectively.
areas were calculated using the program NACCESS [32], using a probe radius of 1.4 Å and a slice width of 0.05 Å. This program allowed us to calculate the accessible surface areas according to the algorithm-defined by Lee and Richards [33]. Using the ΔASA values calculated, the ΔC_p obtained according to Eq. (3) is approximately −253 cal·K⁻¹·mol⁻¹. This theoretical value correlated well with the experimental value for binding of ANS (−295 cal·K⁻¹·mol⁻¹). However, this theoretical value is less negative than that obtained for BS. This deviation (≈50%) can be explained due to a stronger hydrophobic contribution and, in this case, the BS−Sj26GST interaction would be accompanied by conformational changes in the binding interface. The larger hydrophobic contribution in the binding of BS could be explained in terms of the different ligand size, since ASA for BS is larger than that for ANS (see above). This implies a larger dehydration of the buried water molecules in the L-site produced by the binding, thus providing a larger entropy contribution.

As it has been indicated before, the entropy change for ANS binding was negative at all temperatures studied, whereas the entropy change for the binding of BS was positive at temperatures below 30 °C. Since the ΔS° for the binding of BS is comprised of both favourable and unfavourable entropic contributions, it follows that at ~305.3 K (~32 °C) the former is balanced by the latter one. Favourable entropic changes include solvent displacement (i.e., release of “frozen” water molecules in the bulk phase, often referred to as the hydroscopic effect; ΔS_{HE}° and an increase in vibrational modes (vibrational entropy; ΔS_v°), whereas the unfavourable contributions include the loss in rotational and translational entropy (ΔS_t°) and conformational entropy (ΔS_{conf}°) (Eq. (5)).

\[
ΔS = ΔS_{HE} + ΔS_v + ΔS_t + ΔS_{conf}
\]

Several investigators have predicted and/or calculated the individual entropic changes for interactions of a variety of ligands with their cognate proteins. However, such predictions have been based on several implicit assumptions. For example, Sturtevant [34] argues that the overall entropic change during protein−ligand interactions is dominated by hydrophobic (ΔS_{HE}°) as well as vibrational (ΔS_v°) entropic contributions. According to Sturtevant [34], large temperature-dependent entropic changes during protein−ligand interactions are due to a delicate balance between ΔS_{HE}° and ΔS_v°. In making quantitative predictions, this author assumes that the rotational-translational (ΔS_t°) and conformational (ΔS_{conf}°) entropic changes are negligible. On the other hand, Spolar and Record [35] argue that the contribution of the vibrational entropic changes (ΔS_v°) is negligible during protein−ligand interactions. ΔS_{HE}° can be estimated from the equation given by Spolar and Record [35], ΔS_{HE}° = 1.35ΔC_p ln(T/386) For the BS−Sj26GST complex at 25 °C we obtain ΔS_{HE}° = 202 cal·mol⁻¹·K⁻¹. In a number of bimolecular association reactions, ΔS_{rt}° has been thought to contribute ~50 cal·mol⁻¹·K⁻¹ of rotational and translational entropy [35]. From ΔS°= 14.58 cal·mol⁻¹·K⁻¹ (Table 1), we calculated the remaining entropic loss (ΔS_{conf}°) of −137 cal·mol⁻¹·K⁻¹. This entropic loss may be due to the loss in the conformational restrictions of BS and Sj26GST molecules. Similar calculations were performed at the other temperatures for both ANS and BS (Table 2).

According to data shown in Table 2, the entropic gain due to solvent displacement, ΔS_{HE}°, as a consequence of the binding of BS is larger than that obtained for ANS. Therefore, the conformational entropic loss ΔS_{conf}° for the binding of BS is approximately twofold larger than that for ANS binding at all temperatures studied (Table 2). This result implies larger conformational changes in the vicinity of the L-site for BS than those for ANS. However, as it was mentioned before, these conformational changes do not seem to produce larger modifications in the dimeric structure and the ability to bind ligands to G-sites. Hence, unlike other cases of protein−ligand interactions, the binding of BS cannot be considered as a single “rigid-body” interaction [35].

Although the binding of these two inhibitors (ANS or BS) to Sj26GST requires the dehydration of both the enzyme and the inhibitor, there is a higher entropic gain in the binding of BS as compared to that of ANS, which increases at temperatures below 32 °C. Hence, if the binding of non-substrate ligands to L-site of Sj26GST was dominated completely by hydrophobic forces, ΔG° must be entropically favourable and enthalpically unfavourable [36,37]. In agreement to this, it has been reported by several authors that the ANS binding to some proteins, hGSTA1-1 among them, is not completely hydrophobic [7,38,39]. The thermodynamic parameters obtained for these inhibitors reveal that the possible hydrophobic interactions in the binding of these inhibitors must be accompanied by other interactions whose contribution is

<table>
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<th>Table 2</th>
<th>Entropic contributions corresponding to the binding of ANS or BS to S. japonicum-GST at different temperatures</th>
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<tr>
<td><strong>Ligand</strong></td>
<td><strong>T (K)</strong></td>
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<td></td>
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<tr>
<td>ANS</td>
<td>298.4</td>
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<td></td>
<td>303.2</td>
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<td></td>
<td>308.2</td>
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a Data from the Table 1.

b Calculated from the equation ΔS_{HE}° = 1.35ΔC_p ln(T/386).

c ΔS_{HE}° and ΔS_{conf}° are the differences in each of the values with BS from those with ANS.

d Calculations were done using the Eq. (5) with ΔS_v°= 0 and ΔS_v°= −50 cal·mol⁻¹·K⁻¹. The procedure is summarized briefly in the text.
enthalpic. Van der Waals interactions and hydrogen bonding are generally considered to be the major potential sources for negative $\Delta H$ values [34]. Binding of some non-substrate ligands (such as BS) to L-site of hGSTP1-1 involves a lot of van der Waals contact between the inhibitor and the protein [16]. Similar interactions may involve a lot of van der Waals contact between the non-substrate ligands (such as BS) to L-site of hGSTP1-1 groups (ANS has one and BS has two; Fig. 1) that will be deprotonated at pH 6.5. These groups were hydrogen bonded to water before binding. Their dehydration will be produced by the binding process, thus allowing other new interactions. It is known that the binding of ligands, such as ANS (with sulfonate groups), depends primarily on ion pair formation between the sulfonate anion and cationic groups of water-soluble proteins (histidine, arginine, etc.). Furthermore, the overall enthalpies of ANS binding to some proteins, obtained by titration calorimetry, were approximately the arithmetic sums of individual ANS–polyamino acid sidechain binding enthalpies [40]. Thus, contrary to what is sometimes assumed—that the hydrophobic contribution was the only force on the binding of ANS to proteins—it was also demonstrated that ion pairs formation (electrostatic interactions) must equally be considered [40]. According to these considerations, the thermodynamic parameters (Table 1) must include the contribution which is due to some electrostatic interactions. It is well known that the existence of electrostatic interactions provides both positive $\Delta H$ and positive $\Delta S$ values. Alternatively, it can also provide less negative $\Delta H$ and $\Delta S$ values, if other interactions with opposed signs such as H-bonds, van der Waals, etc., are also implicated in the binding reaction. Since BS has two sulfonate groups whereas ANS has only one, the electrostatic interaction contribution must be higher in the complex formation BS–SJ26GST than those in the ANS–SJ26GST complex. This explanation could partly justify that: (i) the enthalpy changes were less negative for the BS than those for the ANS (Table 1), and (ii) the entropy changes were also less negative for the BS (positive to some temperatures) that those for ANS.

Thus, the hydrophobic contribution is not the only force which participates in the binding of these ligands to proteins. Therefore, the L-site of SJ26GST is not fully hydrophobic, the binding process to the L-site being dependent on the size, structure and nature of the ligand.

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