Thermodynamic analysis of the binding of glutathione to glutathione S-transferase over a range of temperatures

Emilia Ortiz-Salmerón, Zeyad Yassin, M. Jose Clemente-Jimenez, F. Javier Las Heras-Vazquez, Felipe Rodriguez-Vico, Carmen Barón and Luis García-Fuentes

Dpto. Química Física, Bioquímica y Q. Inorgánica, Facultad de Ciencias Experimentales, Universidad de Almería, Almería, Spain

The binding properties of a glutathione S-transferase (EC 2.5.1.18) from Schistosoma japonicum to substrate glutathione (GSH) has been investigated by intrinsic fluorescence and isothermal titration calorimetry (ITC) at pH 6.5 over a temperature range of 15–30 °C. Calorimetric measurements in various buffer systems with different ionization heats suggest that protons are released during the binding of GSH at pH 6.5. We have also studied the effect of pH on the thermodynamics of GSH–GST interaction. The behaviour shown at different pHs indicates that at least three groups must participate in the exchange of protons. Fluorimetric and calorimetric measurements indicate that GSH binds to two sites in the dimer of 26-kDa glutathione S-transferase from Schistosoma japonicum (SjGST). On the other hand, noncooperativity for substrate binding to SjGST was detected over a temperature range of 15–30 °C.

Among thermodynamic parameters, whereas ΔG° remains practically invariant as a function of temperature, ΔH and ΔS° both decrease with an increase in temperature. While the binding is enthalpically favorable at all temperatures studied, at temperatures below 25 °C, ΔG° is also favoured by entropic contributions. As the temperature increases, the entropic contributions progressively decrease, attaining a value of zero at 24.3 °C, and then becoming unfavorable. During this transition, the enthalpic contributions become progressively favorable, resulting in an enthalpy–entropy compensation. The temperature dependence of the enthalpy change yields the heat capacity change (ΔCp) of −0.238 ± 0.04 kcal per K per mol of GSH bound.

Keywords: glutathione S-transferase; Schistosoma japonicum; glutathione; binding; microcalorimetry.

The glutathione transferases (EC 2.5.1.18) (GSTs) are a family of enzymes involved in the mechanism of cellular detoxification. They catalyze the nucleophilic attack of glutathione on the electrophilic centre of a number of toxic compounds and xenobiotics [1]. The cytosolic enzymes have been grouped into at least five main species-independent classes: (alpha, mu, pi [2], theta [3] and sigma [4,5]) on the basis of N-terminal sequence, substrate specificity, and immunological properties [6]. They are characterized by low sequence homology (less than 30%). Despite this heterogeneity, the overall polypeptide fold is very similar among the crystal structures so far obtained [7], and all GSTs showed high selectivity toward the reduced glutathione (GSH) molecule.

The homodimeric (26 kDa per subunit) glutathione S-transferase from Schistosoma japonicum (SjGST) is a member of one of the most important supergene families of enzymes involved in the phase II metabolism of electrophilic compounds [8]. The glutathione-conjugates have greater solubility in water, facilitating their export from the cell, where they are metabolized via the mercapturate pathway and eventually excreted. SjGST first gained attention when it was identified by Smith et al. [9] as a major antigen capable of inducing host-protective immunity in mice.

Crystal structures of SjGST have been solved [10,11] and it has been confirmed that each subunit in homodimeric SjGST contains two structural domains, an N-terminal and a C-terminal domain. The dimeric structure is required for the formation of two functional active sites (one per subunit).

This protein can be purified easily in large amounts using glutathione affinity chromatography, leading to the development of the pGEX gene fusion expression system, which expresses the fully functional and highly soluble SjGST. Today, the pGEX system is used widely for the overexpression in Escherichia coli of a wide variety of proteins fused to the C-terminus of SjGST.

It is important to point out the physiological role of the SjGST as a detoxification protein and vaccine and drug target, with a wide application in protein biotechnology. This work reports the results of studies of the binding of GSH (γ-Glu-Cys-Gly) to the parasitic helminth SjGST using fluorescence quenching and high sensitivity titration microcalorimetry. To our knowledge, this is the first time that a calorimetry study has been carried out on GST from S. japonicum. ΔH was found to depend on buffer ionization.
heats, thus implying a proton exchange upon binding. The dependence on pH shows the existence of several groups in the active site capable of exchanging protons. \( \Delta H \) on association has been measured at several temperatures to obtain the heat capacity change.

**MATERIALS AND METHODS**

**Reagents and buffers**

All chemicals were of analytical grade and were used without further purification. GSH was purchased from Sigma; dithiothreitol and EDTA were from Pharmacia Biotech and Riedel-Haen, respectively. Buffer reagents were from Sigma. All solutions were made with distilled and deionized (Milli Q) water.

**Expression and purification**

The pGEX-2T plasmid (Amersham Pharmacia Biotech) was used to overexpress the SjGST in *E. coli* strain BL21 [12]. A single colony of *E. coli* was incubated for 12–15 h at 37 °C with 200–250 r.p.m. shaking in Luria–Bertani culture medium containing 100 \( \mu \)g-mL\(^{-1}\) of ampicillin. This culture was diluted 100 times in fresh Luria–Bertani medium with 50 \( \mu \)g-mL\(^{-1}\) of ampicillin and grown at 37 °C until the absorbance at 600 nm reached 0.2–0.3. At this moment isopropyl thio-

Buffer reagents were from Sigma. All solutions were made with distilled and deionized (Milli Q) water.

**Enzyme assay**

The GSH transferase activity was measured spectrophotometrically by monitoring the increase in absorbance at 340 nm. The enzyme assay was composed of 1 mL of 1 m phosphate buffer pH 6.5 containing 1 mm GSH and 100 mm 1-chloro-2,4-dinitrobenzene [13]. The absorbance was measured during four minutes. The results revealed that the specific activity for the samples was between 8 and 12 U·mg\(^{-1}\).

Protein concentrations were determined from absorbance measurements at 278 nm, using a molar extinction coefficient of 7.01 \( \times 10^4 \) m\(^{-1}\)·cm\(^{-1}\) [14]. Absorbance measurements were carried out using a Beckman DU-7400 spectrophotometer with cells maintained at 25 °C.

**Fluorescence measurements**

Titrations monitoring the tyrosine and tryptophan fluorescence of *Schistosoma japonicum* GST protein were performed with a Perkin Elmer LS50B spectrophotometer interfaced to a computer for data collection and analysis. The excitation wavelength was 278 nm, and fluorescence was monitored at 339 nm. The temperature of the sample was controlled at 25.0 ± 0.2 °C using a thermostatted cuvette holder and a Frigiterm 6000382 Selecta refrigerated circulating water bath. A 2.0-mL solution of GST in a 4.0-mL quartz fluorescence cell was stirred after each addition of ligand.

The fluorescence measurements were corrected for dilution. They were also corrected for inner filter effects using the following relationship: \( F^* = F_{\text{obs}} \text{antilog}(A_{278}/2) \) [15], which is valid for absorbances ≤ 0.1.

It was assumed that the dimeric protein has two equal [10,11] and independent sites for the substrate GSH, with a characteristic microscopic association constant, \( K \). The saturation fraction, \( Y \), can be expressed as:

\[
Y = \frac{\text{[GSH]} \times K}{1 + \text{[GSH]} \times K}
\]

(1)

where [GSH] stands for the free concentration of glutathione. On the other hand, the free concentration of glutathione can be expressed as:

\[
\text{[GSH]} = \text{[GSH]}_{1} - 2Y \times \text{[SjGST]}
\]

(2)

where \( \text{[GSH]}_{1} \) and \( \text{[SjGST]} \) are the total glutathione and protein concentrations, respectively. Using Eqs (1) and (2) we can calculate the free concentration of glutathione after each addition according to:

\[
\text{[GSH]} = \sqrt[2]{(1 - K \times \text{[GSH]}_{1} + 2K \times \text{[SjGST]})^2 + 4K \times \text{[GSH]}_{1} - K \times \text{[GSH]}_{1} - 2K \times \text{[SjGST]}} - 1
\]

(3)

Moreover, the saturation fraction, \( Y \), can be calculated as,

\[
Y = \frac{\Delta F_{\text{corr}}}{\Delta F_{\text{max,corr}}} = \frac{F(\text{GSH}) - F(0)}{F(\infty) - F(0)}
\]

(4)

where \( F(0), F(\text{GSH}) \) and \( F(\infty) \) account for the corrected fluorescence intensity for the protein solution without ligand, at a concentration of ligand equal to GSH and at saturating ligand concentration, respectively. Using Eqs

The purification yield was ≈ 80 mg of apparently pure protein per L of culture.

Solutions of protein were prepared by dialysis of the enzyme against several changes of 1 mm of EDTA, 2 mm of dithiothreitol and 20 mm of the corresponding buffer solutions [2-glycerophosphate, phosphate, Pipes, Mops and 2-[(2-amino-2-oxoethyl)-amino]ethanesulfonic acid (Aces)] at pH 6.5 and 4 °C.
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(1),(2) and (4), both the association constant $K$ and $F(\infty)$ can be calculated from the experimental values. The data were analyzed using MICROMATH (Scientific Software).

**Isothermal titration microcalorimetry**

All calorimetric experiments were conducted on an MCS isothermal titration calorimeter (ITC) from Microcal, Inc. (Northampton, MA, USA). A complete description of its predecessor instrument, OMEGA-ITC, experimental strategies, and data analyses are given by Wiseman et al. [16]. The calorimeter was calibrated by known heat pulses as recommended by the manufacturer. During titration, the reference cell was filled with Milli Q water. Prior to the titration experiments, both enzyme and ligand were degassed for 10 min with gentle stirring under vacuum. The sample cell was filled either with 1.8 mL (effective volume: 1.38 mL) of buffer (for control experiment) or with an appropriately diluted enzyme. During the titration, the reaction mixture was continuously stirred at 400 r.p.m.

The background titration profiles, under identical experimental conditions, were obtained by injecting GSH into appropriate buffer solutions. The observed heat effects were concentration-independent and were identical to the heat signals detected after complete saturation of the protein. The raw experimental data were presented as the amount of heat produced per second following each injection of ligand into the enzyme solution (corrected for the ligand heats of dilution) as a function of time. The amount of heat produced per injection was calculated by integration of the area under individual peaks by the ORIGIN software provided with the instrument. The errors are provided by software from the best fit of the experimental data to the model of two equal and independent sites, and correspond to the standard deviation in the fitting of the curves.

ITC measurements were routinely performed in 1 mM EDTA, 2 mM dithiothreitol and 20 mM of sodium phosphate at pH 6.5. Heat contributions due to coupled protonation events upon binding were evaluated by calorimetric experiments in various buffers and their ionization enthalpies (in kcal·mol$^{-1}$ at 25°C) were as follows: 2-glycerophosphate (−0.17), phosphate (1.22), Pipes (2.74), Mops (5.22) and Aces (7.51) [17]. The pH of the buffer solution (20 mM) was adjusted at the experimental temperature.

**RESULTS AND DISCUSSION**

**Fluorescence experiments**

The binding of GSH to SjGST dimer was observed by intrinsic fluorescence as a function of the substrate concentration at 25°C and pH 6.5 in 20 mM sodium phosphate, 2 mM of dithiothreitol and 1 mM EDTA (Fig. 1). The good fit of the curve to a model with noninteracting sites (see Materials and methods) can be taken as evidence for the existence of noncooperativity in this binding. The fit of the fluorescence data to this model yielded the $K$ value of $(5.7 \pm 0.2) \times 10^3$ M$^{-1}$. Previous equilibrium dialysis studies of GSH to glutathione S-transferase A from rat liver have shown that the binding gave hyperbolic binding isotherms with a stoichiometry of 2 mol per mol of enzyme (i.e. 1 molecule per subunit) [18]. Another finding by equilibrium fluorescence data with human glutathione S-transferase P1-1 assures that this enzyme displays temperature-dependent cooperativity in the absence of cosubstrate 1-chloro-2,4-dinitrobenzene (CDNB). However, no significant cooperativity for substrate binding to GST P1-1 was detected at $\approx 25°C$ [19,20].

**Isothermal calorimetry experiments**

Figure 2 shows the titration of 46.9 μM of SjGST with 25 αlquots (5 μL each) of GSH (stock concentration of 15 mM) in 20 mM phosphate buffer at pH 6.5 and 25.1°C. The top panel of Fig. 2 shows the raw calorimetric data, denoting the amount of heat produced (negative exothermic peaks) following each injection of substrate. The area under each peak represents the amount of heat produced upon the binding of GSH to SjGST. Note that, as the titration progresses, the area under the peaks progressively becomes smaller due to an increased occupancy of the enzyme by glutathione. The bottom panel of Fig. 2 shows the plot of the amount of heat generated per injection as a function of the molar ratio of reduced GSH to enzyme. The smooth solid line represents the best fit of the experimental data to two equal and independent sites with microscopic association constant ($K$), and the standard enthalpy change ($\Delta H_{\text{obs}}$) of $(5.3 \pm 0.4) \times 10^3$ M$^{-1}$, and $-5.87 \pm 0.16$ kcal·mol$^{-1}$, respectively. The differences between the equilibrium constants measured by fluorescence and ITC are of the same order of magnitude.

**Stoichiometry of proton consumption or release upon binding**

It is known that the standard enthalpy changes ($\Delta H_{\text{obs}}$), derived from binding isotherms (see Fig. 2), are not solely...
contributed by physical forces governing the protein–ligand interactions. They often contain contributions from ionization enthalpy of the buffer species, and/or changes in the protein conformations \cite{21,22}. Although the enthalpic contributions of protein conformational changes can be taken as an integral component of the overall binding process, the enthalpy contribution due to protonation-deprotonation of the buffer species must be subtracted from the enthalpy change observed to obtain the binding enthalpy of the complex formed.

Previously, a number of investigators have determined the stoichiometry of protons consumed or released during the course of reaction or protein–ligand interaction by utilizing different buffers with known ionization enthalpies \cite{22±25}. Table 1 summarizes the titration results of SjGST by GSH in different buffers at pH 6.5 and 15.8 \degree C. Moreover, an examination of the data of Table 1 reveals that $\Delta H_{ob}$ is directly related to the ionization enthalpy ($\Delta H_{iz}$) of the buffer species. As the magnitude of $\Delta H_{iz}$ increases, $\Delta H_{ob}$ decreases, as demonstrated by the plot of Fig. 3. These results suggest that the binding of GSH to SjGST at pH 6.5 involves the release of protons to the buffer media. In order to quantify the amount of protons released at pH 6.5, the data of Fig. 3 were analyzed according to the following linear relationship

$$
\Delta H_{ob} = \Delta H_b + n \cdot \Delta H_{iz}
$$

where $n$ is the number of protons exchanged with the buffer media to stabilize the complex at a given pH and temperature. The number of protons exchanged at each temperature has been calculated in the same way. The number of protons released by the enzyme during GSH binding at pH 6.5 involves the release of protons to the buffer media. In order to quantify the amount of protons released at pH 6.5, the data of Fig. 3 were analyzed according to the following linear relationship \cite{26},

$$
\left( \frac{\partial \Delta H_b}{\partial pH} \right)_T = -2.3RT^2 \left( \frac{\partial n}{\partial T} \right)_{pH}
$$

Table 1. Effect of buffers on the binding of glutathione to SjGST at 15.8 \degree C and pH 6.5. The uncertainties are standard errors in the fit of the curves.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>$K \times 10^{-4}$ (M$^{-1}$)</th>
<th>$\Delta H_{ob}$ (kcal-mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Glycerophosphate</td>
<td>1.05 ± 0.80</td>
<td>–2.83 ± 0.10</td>
</tr>
<tr>
<td>Phosphate</td>
<td>0.86 ± 0.40</td>
<td>–3.58 ± 0.08</td>
</tr>
<tr>
<td>Mops</td>
<td>0.88 ± 0.70</td>
<td>–4.99 ± 0.20</td>
</tr>
<tr>
<td>Aces</td>
<td>0.55 ± 0.25</td>
<td>–5.27 ± 0.13</td>
</tr>
</tbody>
</table>
Table 2. GSH binding heats of SjGST in different buffers and at different conditions to yield the binding enthalpies and linked protons at 25 °C.

<table>
<thead>
<tr>
<th>pH</th>
<th>Buffer</th>
<th>(\Delta H_f) (kcal·mol(^{-1}))</th>
<th>(\Delta H_{obs}) (kcal·mol(^{-1}))</th>
<th>(\Delta H_b) (kcal·mol(^{-1}))</th>
<th>Linked protons</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Acetate</td>
<td>0.12</td>
<td>-6.02</td>
<td>-6.05</td>
<td>0.24</td>
</tr>
<tr>
<td>5</td>
<td>Mes</td>
<td>3.72</td>
<td>-5.17</td>
<td>-6.05</td>
<td>0.24</td>
</tr>
<tr>
<td>5.5</td>
<td>Acetate</td>
<td>0.12</td>
<td>-6.54</td>
<td>-6.55</td>
<td>0.07</td>
</tr>
<tr>
<td>5.5</td>
<td>Mes</td>
<td>3.72</td>
<td>-6.28</td>
<td>-6.55</td>
<td>0.07</td>
</tr>
<tr>
<td>6</td>
<td>Mes</td>
<td>3.72</td>
<td>-6.72</td>
<td>-6.05</td>
<td>-0.18</td>
</tr>
<tr>
<td>6</td>
<td>Mops</td>
<td>5.22</td>
<td>-7.01</td>
<td>-6.05</td>
<td>-0.18</td>
</tr>
<tr>
<td>6.5</td>
<td>Phosphate</td>
<td>1.22</td>
<td>-5.39</td>
<td>-5.08</td>
<td>-0.26</td>
</tr>
<tr>
<td>6.5</td>
<td>Mops</td>
<td>5.22</td>
<td>-6.40</td>
<td>-5.08</td>
<td>-0.26</td>
</tr>
<tr>
<td>7</td>
<td>Phosphate</td>
<td>1.22</td>
<td>-6.22</td>
<td>-5.90</td>
<td>-0.27</td>
</tr>
<tr>
<td>7</td>
<td>Mops</td>
<td>5.22</td>
<td>-7.29</td>
<td>-5.90</td>
<td>-0.27</td>
</tr>
<tr>
<td>7.5</td>
<td>Phosphate</td>
<td>1.22</td>
<td>-6.56</td>
<td>-6.39</td>
<td>-0.14</td>
</tr>
<tr>
<td>7.5</td>
<td>Mops</td>
<td>5.22</td>
<td>-7.14</td>
<td>-6.39</td>
<td>-0.14</td>
</tr>
<tr>
<td>8</td>
<td>Heps</td>
<td>5.02</td>
<td>-5.42</td>
<td>-5.50</td>
<td>0.02</td>
</tr>
<tr>
<td>8</td>
<td>Tris</td>
<td>11.35</td>
<td>-5.30</td>
<td>-5.50</td>
<td>0.02</td>
</tr>
<tr>
<td>8.5</td>
<td>Heps</td>
<td>5.02</td>
<td>-5.84</td>
<td>-7.13</td>
<td>0.26</td>
</tr>
<tr>
<td>8.5</td>
<td>Tris</td>
<td>11.35</td>
<td>-4.22</td>
<td>-7.13</td>
<td>0.26</td>
</tr>
</tbody>
</table>

at pH 6.5 and within the temperature range studied, the slope of a plot of \(\Delta H_b\) vs. pH must be practically zero. This result is corroborated by the data obtained for the enthalpy change in function of pH at 25 °C (Table 2). As can be observed, the enthalpy change seems to reach a maximal value at pH 6.5. Therefore, the slope of a plot of enthalpy change vs. pH will be practically zero at this particular pH.

The protonation changes for GSH-SjGST binding can be viewed as arising from a shift in the p\(K_a\) of one or more groups during complex formation. Alterations in the protonation state of certain residues in the vicinity of the substrate binding site may explain the variation in the number of protons at the four temperatures examined. This means that one or several p\(K_a\) values, corresponding to some donor proton groups of the ligand and/or enzyme, decrease (i.e. become more acidic). As approximately 0.3 H\(^+\) per monomer are released, few groups will be able to change their p\(K_a\) value. In fact, a common feature of all GSTs, which also have very similar GSH binding sites (G-sites), is that they lower the apparent p\(K_a\) of the bound GSH from 9.1 to about 6.0–6.6. The hydroxyl group of a conserved tyrosine (for instance, Tyr6 in this transferase, Tyr7 in the Pi class GST P1-1 and Tyr9 in the Alpha class GST A1-1), which forms H-bonds to the sulfur atom of GSH, probably stabilizes and orients the thiolate in a productive fashion [27]. Recently, it has been suggested that the Glu-carboxylate of GSH itself could act as proton acceptor by raising its normal p\(K_a\) upon binding to the enzyme; in that case the proton may leave the active site when the product is released [28]. On the other hand, Caccuri et al. [20,29] performed a kinetic analysis with GST P1-1 to show the role of the thiol proton of GSH, and they demonstrated that this proton is released during the binding of substrate. At pH 6.5, approximately 0.27 proton equivalents per active site were released for *Lucina cuprin*a GST [20].

**Effect of pH on SjGST-glutathione**

We have investigated the effect of pH on the binding isotherms for the titration of SjGST by glutathione. A series of titration calorimetry binding measurements was made for glutathione binding to SjGST as a function of pH. The data are presented in Table 2. The binding isotherms were made at each pH in the presence of two different buffers and the number of protons at each pH was calculated. Figure 4 shows a bell-shaped curve of protons exchanged as a function of pH. As can be observed in Table 2, the protons are taken up at = pH 5 and 8.5, and are practically zero at pH 8, whereas they are released between pH 5.5 and 8. This trend can only be explained if at least three groups participate in the exchange of protons. It follows that, if the p\(K_a\) values of two groups, one with a low p\(K_a\) and another with a high p\(K_a\), increase as a consequence of binding, then those groups will take up protons from the buffer media. On the other hand, a third group is also necessary with an intermediate p\(K_a\) value compared to the two previous groups. This third group should decrease its p\(K_a\) as a consequence of binding, resulting in a release of protons to the buffer media. Notice in Eqn (5) that while the binding enthalpy was corrected for the enthalpy of buffer ionization, \(\Delta H_b\) retains contributions from the ionization enthalpies of groups on the protein, the ligand or both. Thus, \(\Delta H_b\) can be the sum of one term that does not depend on the ionization of groups, and that corresponds to the intrinsic enthalpy, and another term proportional to the change of ionization of SjGST and/or GSH. At this point, it is interesting to consider that the sulfhydryl group of the substrate could be the group that released protons, in concordance with other findings [20]. Moreover, several residues of the active site must participate in the uptake of protons. In this context, we note that in the active site of GST from *S. japonicum*, a residue structurally conserved as tyrosine, Tyr6 (p\(K_a\) 9.5) assists the proton abstraction from GSH or as hydrogen bond donator to stabilize the presumed thiolate anion (G-S\(^-\) … H-O-Tyr). On the other hand, Asp100 is a residue with low p\(K_a\) (4.6), also present in the active site. Thus, Tyr6 or some other proton-acceptor residue at pH 8.5, may be responsible for the increase in p\(K_a\) explaining the behaviour found at pH 8.5, whereas a group such as Asp100 may explain the number of protons exchanged at
Dpractically invariant with changes in temperature, both by the buffer ionization heat, are presented in Table 3. The derived from the temperature-dependent titration, corrected of which have appropriate pK\(_a\) values to participate in the exchange of protons, we could consider the intrinsic binding enthalpy to be approximately \(-5.5\) kcal\(\cdot\)mol\(^{-1}\). This value for intrinsic enthalpy combined with a proton exchange of protons, we could consider the intrinsic enthalpy and entropy.

Heat capacity change \(\Delta C_p\) and temperature dependence of the thermodynamic parameters

We analyzed the interaction between GSH and SjGST in different buffers at pH 6.5 as a function of temperature between 15 and 30 °C. The thermodynamic parameters derived from the temperature-dependent titration, corrected by the buffer ionization heat, are presented in Table 3. The data in Table 3 reveals that, whereas \(\Delta G^o\) remains practically invariant with changes in temperature, both \(\Delta H_b\) and \(\Delta S^o\) decrease with an increase in temperature. \(\Delta H_b\) depends linearly on temperature in the range of 15–30 °C. \(\Delta C_p = -0.238 \pm 0.04\) kcal\(\cdot\)K\(^{-1}\)\(\cdot\)mol\(^{-1}\) was obtained from the slope of \(\Delta H_b\) vs. temperature as is displayed in Fig. 5. A negative \(\Delta C_p\) value is normal in such binding studies [25,30,31], and is a distinctive feature of site-specific binding [32–35]. \(\Delta C_p\) values apparently equal to zero are obtained when the enthalpy changes are calculated using the variation of the equilibrium constant with temperature by the van’t Hoff equation (\(\Delta H_{vH}\)), and (a) the magnitude for \(\Delta C_p\) is relatively small and temperature range is small, or (b) the temperature range under study is far from the maximum in a van’t Hoff plot. Hence, there could be significant discrepancies between the heat capacity change obtained by a van’t Hoff plot and true calorimetric measurements [36,37]. Our data belong to scenario (a) mentioned above.

Table 3 also shows that \(\Delta S^o\) is positive at 15.8 and 20.5 °C, changing sign near 25 °C. Clearly, as may be observed in Fig. 5, the intersection point, \(\Delta G^o = \Delta H_b\) (24.3 °C and \(-5.04\) kcal\(\cdot\)mol\(^{-1}\)), implies that the entropic contributions to SjGST-GSH binding are equal to zero. Hence, at the latter temperature, \(\Delta G^o\) of SjGST-GSH binding is exclusively contributed by \(\Delta H_b\). On the other hand, at 15.8 °C the binding is driven almost equally by enthalpy and entropy.

The entropy change was calculated from the \(\Delta H_b\) obtained using the value of \(\Delta G^o\) calculated from the microscopic binding constant at each temperature (\(\Delta G^o = \Delta H_b - T\Delta S^o = -RT\ln K\)). The standard state is that of 1 mol\(\cdot\)L\(^{-1}\). The calculation of thermodynamic functions implies the usual approximation of setting standard enthalpies equal to the observed ones.

\begin{table}
\centering
\caption{Temperature dependence of thermodynamic parameters for binding of glutathione to SjGST. The uncertainties are standard errors in the fit of the curves}
\begin{tabular}{lccc}
\hline
Temperature (°C) & \(\Delta G^o\) (kcal\(\cdot\)mol\(^{-1}\)) & \(\Delta H_b\) (kcal\(\cdot\)mol\(^{-1}\)) & \(7\Delta S^o\) (kcal\(\cdot\)mol\(^{-1}\)) \\
\hline
15.8 & \(-5.08\pm0.15\) & \(-3.06\pm0.20\) & \(2.56\pm0.16\) \\
20.5 & \(-5.10\pm0.12\) & \(-3.84\pm0.37\) & \(1.26\pm0.12\) \\
25.1 & \(-4.87\pm0.16\) & \(-5.70\pm0.17\) & \(-0.83\pm0.16\) \\
30.1 & \(-5.14\pm0.13\) & \(-6.19\pm0.17\) & \(-1.08\pm0.13\) \\
\hline
\end{tabular}
\end{table}
The binding of substrate is noncooperative in the temperature range studied, which suggests that the interaction does not produce a conformational change affecting the other subunit. Therefore, its thermodynamic parameters may be attributed to intrinsic binding [38] and slight changes in the binding site region. This agrees with the observation that the structure of SjGST bound to substrate was nearly identical to the crystal structure of free GST [11], and that the binding of substrate does not significantly change the protein’s conformation [39]. A low negative $\Delta C_p^\circ$ value, as we have obtained in this study, is usual for these types of processes [24,30]. The cooperative binding process and the induced structural change responsible for this cooperativity are usually accompanied by positive $\Delta C_p^\circ$ values [40].

The experimental values of enthalpy, entropy change (Table 2), and the heat capacity change are the result of contributions of different signs: polar contributions arising from net formation of hydrogen bonds and electrostatic interactions; van der Waals’ interaction between polar and apolar groups; contributions arising from hydration of ligand and protein groups; the ionization or protonation of groups of the ligand and/or protein.

Crystallographic studies showed some hydrogen bonding interaction between SjGST and GSH [11], which may be taken as structural sources for negative $\Delta H_b$ values. Generally, there are four types of interaction with glutathione in the binding site: stabilization and orientation of the $\gamma$-glutamate of glutathione; alignment of glutathione peptide backbone; stabilization of the terminal carboxylate of glycine; and interaction with the sulphhydryl of cysteine for enzymatic activation. Association and orientation of the $\gamma$-glutamate is accomplished by either a Ser-Gln or Thr-Gln sequence that forms hydrogen bonds with the carboxyl group (atoms O\textsubscript{\alpha}1 and O\textsubscript{\alpha}2). Moreover, this configuration is further stabilized by a salt bridge between the atom N of $\gamma$-glutamate and Asp100 of subunit B. Other hydrogen bonds to the glutathione peptide are produced by another important dipeptide sequence, Leu-Asn, and the stabilization produced by Tyr6, which can act as a base or as a hydrogen bond acceptor (GS-H...O-Tyr) to assist the proton abstraction from GSH, or as a hydrogen bond donor to stabilize the presumed thiolate anion (G-S’...H-O-Tyr).

The net formation of hydrogen bonds produces a negative enthalpy change and should be accompanied by a negative $\Delta S^\circ$. However, one should consider that the formation of hydrogen bonds between glutathione and the protein results in the release of water molecules. These molecules, before binding, were hydrogen bonded to protein and/or ligand; one could therefore expect unfavourable enthalpic and favourable entropic components due to the dissociation of water from protein, ligand or both [41].

The positive entropy change can be explained by hydrophobic effects and electrostatic interactions. In the binding reaction the complex formation requires the dehydration of both the protein and the ligand, and is therefore accompanied by an entropic gain from the transfer of interfacial water into the bulk solvent. Negative heat-capacity change values are usually interpreted as arising from the burying of apolar groups from water [30,32,42–44]. Several authors [32,44,45] have suggested that the $\Delta C_p^\circ$ may be described as a phenomenon in hydration terms, pointing out that changes in vibrational modes apparently contribute little to $\Delta C_p^\circ$. On the basis of the X-ray crystallographic data of several proteins, the changes in the water accessible surface areas of both nonpolar ($\Delta A_{np}$) and polar ($\Delta A_p$) residues, on protein folding, have been calculated. Such calculations reveal that the ratio $\Delta A_{np}/\Delta A_p$ varies between 1.2 and 1.7 [44]. This range is comparable to a value for the ratio of $\Delta A_{np}/\Delta A_p$ of 2.4, calculated for the binding of glutathione to SjGST. The application of Murphy’s approach [44] to the experimentally determined values (Table 3) indicates that the surface area buried on complex formation comprises 70% nonpolar surface (approximately 1720 Å\textsuperscript{2}) and 30% polar surface (approximately 720 Å\textsuperscript{2}).

Our results show that the binding of glutathione to SjGST is enthalpy driven between 15 and 30 °C and is accompanied by a net release of protons at pH 6.5. At temperatures below 25 °C, the entropy change is positive, favouring GSH binding. Calorimetric studies at various pHs show that the $pK_a$ values of at least three groups change during the binding. The dependence of $\Delta H_b$ and the number of protons exchanged on the pH is consistent with a decrease in the p$K_a$ of the thiol group of ligand and an increase in p$K_a$ values of groups Asp100 and Tyr6 existent in the active site. In addition, our data are in agreement with the observation that the binding of glutathione does not induce a profound conformational change in SjGST dimer, only slight modifications in the active site region. Moreover, the affinity of this substrate for the enzyme is practically the same in the studied temperature range. The enthalpy change of binding is not strongly temperature-dependent, arising from a small negative $\Delta C_p^\circ$ of binding, which suggests only moderate changes in the apolar surfaces accessible to the solvent.

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