

A calorimetric study of the binding of *S*-alkylglutathiones to glutathione *S*-transferase

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Abstract

The binding of three competitive glutathione analogue inhibitors (*S*-alkylglutathione derivatives) to glutathione *S*-transferase from *Schistosoma japonicum*, SjGST, has been investigated by isothermal titration microcalorimetry at pH 6.5 over a temperature range of 15–30°C. Calorimetric measurements in various buffer systems with different ionization heats suggest that no protons are exchanged during the binding of *S*-alkylglutathione derivatives. Thus, at pH 6.5, the protons released during the binding of substrate may be from its thiol group. Calorimetric analyses show that *S*-methyl-, *S*-butyl-, and *S*-octylglutathione bind to two equal and independent sites in the dimer of SjGST. The affinity of these inhibitors to SjGST is greater as the number of methylene groups in the hydrocarbon side chain increases. In all cases studied, ΔG^0 remains invariant as a function of temperature, while ΔH_b and ΔS^0 both decrease as the temperature increases. The binding of three *S*-alkylglutathione derivatives to the enzyme is enthalpically favourable at all temperatures studied. The temperature dependence of the enthalpy change yields negative heat capacity changes, which become less negative as the length of the side chain increases. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Glutathione *S*-transferases (EC 2.5.1.18) (GSTs) play an important role in the biotransformation of xenobiotics, i.e. compounds which are foreign to living cells [1]. This role involves conjugating electrophilic substrates to the tripeptide glutathione (GSH, γ -Glu-Cys-Gly), which often makes them less toxic and more readily excretable from the body. 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 se-

Abbreviations: GSH, reduced glutathione; SjGST, 26 kDa glutathione *S*-transferase from *Schistosoma japonicum*; ITC, isothermal titration calorimetry; MeSG, *S*-methylglutathione; BuSG, *S*-butylglutathione; OcSG, *S*-octylglutathione; MOPS, 3-(*N*-morpholino)propanesulphonic acid; ACES, 2-[(2-amino-2-oxoethyl)amino]ethanesulphonic acid; DTT, dithiothreitol; DTNB, 1-chloro-2,4-dinitrobenzene

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quence, substrate specificity, and immunological properties [6]. They are characterized by a 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 are highly selective for the GSH molecule.

The homodimeric (26 kDa/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 drew 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]. Each subunit in homodimeric SjGST contains two structural domains, an N-terminus domain and a C-terminus domain. The dimeric structure is required for the formation of two functional active sites (one per subunit).

It is important to point out the physiological role of SjGST as a detoxification protein and vaccine and drug target, with a wide application in protein biotechnology.

It is known that the binding of *S*-substituted glutathiones inhibits the enzyme, and that the extent of inhibition correlates to the length of the alkyl side chain of the inhibitor [12–14]. However, the forces and contributions responsible for the association between these inhibitors and SjGST are unknown. The present investigation reports the results of studies of the binding to SjGST of three *S*-alkylglutathione derivatives, by high sensitivity titration microcalorimetry. To our knowledge, this is the first time that a calorimetry study with these inhibitors has been carried out on GST. On increasing the length of the alkyl group of the inhibitor, a higher affinity constant was obtained, stemming from a more favourable enthalpic contribution. The binding of GSH to SjGST is accompanied by a net release of protons [15,16]. Our results show that the replacement of the thiolic proton by the alkyl group eliminates the release of protons during binding. The ΔH values measured are independent of buffer ionization heats for

these inhibitors. The calorimetric studies have been performed at several temperatures in order to obtain the heat capacity changes for each inhibitor.

2. Materials and methods

2.1. Reagents and buffers

All chemicals were of analytical grade and were used without further purification. *S*-Methylglutathione, *S*-butylglutathione, and *S*-octylglutathione were purchased from Sigma; DTT and EDTA were from Pharmacia-Biotech and Riedel-de Haen, respectively. Buffer reagents were from Sigma. All solutions were made with distilled and deionized (Milli Q) water.

2.2. Expression and purification

The pGEX-2T plasmid (Amersham Pharmacia Biotech) was used to overexpress the SjGST in *Escherichia coli* strain BL21 [17]. A single colony of *E. coli* cells was incubated for 12–15 h at 37°C with 200–250 rpm shaking in Luria–Bertani (LB) culture medium containing 100 µg/ml of ampicillin. This culture was diluted 100 times in fresh LB medium with 50 µg/ml of ampicillin and grown at 37°C until the absorbance at 600 nm reached 0.2–0.3. Isopropyl β-D-thiogalactosidase was then added at a final concentration of 0.2 mM to induce overexpression of SjGST. After growing for 3 h at 37°C, the cells were centrifuged at 7000 rpm in a Beckman JA14 rotor for 10 min at 4°C. The cells' pellet was resuspended in phosphate-buffered saline (PBS) and lysed with 10 mg/ml lysozyme, followed by freezing under liquid nitrogen and defrosting at 37°C seven times. The supernatant was then collected by centrifugation at 10 000 rpm in a Beckman JA20 rotor for 10 min at 4°C, after which it was applied directly to a glutathione affinity column. After the SjGST protein was bound to the matrix, it was washed with PBS buffer to remove non-specifically bound proteins. In order to use the intact SjGST for calorimetry, an additional nine-residue peptide at its C-terminus was removed by adding 10 units/mg of thrombin protease to the matrix bound to SjGST. After incubating the suspension at room temperature for 15 h, SjGST was eluted with

10 mM of reduced glutathione. The enzyme showed a single band pattern in polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. Purified protein was stored at -80°C . The purification yield was around 80 mg of apparently pure protein per litre of culture.

Solutions of protein were prepared by dialysis of the enzyme against several changes of 1 mM of EDTA, 2 mM of DTT and 20 mM of the corresponding buffer solutions (phosphate, MOPS and ACES) at pH 6.5 and 4°C .

Protein concentrations were determined from absorbance measurements at 278 nm, using the absorbance coefficient of $7.01 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ [18]. Absorbance measurements were carried out in a Beckman DU-7400 spectrophotometer with cells maintained at 25°C .

2.3. Isothermal titration microcalorimetry

All calorimetric experiments were conducted on an MCS isothermal titration calorimeter (ITC) from Microcal (Northampton, MA). A complete description of its predecessor instrument, OMEGA-ITC, experimental strategies, and data analyses are given by Wiseman et al. [19]. 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 ligands 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 titration, the reaction mixture was continuously stirred at 400 rpm.

The background titration profiles, under identical experimental conditions, were obtained by injecting the ligand into appropriate buffer solutions. The observed heat effects were concentration-independent and 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.

ITC measurements were routinely performed in 1 mM EDTA, 2 mM DTT 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, whose ionization enthalpies (in kcal mol^{-1} at 25°C) are: phosphate (1.22), MOPS (5.22) and ACES (7.51) [20]. The pH of the buffer solution (20 mM) was adjusted at the experimental temperature.

3. Results and discussion

3.1. Isothermal calorimetry experiments

Fig. 1 shows the titration of $41 \mu\text{M}$ of SjGST with 25 aliquots (5 μl each) of *S*-methylglutathione (MeSG) (stock concentration of 10.7 mM) in 20 mM phosphate buffer at pH 6.5 and 20°C . The top panel of Fig. 1 shows the raw calorimetric data, denoting the amount of heat produced (negative exothermic peaks) following each injection of inhibitor. The area under each peak represents the amount of heat produced upon binding of MeSG to SjGST. Note that, as the titration progresses, the areas under the peaks progressively become smaller due to increased occupancy of the enzyme by MeSG. The bottom panel of Fig. 1 shows the plot of the amount of heat generated per injection as a function of the molar ratio of MeSG to the 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 (ΔH_{obs}) of $7.1 \times 10^3 \text{ M}^{-1}$, and -5.7 kcal/mol , respectively. Identical experiments to those described in phosphate were carried out in two other buffers with different ionization enthalpies, viz. MOPS and ACES, at 15, 20, 25 and 30°C . The calorimetric titration of SjGST with MeSG in the three buffers studied indicates that no protons are exchanged during the binding reaction. The number of protons (n) at each temperature has been obtained by fitting the thermodynamic binding parameters at each temperature and for each buffer by the equation

$$\Delta H_{\text{obs}} = \Delta H_{\text{b}} + n\Delta H_{\text{iz}}$$

where ΔH_{b} is the intrinsic binding enthalpy, and ΔH_{iz} the buffer ionization heat.

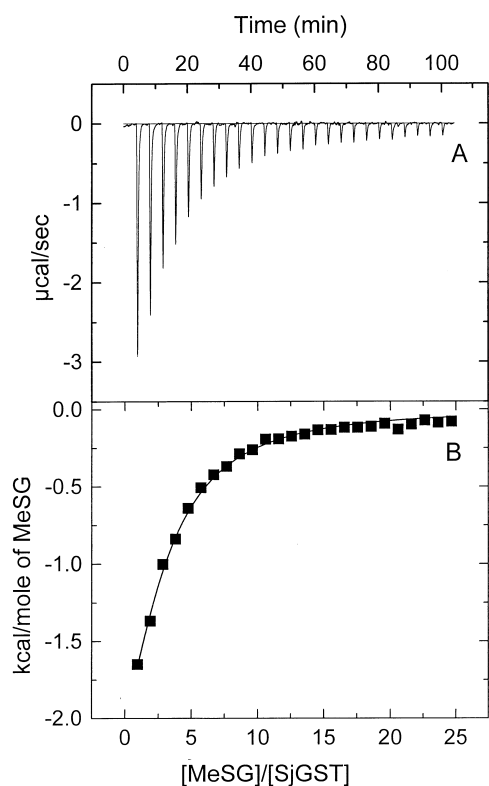


Fig. 1. Titration of S-jGST by MeSG in 20 mM phosphate buffer (pH 6.5) containing 2 mM DTT and 1 mM EDTA at 20°C. Panel A shows the raw data, generated by titration of 1.8 ml of 41 μ M by twenty-five 5 μ l injections of 10.7 mM MeSG. The area under each peak was integrated and plotted against the molar ratio of MeSG to S-jGST in panel B. The smooth solid line represents the best fit of the experimental data to a model with two non-interacting sites.

The number of protons exchanged by the enzyme during MeSG binding was 0.04, 0.04, 0.03, and 0.03 at 15, 20, 25, and 30°C, respectively.

To study the influence of the alkyl groups of the inhibitor on the thermodynamic parameters, similar experiments to those mentioned above were performed with *S*-butyl- and *S*-octylglutathione in the same temperature range (15–30°C). As with MeSG, the number of protons exchanged was also practically zero at all temperatures studied. These results show that there is no exchange of protons for *S*-alkylglutathione derivatives at pH 6.5, which seems to indicate that the protons released during the binding of substrate could come from the interaction of the thiol group with the enzyme at this pH [15,16]. Fig. 2 shows data from the calorimetric titration of 1.38 ml of 63 μ M S-jGST in the sample cell at 25.1°C

with 4 mM solution of *S*-octylglutathione in the same buffer (20 mM phosphate, 2 mM DTT, 1 mM EDTA at pH 6.5). The sample data represent 22 injections of 5 μ l, each spaced at 4 min intervals. The comparison of the curves depicted in Figs. 1B and 2B, revealing the different affinities for both inhibitors, is of particular interest. An intermediate behaviour to that shown for *S*-methyl- and *S*-octyl derivatives was found for *S*-butylglutathione (Table 1). A plot of these data as accumulated heat shows hyperbolic curves, which are typical for an equal and independent sites model (non-cooperative model).

The affinity of the inhibitor increases as the length of the alkyl group increases, in concordance with previous inhibition studies [12]. Thus, as is displayed in Table 1, the binding equilibrium constant for MeSG is one and two orders of magnitude lower

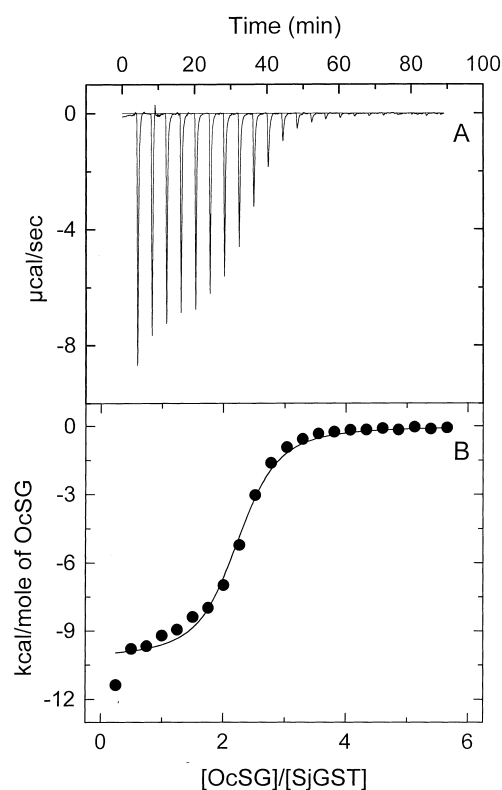


Fig. 2. Titration of S-jGST by OcSG in 20 mM phosphate buffer (pH 6.5) containing 2 mM DTT and 1 mM EDTA at 25.1°C. Panel A shows the raw data, generated by titration of 1.8 ml of 61 μ M by twenty-five 5 μ l injections of 4 mM OcSG. The area under each peak was integrated and plotted against the molar ratio of OcSG to S-jGST in panel B. The smooth solid line represents the best fit of the experimental data to a model with two non-interacting sites.

than those for *S*-butylglutathione and *S*-octylglutathione, respectively.

3.2. Heat capacity changes ΔC_p^0 and temperature dependence of the thermodynamic parameters

Since the number of protons exchanged on binding for the three inhibitors is practically zero, we analysed the interaction between three *S*-alkylglutathione derivatives and SjGST in phosphate buffer, as a function of temperature between 15 and 30°C at pH 6.5. The thermodynamic parameters derived from the temperature-dependent titration are presented in Table 1.

The entropy change was calculated from the ΔH_b obtained using the value of ΔG^0 calculated from the microscopic binding constant at each temperature ($\Delta G^0 = \Delta H_b - T \Delta S^0 = -RT \ln K$). The standard state is that of 1 mol/l. The calculation of thermodynamic functions implies the usual approximation of setting standard enthalpies equal to those observed.

A different behaviour is observed for the three inhibitors studied. In all cases, whereas ΔG^0 remains practically invariant with changes in temperature, both ΔH_b and ΔS^0 decrease as the temperature increases. ΔH_b depends linearly on temperature in the range studied, and the heat capacity change is calculated from the slope. Table 1 also shows that ΔS^0 is positive at 15 and 20°C for MeSG, changing signs around 25°C. Clearly, the intersection point, $\Delta G^0 = \Delta H_b$ (20.8°C and -5.03 kcal/mol), implies that the entropic contributions to MeSG–SjGST

binding are equal to zero. Hence, ΔG^0 of MeSG–SjGST binding is exclusively contributed by ΔH_b at 20.8°C.

Van der Waals interactions and hydrogen bonding are usually considered to be the major potential sources of negative ΔH values [21]. As can be seen from the thermodynamic parameters displayed in Table 1, we suggest that van der Waals interactions and hydrogen bonds play a fundamental role in the interactions between these inhibitors and SjGST.

Before binding, the inhibitor might be forming H-bonds with the water molecules of the solvent. After binding, the inhibitor might also be forming hydrogen bonds with the groups of amino acids of the active site. Although the three-dimensional structures of these inhibitors bonded to this enzyme have not been determined, the structure of substrate–enzyme is known [11]. In this structure several H-bonds have been described which may also exist in the enzyme–inhibitor complexes. These H-bonds are formed in a more apolar medium than water and may be the major contribution to the intrinsic enthalpy change obtained. An increase in the length of the apolar side chain of the inhibitor produces a more hydrophobic environment, which would explain the fact that the enthalpy change is more negative. Since the increase in affinity is caused by a more favourable enthalpic contribution, the H-bonds in a more hydrophobic environment may explain why affinity is higher for the *S*-octyl derivative.

On the other hand, the sign of the entropic change on binding provides some clues to the kinds of phys-

Table 1
Temperature dependence of thermodynamic parameters

Inhibitor	Temperature (K)	K (M^{-1})	ΔG^0 (kcal/mol)	ΔH_b (kcal/mol)	ΔS^0 (cal mol $^{-1}$ K $^{-1}$)	ΔC_p^0 (cal mol $^{-1}$ K $^{-1}$)
MeSG	288.0	8.9×10^3	−5.21	−3.13	7	−328
	293.0	6.2×10^3	−5.10	−4.77	2	
	298.0	4.2×10^3	−4.94	−6.42	−5	
	303.0	2.2×10^3	−4.63	−8.05	−12	
BuSG	288.8	2.5×10^5	−7.13	−7.57	−2	−240
	294.0	1.8×10^5	−7.10	−8.97	−6	
	298.5	1.1×10^5	−6.88	−9.55	−9	
	303.2	1.2×10^5	−7.02	−11.30	−14	
OeSG	290.0	9.5×10^5	−7.93	−8.10	−1	−200
	293.5	7.2×10^5	−7.87	−8.81	−3	
	298.2	3.7×10^5	−7.94	−10.02	−9	
	303.3	7.0×10^5	−8.11	−10.70	−9	

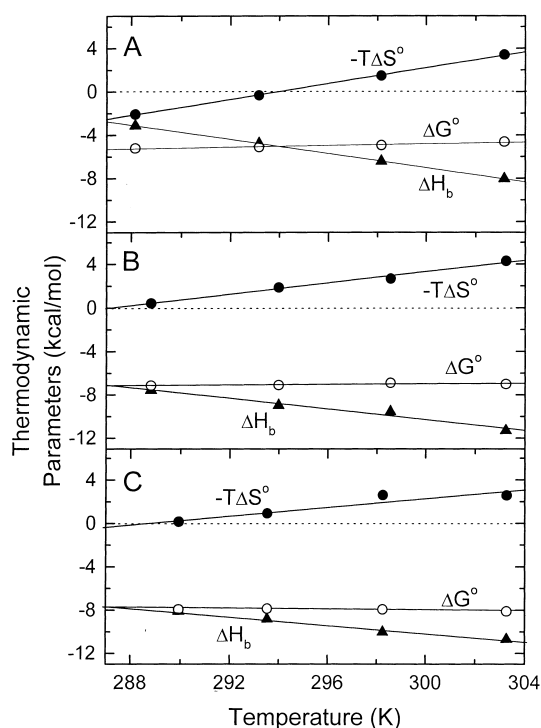


Fig. 3. Temperature dependence of the thermodynamic parameters for the binding of SjGST to (A) MeSG, (B) BuSG and (C) OcSG. The heat capacity change associated with the binding of these inhibitors to SjGST was determined from the slope of linear regression analysis of the ΔH_b vs. temperature data.

ical processes involved. Above 20.8°C the entropy changes on binding to SjGST are negative for all inhibitors (Fig. 3A–C; Table 1). Processes for which there is a negative entropy change may be attributed to hydrogen bond formation, a decrease in the number of isoenergetic conformations, and a decrease in soft internal vibrational modes [21]. For *S*-butyl- and *S*-octylglutathione the binding entropy remains negative at all temperatures studied, but for *S*-methylglutathione the entropy change is increased positively at temperatures below 293.9 K. Processes for which a positive entropy change is expected may arise from the burial of electrostatic charges or hydrophobic groups from water [21]. The entropy change values obtained for the three inhibitors (Table 1) seem to indicate that there is no significant increase in hydration water molecules released to increase the length of the apolar side chain of the inhibitor.

The binding of SjGST to *S*-methyl-, *S*-butyl- and *S*-octylglutathione involves negative changes in heat capacity (Table 1), which are frequent in binding

studies [21–23], and are a distinctive feature of site-specific binding [24–27]. The values obtained are different for the three inhibitors studied. While the MeSG–SjGST interaction results in a ΔC_p^0 of $-328 \text{ cal mol}^{-1} \text{ K}^{-1}$, the interaction involving *S*-butylglutathione (BuSG) and *S*-octylglutathione (OcSG) yields -240 and $-200 \text{ cal mol}^{-1} \text{ K}^{-1}$, respectively. This may suggest differences in the binding processes between these inhibitors. Ladbury et al. [28] indicate that high affinity binding and large negative ΔC_p^0 are not necessarily correlated. Our results support their conclusion, showing that the higher binding affinity is obtained with lower heat capacity change. Ladbury et al. also suggest that a large negative ΔC_p^0 may be a consequence of high specificity binding, whether at high or low affinity. They propose that a large negative heat capacity change results from the formation of an ‘intimate complementary interface of a “specific” complex’ and is ‘not a consequence of a high affinity reaction per se’.

Crystallographic studies of human glutathione transferase bound to two inhibitors (*S*-hexylglutathione and γ -glutamyl-(*S*-benzyl)cysteinyl-D-phenylglycine) indicate that both the hexyl and benzyl groups occlude the highly hydrophobic H-site [29]. Moreover, other structural studies on a complex between human GST and nonylglutathione (PDB entry 12GS) show that the alkyl chain is nicely embedded inside the H-site of the protein. A similar behaviour could be expected for the interaction of inhibitors studied with SjGST. This result seems to suggest that the hydrophobic interactions will increase as the inhibitor’s side chain lengthens. However, the thermodynamic parameters obtained show that the possible hydrophobic interactions on the binding of these inhibitors must be accompanied by other interactions whose contribution is enthalpic. If the hydrophobic interactions drive the binding, ΔG^0 must be entropically favourable and enthalpically unfavourable [30,31]. Moreover, the enthalpy and entropy changes should be higher and the heat capacity changes should be more negative (Table 1) as the length of the side chain increases. The binding of the three inhibitors to the enzyme is enthalpically favourable, and we believe that the major contribution to this fact is probably the H-bonds formed between the inhibitor and groups of amino acids of the protein.

Negative heat capacity change values are usually interpreted as arising from the burying of apolar groups from water [21,24,28–34]. Murphy and Freire [35] and Spolar and Record [24] have suggested that the ΔC_p^0 may be described as a phenomenon in hydration terms, pointing out that changes in vibrational modes contribute little to ΔC_p^0 . On the basis of the X-ray crystallographic data of several proteins, the changes in the water accessible surface areas of both non-polar (ΔA_{np}) and polar (ΔA_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 [35]. This range is comparable to values for the ratio of $\Delta A_{np}/\Delta A_p$ of 1.4, 1.3 and 1.2, calculated for the binding of *S*-methyl-, *S*-butyl- and *S*-octylglutathione, respectively. The application of Murphy's approach [35] to the experimentally determined values (Table 1) indicates that the surface area buried on complex formation comprises 60%, 54% and 50% non-polar surface for *S*-methyl-, *S*-butyl- and *S*-octylglutathione, respectively. These differences are minimal and are within experimental error for these theoretical values. Therefore, as Spolar and Record [24] indicate, these values can be taken as the 'rigid body' interactions.

The binding of these inhibitors was non-cooperative in the temperature range studied, which suggests that the interaction does not perform a conformational change affecting the other subunit. Therefore, its thermodynamic parameters may be attributed to intrinsic binding [36] 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 the binding of an inhibitor does not significantly change the protein's conformation [37]. Negative ΔC_p^0 values, such as those obtained in this study, are usual for this type of process [21–23,38]. The cooperativity binding process and the induced structural change responsible for this cooperativity are usually accompanied by positive ΔC_p^0 values [39].

Our results show that during the binding of *S*-alkylglutathione derivatives to SjGST no protons are exchanged with the buffer medium. This suggests that the protons released during the binding of substrate (GSH) [15,16] might come from the thiol group of sulfhydryl at pH 6.5. The binding is enthalpy driven between 15 and 30°C for the three inhib-

itors studied. The negative value of enthalpy change may be attributed mainly to the H-bonds formed in the enzyme–inhibitor complex, which are stronger as the apolarity of the active site increases. The affinity constant, which increases as the length of the side chain of the inhibitor increases, is two orders of magnitude higher for *S*-octylglutathione than for *S*-methylglutathione. These results seem to indicate that the active site of the complex is more apolar for the *S*-octyl derivative. In addition, our data are in agreement with the observation that the binding of these inhibitors does not induce a profound conformational change in SjGST dimer, only slight modifications in the active site region. The dependence of the enthalpy changes of binding on temperature arises from the negative ΔC_p^0 of binding, which suggests only slight changes in the apolar surfaces accessible to the solvent.

Acknowledgements

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