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Role of mutation Y6F on the binding properties of *Schistosoma japonicum* glutathione S-transferase

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Abstract

The role of the hydroxyl group of tyrosine 6 in the binding of *Schistosoma japonicum* glutathione S-transferase has been investigated by isothermal titration calorimetry (ITC). A site-specific replacement of this residue with phenylalanine produces the Y6F mutant, which shows negative cooperativity for the binding of reduced glutathione (GSH). Calorimetric measurements indicated that the binding of GSH to Y6F dimer is enthalpically driven over the temperature range investigated. A concomitant net uptake of protons upon binding of GSH to Y6F mutant was detected carrying out calorimetric experiments in various buffer systems with different heats of ionization. The entropy change is favorable at temperatures below 26 °C for the first site, being entropically favorable at all temperatures studied for the second site. The enthalpy change of binding is strongly temperature-dependent, arising from a large negative $\Delta C^{\circ}_{p1} = -3.45 \pm 0.62\,\mathrm{kJ\,K^{-1}\,mol^{-1}}$ for the first site, whereas a small $\Delta C^{\circ}_{p2} = -0.33 \pm 0.05\,\mathrm{kJ\,K^{-1}\,mol^{-1}}$ for the second site was obtained. This large heat capacity change is indicative of conformational changes during the binding of substrate.

Keywords: Glutathione S-transferase; *Schistosoma japonicum*; Glutathione; Unfolding; Fluorescence; Binding; Microcalorimetry; *S*-Methylglutathione

1. Introduction

The glutathione S-transferases (GSTs) are a family of dimeric detoxication enzymes, which catalyze the conjugation of GSH to a variety of endogenous and exogenous electrophiles. The mammalian cytosolic GSTs are separated into seven gene classes based on crystal structure and substrate specificity: alpha (α), pi (π), mu (μ), theta (θ), kappa (κ), sigma (σ), and zeta (ζ) [1–5]. X-ray crystallographic and site-directed mutagenesis studies illustrate that each GST contains a conserved tyrosine or serine residue which hydrogen bonds to, and effectively deprotonates, GSH to the nucleophilic thiolate (GS) [6–9]. Because the thiolate anion is a more reactive nucleophile than the protonated thiol, the catalytic advantage is obvious [10]. Crystal structures indicate that each GST has the same basic protein fold, which consists of two domains; one domain provides the binding site for GSH (G-site), and the other contributes to the binding of the hydrophobic ligand (H-site) [11]. Although the G-site is highly homologous in all of GSTs, there is a great deal of variability in the H-site, which confers the differing substrate selectivity and catalytic properties for each gene class.

Site-directed mutagenesis studies have revealed the importance of specific residues in the glutathione binding site. Manoharan et al. [12] showed that the substitution of binding site residues Arg 13, Gln 62, and Asp 96 in π GST resulted in 20–50-fold decreases of both the catalysis and glutathione binding efficiencies in comparison to the wild-type. However, the substitution of Tyr^7 by phenylalanine still resulted in 27% of the wild-type capacity to bind glutathione, whilst the enzymatic catalysis was reduced to less than 1%. This substantial reduction in the catalytic activity due to the substitution of tyrosine was also obtained for μ GST [7]. Furthermore, there are different views on how the thiol anion of glutathione is created to promote high

Abbreviations: GSH, reduced glutathione; Y6F, glutathione S-transferase Y6F mutant from *Schistosoma japonicum*; DTT, dithiothreitol; CDNB, 1-chloro-2,4-dinitrobenzene; ITC, isothermal titration calorimetry; Mops, 3-(*N*-morpholino) propanesulfonic acid; Aces, 2-[(2-amino-2-oxoethyl)-amino]ethanesulfonic acid

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nucleophilic reactivity, i.e. either the thiol of glutathione is deprotonated [7,13] or tyrosine OH group is deprotonated [14].

The properties and crystal structure of the homodimeric glutathione S-transferase from *Schistosoma japonicum* (wild-type enzyme) reveal a number of significant structural and functional differences compared to the other GST classes. The crystal structure of the binary complex GSH-wild-type enzyme [15] has suggested that the sulfur of GSH (or GS^-) is located at 3 Å from the phenolic oxygen of Tyr^6 in the active site, thus forming a hydrogen bond. In order to assess the influence of the phenolic hydroxyl group of Tyr^6 on the properties of bound GSH and its contribution to catalysis, an expression plasmid encoding the Y6F mutant of GST of *S. japonicum* was constructed, and the mutant enzyme was expressed and purified. In this paper, we have studied the conformational stability of the Y6F mutant using urea as a denaturant, as well as the binding of reduced glutathione to Y6F by ITC.

2. Materials, methods and experimental data analysis

2.1. Chemicals

GSH, CDNB and urea were ultrapure grade from Sigma. DTT was from Pharmacia. Phosphate, Mops and Aces buffers were purchased from Merck and Sigma. Centriprep 30 concentrators were from Amicon. All other chemicals were of analytical grade of the highest purity available. All solutions were made with distilled and deionized (Milli Q) water. All solutions were degassed and clarified through a 0.45-μm Millipore filter before use.

2.2. Enzyme

The recombinant Y6F mutant was expressed in *Escherichia coli* and purified as previously described elsewhere [16]. The protein was purified by glutathione affinity chromatography. The enzyme showed a single-band pattern in polyacrylamide gel electrophoresis in presence of sodium dodecyl sulfate. Purified protein was stored at −80 °C in 20 mM sodium phosphate, pH 7, and containing 2 mM DTT. Protein concentration was calculated from the absorbance at 280 nm assuming an $\varepsilon_{1\,mg/ml}$ of 1.02. The extinction coefficient was calculated on the basis of the amino acid sequence as reported by Gill and von Hippel [17] and confirmed by bicinchoninic acid method (Pierce). A molecular mass of 26 kDa per subunit was used in the calculations [18]. Absorbance measurements were carried out using a Beckman DU-7400 spectrophotometer with cells maintained at 25 °C.

2.3. Enzyme assay

The catalytic activity of Y6F mutant was determined 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 CDNB [19] and corrected for the corresponding non-enzymatic controls. The specific activity of Y6F was compared to those obtained for the wild-type enzyme in the same conditions.

2.4. Aggregation state

In order to examine the aggregation state of the Y6F mutant and the GSH–Y6F complex at 25 °C gel filtration chromatography with Superdex S-200 10/30 (Pharmacia) was used. Analytical HPLC was carried out on a Waters apparatus. Effluents were monitored with a UV-Vis detector from Waters, set at 280 nm. The column (1 cm × 30 cm) was equilibrated in 20 mM sodium phosphate, 1 mM EDTA and 2 mM DTT at pH 6.5 with and without 15 mM of GSH. The calibration of the column was performed with aprotinin (6.5 kDa), cytochrome *c* (12.4 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa) and Blue Dextran (2000 kDa) all of them from Sigma. Samples were applied in a volume of 0.2 ml at 1–2 mg/ml. Absorbance at 280 nm was measured by a Beckman DU-7400 spectrophotometer for detection of protein.

2.5. Unfolding studies

Folding/unfolding studies of Y6F were performed with a Perkin-Elmer LS50B spectrofluorometer interfaced to a computer for data collection and analysis. The temperature of the sample was controlled using a thermostatted cuvette holder and a Frigiterm 6000382 Selecta refrigerated circulating water bath.

To study the unfolding/refolding of Sj26GST under equilibrium conditions, tryptophan and tyrosine fluorescence changes were used as structural and functional probes to monitor changes. The enzyme contains four tryptophan residues per subunit (Trp 7, Trp 40, Trp 200 and Trp 205). The tryptophan emission spectrum for the folded protein (excitation at 295 nm) has a maximal emission at 335 nm, which shifts to 355 nm when the protein unfolds. Refolding of the denatured protein by a 10-fold dilution of the denaturant resulted in both an increase and a decrease of fluorescence at 335 and 355 nm, respectively. No aggregation was detected for the results shown. Urea concentrations were determined from refractive index measurements [20].

Denaturation curves were evaluated according to the linear extrapolation method [21] for dimeric proteins [22] as described elsewhere [20]. The dimeric state must, therefore, be significantly populated in the transition zone. The equilibrium constant, K_U, corresponding to this reaction:

$$A_2 \overset{K_U}{\rightleftharpoons} 2U$$

was calculated at each point in the transition region of the denaturation curve by

$$K_U = \frac{[U]^2}{[A_2]} = 2M^{total}\frac{f_U^2}{1 - f_U} \quad (1)$$

where M^{total} being the total protein concentration and f_U the fraction of unfolded protein. If this model provides an appropriate thermodynamic description of the denaturation reaction, then one should calculate the same value for K_U or ΔG_U (calculated as $-RT\ln K_U$) from experiments performed at different protein concentrations. A linear dependence of the Gibbs free energy of unfolding on the denaturant is assumed [23]:

$$\Delta G_U = \Delta G_U^W - m \times [\text{Urea}] \quad (2)$$

where ΔG_U^W represents the difference in Gibbs free energy between the unfolded and folded protein in the absence of denaturant.

The conformational stability parameters ΔG_U^W and m were calculated by iterative fitting of the denaturation curves to the above equations using Scientist (MicroMath Scientific Software, Salt Lake City, UT). Values of $C_{1/2}$ (the denaturant concentration at which half the population of protein molecules are unfolded) were obtained from the midpoints of the unfolding transitions.

2.6. Isothermal titration microcalorimetry (ITC)

ITC experiments were performed using an MCS ITC from Microcal, Inc. (Northampton, MA, USA). A complete description of its predecessor instrument, OMEGA-ITC, experimental strategies, and data analyzes are given by Wiseman et al. [24] and Schwarz et al. [25]. Prior to the titration experiments, both the enzyme and the 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 the control experiment) or of an appropriately diluted enzyme. During the titration, the reaction mixture was continuously stirred at 400 rpm. The experiments were carried out by titrating Y6F solutions with GSH.

The background titration profiles, under identical experimental conditions, were obtained by injecting GSH or inhibitor into appropriate buffer solutions. The observed heat effects for the background titrations were concentration-independent and were identical to the heat signals detected after complete saturation of the protein. The raw experimental data are presented as the amount of heat evolved per second following each injection of ligand into the enzyme solution (after correction for the ligand heat of dilution) as a function of time. The amount of heat produced per injection was calculated by integrating the area under individual peaks using the Origin software provided with the instrument. A two equal and interacting sites model was used to fit the data. The partition function, P, in Eq. (3), which specifies two sites for the binding of GSH to the Y6F dimer, was used to analyze the ITC isotherms:

$$P = 1 + 2K_1X + K_1K_2X^2 \quad (3)$$

where K_1 and K_2 are the microscopic equilibrium association "binding" constants for GSH binding to the first and the second site on Y6F dimer, respectively, and X is the free GSH concentration.

For this model, the total heat after the ith injection, Q_i^{tot}, is given by Eq. (4):

$$Q_i^{tot} = \frac{M_i^{tot}V_o}{P}[\Delta H_1 \times 2K_1X_i + (\Delta H_1 + \Delta H_2)K_1K_2X_i^2] \quad (4)$$

where X_i is the free GSH concentration after the ith injection, is obtained by solving Eq. (5):

$$X_i^{tot} = X_i + \nu M_i^{tot} = X_i + M_i^{tot}\left(\frac{2K_1X_i + 2K_1K_2X_i^2}{P}\right) \quad (5)$$

being ν the degree of binding (moles of ligand bound per mole of protein) which is given by

$$\nu = \left(\frac{\partial \ln P}{\partial \ln X}\right)_{T,P} \quad (6)$$

ΔQ_i (the heat for the ith injection) is given by Eq. (7):

$$\Delta Q_i = Q_i^{tot} - Q_{i-1}^{tot} + \frac{dV_i}{2V_o}(Q_i^{tot} + Q_{i-1}^{tot}) \quad (7)$$

In Eqs. (4), (5) and (7), X_i^{tot} and M_i^{tot} are the total cell concentrations of GSH and Y6F dimer, respectively. V_o is the volume of the calorimetric cell (1.38 ml).

Values of ΔH_{obs} determined by ITC can have contributions from the heat of ionization of the buffer if there is any net protonation or deprotonation of the interacting species upon formation of the protein–GSH complex [26–28]. Experimental values of the ΔH_{obs} determined in a particular buffer may include contributions from the heat of ionization of the buffer. Values of ΔH^b (binding enthalpy) have been corrected for the contribution due to the heat of ionization of the buffer using Eq. (8):

$$\Delta H_{obs} = \Delta H^b + \Delta n\,\Delta H_{ion} \quad (8)$$

where Δn is the number of protons released (negative value) by or absorbed (positive value) by the protein–ligand complex as a result of binding, respectively. We note, however, that even after this correction, ΔH^b still includes the contributions from the heat of protonation of the ionizable group (or groups) on the protein or GSH.

By a combined analysis to Eqs. (4), (5), (7) and 8 using the data from several buffers with different enthalpies of ionization, we can obtain the enthalpy changes (ΔH_1^b and ΔH_2^b) and the number of protons exchanged (Δn_1 and Δn_2) to the first and second sites, respectively.

To determine the heat contributions due to coupled protonation events upon binding the buffers used and their ionization enthalpies (in kJ mol^{-1} at 25 °C) were as follows: Aces (31.40), Mops (21.82) and phosphate (5.10) [29]. To calculate the ionization enthalpy at each experimental temperature, we have used the heat capacity change for each buffer [29].

A complete thermodynamic description of the binding is obtained including the free energy of binding (ΔG°) and the change in entropy (ΔS°) calculated using: $\Delta G^\circ = -RT \ln K = \Delta H^b - T\,\Delta S^\circ$, where R is the gas constant and T is the absolute temperature. The standard state is that of 1 mol l^{-1}, and these calculations imply the usual approximation of setting standard enthalpies equal to the observed ones.

3. Results and discussion

3.1. Kinetic studies

The activity assay for the Y6F mutant was performed as described in Section 2. A very low specific activity, compared to those obtained for the wild-type enzyme, was obtained at 25 °C (Y6F has a turnover number at pH 6.5 of approximately 1.5% that of the wild-type enzyme). This behavior seems to suggest that the amino acid Tyr^6 is very important for catalysis produced by this transferase. However, although the activity is very low, it retains the ability to bind GSH, as is demonstrated later by calorimetry. Reinemer et al. [30] have considered the possibility that the tyrosine may act either as a general base or hydrogen bond acceptor (GS–H · · · $^-$O–Tyr) to assist in the removal of a proton from GSH or as a hydrogen bond donor to stabilize the thiolate (GS^- · · · H–O–Tyr) in the binary E–GSH complex. Thermodynamic considerations would suggest that the latter is the more likely possibility [13]. In either case, the proximity of the active site tyrosine to the sulfur of GSH, and analogues in the crystal structures of GSTs, suggests that it may have a significant influence on the pK_a or orientation of the thiol in the E–GSH complex and hence affect catalysis. A similar behavior was found for isoenzyme 3–3 [7].

3.2. Unfolding experiments at different temperatures

Individual samples of Y6F at a final concentration of 0.2 μM, were kept incubated in urea solutions (0–8 M) for a sufficient time to reach the denaturation equilibrium. Profiles of unfolding fraction, f_U, versus urea concentration were obtained at several temperatures within the range of 25–45 °C (see Fig. 1A) in 20 mM phosphate buffer, containing 1 mM EDTA and 2 mM DTT at pH 6.5. These profiles were analyzed according to the two-state denaturation model shown in Section 2. A refolding profile is also shown (as inset) in Fig. 1B. The fitting curves allowed us to estimate the conformational stability of the Y6F mutant in the absence of denaturant, ΔG_U^W, and the m-value, at different temperatures. It is known that the size of the protein, or the amount of its surface area exposed to solvent upon unfolding, is the

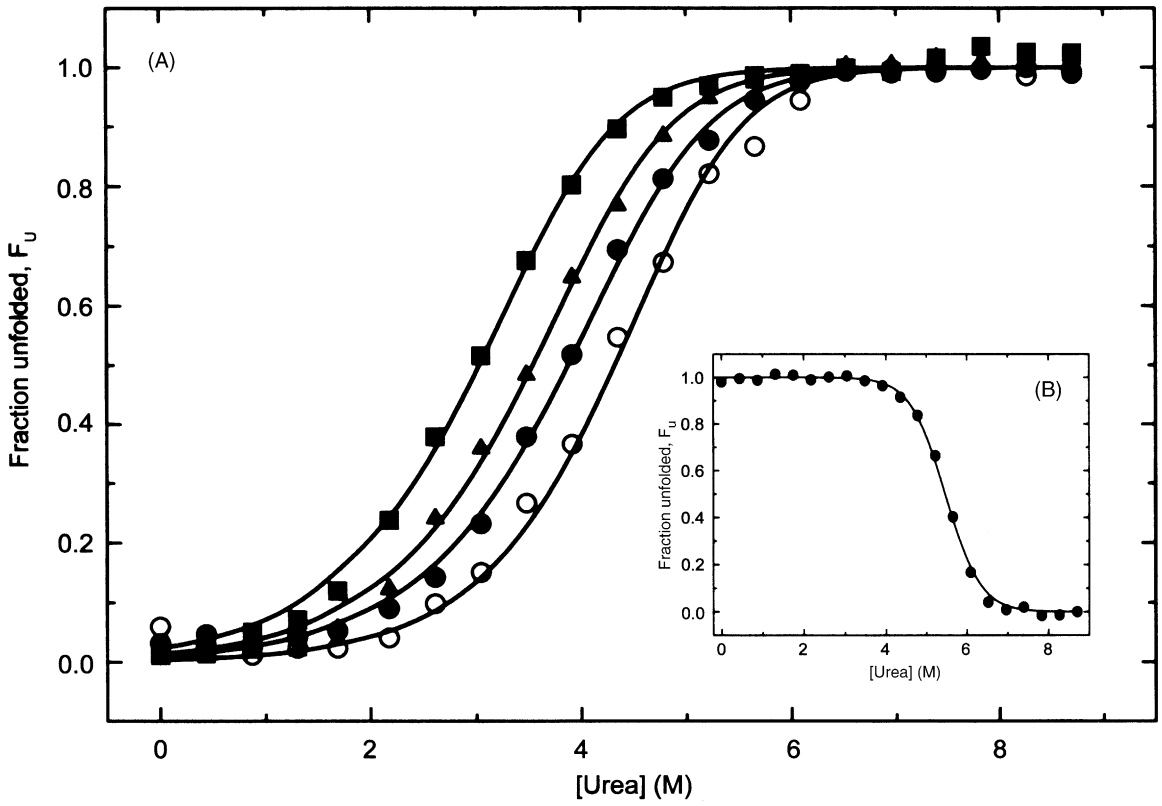

Fig. 1. Urea-unfolding equilibrium profiles for Y6F mutant. Fraction unfolded, f_U, is plotted vs. urea concentration at 25 °C (○), 30 °C (●), 35 °C (▲) and 45 °C (■) in 20 mM sodium phosphate, 1 mM EDTA and 2 mM DTT at pH 6.5 (Panel A). The solid lines represent the best fits of the experimental data to a two-state model. In panel B (inset) a profile corresponding to a refolding of Y6F was shown in the same experimental conditions and at 25 °C.

Table 1
Unfolding data for *S. japonicum* Y6F-glutahione S-transferase at pH 6.5 and at different temperatures

T (°C)	m (kJ mol^{-1} M^{-1})	ΔG_U^W (kJ mol^{-1})	ΔASA[a] (Å^2)	$C_{1/2}$ (M)
25	5.15 ± 0.25	25.91 ± 1.11	9859 ± 543	5.04 ± 0.08
30	5.41 ± 0.13	25.01 ± 0.52	8422 ± 292	4.62 ± 0.02
35	5.68 ± 0.17	24.33 ± 0.67	9018 ± 369	4.27 ± 0.01
45	5.93 ± 0.21	22.24 ± 0.63	9564 ± 455	3.74 ± 0.02

[a] Calculated by empirical equation, $m = 1538 + 0.46 \times (\Delta ASA) \cdot$(Myers et al. [42]), with m in J mol^{-1} M^{-1}. The uncertainties are standard error in fitting of the curves.

major structural determinant factor for the *m*-value [4,23]. The *m*-values obtained (Table 1) practically do not change with increasing temperature, which indicates the area exposed to solvent during unfolding is practically the same over this temperature range (see Table 1). A different behavior was found for the wild-type enzyme, where the *m*-values decrease with increasing temperature, reducing therefore the area exposed to solvent [31]. This result might indicate that the Y6F mutant is less sensible to temperature than the wild-type enzyme. On the other hand, the ΔG_U^W values and $C_{1/2}$ diminish progressively as the temperature increases, moving the denaturation curves as a result, towards the left. These different results found between Y6F mutant and the wild-type enzyme might be indicative of some structural differences in both enzymes, with which one would expect a different behavior in their binding properties (see below).

3.3. Isothermal titration microcalorimetry

Direct calorimetric measurements were performed in order to obtain independent estimates of the thermodynamic parameters governing the binding of substrate GSH to GST–Y6F. Fig. 2A shows a typical ITC profile for the binding of GSH to dimeric Y6F in a phosphate buffer at pH 6.5 and 25.3 °C. The sample data displays 19 equivalent 5 μl injections preceded by a 2 μl pre-injection (spaced at 4 min intervals) of GSH solution into the enzyme solution. The negative sign of the measured heat indicates that the enthalpy change for each injection was negative and that the process of binding under these conditions was exothermic. Control experiments which involve the same type of injections of substrate solution into the same buffer were also carried out in order to measure the heat of dilution of GSH.

On the other hand, by using size exclusion chromatography, it was demonstrated that the Y6F mutant is a dimer, in the conditions described in the paper, with an estimated molecular mass of approximately 50 kDa. A non-cooperative model is unable to fit the calorimetric data appropriately, creating χ^2 values unacceptable. However, a model of two equal and interacting sites is able to fit all the calorimetric experiments. A representative ITC experiment, in which injections of 0.026 μmol of GSH per 5 μl addition were inserted into 47.1 μM Y6F mutant at 25.3 °C in 20 mM sodium

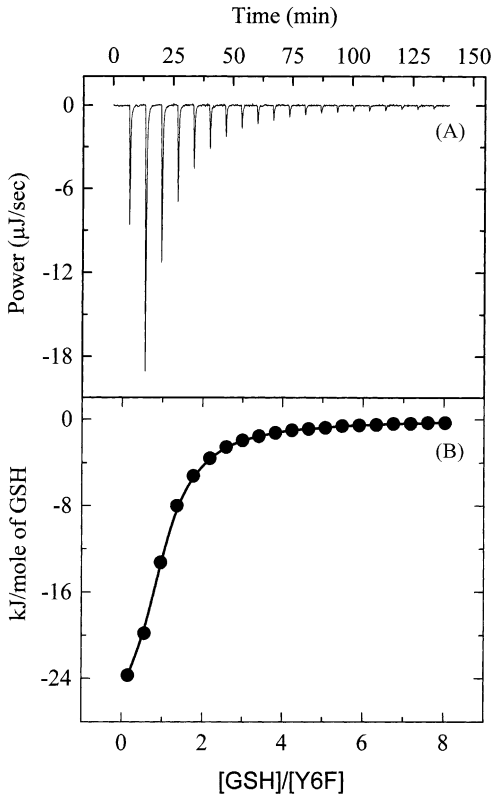

Fig. 2. ITC results for the binding of 47.1 μM Y6F mutant by 5.21 mM GSH in 20 mM sodium phosphate (pH 6.5), 1 mM EDTA and 2 mM DTT at 25.3 °C. Panel A shows the raw data for the titration of 1.8 ml of enzyme with nineteen 5 μl injections of GSH. A pre-injection of 2 μl was performed at the beginning. The area under each peak was integrated and plotted in panel B against the molar ratio of GSH to Y6F inside the cell. The solid smooth line represents the best fit of the experimental data to a model of two equal and interacting sites.

phosphate (pH 6.5) is shown in Fig. 2. Identical experiments were carried out at 20.1 and 30.2 °C. As the temperature rises the binding enthalpies become more exothermic. To measure ionization changes on binding, the same calorimetric titrations were done in three buffers with different ionization enthalpies, viz., 20 mM phosphate, 20 mM Mops, 20 mM Aces containing 1 mM EDTA and 2 mM DTT at pH 6.5. Since the reaction is less exothermic in Aces buffer than it is in Mops or phosphate at the three temperatures studied, we conclude that there is a net proton uptake during the binding reaction for the first site.

It is very important to underline that thermodynamic parameters shown in Table 2 have been obtained by a global fit using all the calorimetric data measured in different buffers at each temperature. Thus, negative cooperativity was found for the binding of GSH to Y6F over the temperature range of 20–35 °C.

The binding equilibrium constant for the first site, K_1, is one order of magnitude higher than that for the second site, K_2 (Table 2). Therefore, the binding of the first molecule of substrate produces a conformational change in the mutant enzyme, in this way decreasing the affinity for the second

Table 2
Apparent thermodynamic parameters for binding of GSH to *S. japonicum* Y6F-glutahione S-transferase at pH 6.5 and at different temperatures

T (°C)	$K_1 \times 10^{-4}$ (M^{-1})	$K_2 \times 10^{-3}$ (M^{-1})	ΔG_1° (kJ mol^{-1})	ΔG_2° (kJ mol^{-1})	ΔH_1^b (kJ mol^{-1})	ΔH_2^b (kJ mol^{-1})	ΔS_1° (J K^{-1} mol^{-1})	ΔS_2° (J K^{-1} mol^{-1})	$-T\,\Delta S_1^\circ$ (kJ mol^{-1})	$-T\,\Delta S_2^\circ$ (kJ mol^{-1})
20.1	7.22 ± 0.50	4.97 ± 0.35	−27.25 ± 1.36	−20.52 ± 0.78	−12.16 ± 0.41	−3.59 ± 0.12	51.45 ± 2.76	58.43 ± 1.76	−15.09 ± 1.02	−17.14 ± 1.32
25.3	7.85 ± 0.45	7.85 ± 0.54	−25.46 ± 1.52	−22.24 ± 1.04	−24.58 ± 0.54	−1.75 ± 0.03	2.96 ± 0.14	68.63 ± 3.45	−0.87 ± 0.04	−20.48 ± 2.32
30.2	5.26 ± 0.36	4.85 ± 0.34	−27.38 ± 1.12	−21.36 ± 0.98	−47.15 ± 0.82	−7.02 ± 0.23	−65.20 ± 2.65	47.27 ± 2.65	19.77 ± 1.42	−14.34 ± 1.06

The errors shown correspond to the standard deviation of the non-linear least squares fit of the data points of the titration curve.

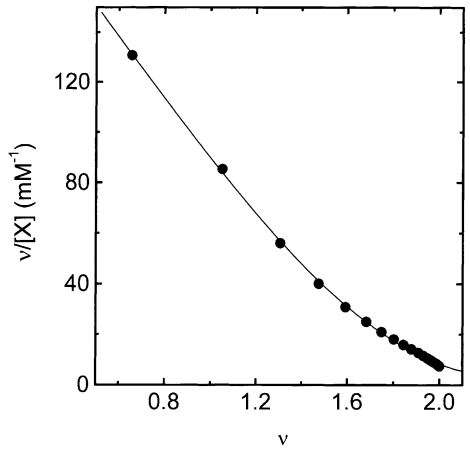

Fig. 3. A Scatchard plot of the ITC data of binding of GSH to Y6F mutant at 20.1 °C. ν and [X] are the degree of binding and the free GSH concentration, respectively.

site. The affinity constant for the first site is higher than those for the wild-type enzyme but similar to those for the second sites.

A Scatchard plot, in which ν/[X] is plotted versus ν, has also been used to investigate the validity of this model (Fig. 3). The Scatchard plot of the ITC data at 20.1 °C is curvilinear. The concave nature of the Scatchard plot as seen in Fig. 3 assures the negative cooperativity of this binding.

On the other hand, the observed enthalpy changes for both the first ($\Delta H_{1,\mathrm{obs}}$) and the second ($\Delta H_{2,\mathrm{obs}}$) sites are negative over range of temperatures studied. Knowing the ΔG_1° and ΔG_2° values for the two sites, along with the ΔH_1^b and ΔH_2^b values (Table 2), allows the calculation of the corresponding entropy of binding ($T\Delta S^\circ$) values for the two sites. This calculation was done at each temperature (Table 2). The difference in global ΔG° for the binding of GSH to Y6F compared with that for wild-type enzyme [16] is mainly entropic. The temperature dependence of ΔH^b and $-T\Delta S^\circ$ for the GSH–Y6F binding exhibits an enthalpic–entropic compensation, and as a consequence, the ΔG° remains relatively constant with temperature (Fig. 4). This enthalpy–entropy compensation was observed for both sites, but is stronger for the first than for the second site. ΔH_1^b are negative at the three temperatures studied and ΔS° is positive at 20.0 and 25.3 °C, changing sign at 25.5 °C. Clearly, as may be observed in Fig. 4, the intersection point, $\Delta G_1^\circ = \Delta H_1^\mathrm{b}$ (25.5 °C and $-26.7\,\mathrm{kJ\,mol^{-1}}$), implies that the entropic contributions to Y6F–GSH binding are equal to zero. Hence, at the latter temperature, ΔG_1° of Y6F–GSH binding is exclusively dominated by ΔH_1^b. On the other hand, around 20.8 °C the binding is driven almost equally by enthalpic and entropic forces. This behavior for the first site is very similar to that observed for the binding of GSH to the wild-type enzyme [16]. The number of protons taken up by the first site during GSH binding was 0.03 ± 0.01; 0.36 ± 0.02 and 0.44 ± 0.02 for 20, 25.3 and 30.2 °C, respectively, increasing with temperature. Nevertheless, the number of protons exchanged by the second site were -0.02 ± 0.01, 0.06 ± 0.04 and -0.05 ± 0.02 for 20.0, 25.3 and 30.2 °C, respectively, i.e. practically equal to zero. The experimental values of ΔH^b ($\Delta H_1^\mathrm{b} + \Delta H_2^\mathrm{b}$) include the enthalpy change for the binding of GSH to the Y6F and the heat of uptaking 0.40 protons from the GSH/Y6F complex. In the wild-type enzyme approximately -0.30 protons were released in the binding of GSH [16], which were assigned to the tiol group of substrate. However, if we accept the existence of conformational changes in this binding, it would be quite difficult to identify which groups will be responsible for this exchanging of protons. Therefore, although the functional groups responsible for proton uptake have not been identified, the current results suggest that the protons could either be uptaken by a group with a pK_a of 5 or lower, or that there are two or more groups, with a pK_a around of 6.5, which can alter their pK_a as a consequence of binding. This latter possibility might be the most pausible if we accept the existence of conformational changes in this binding.

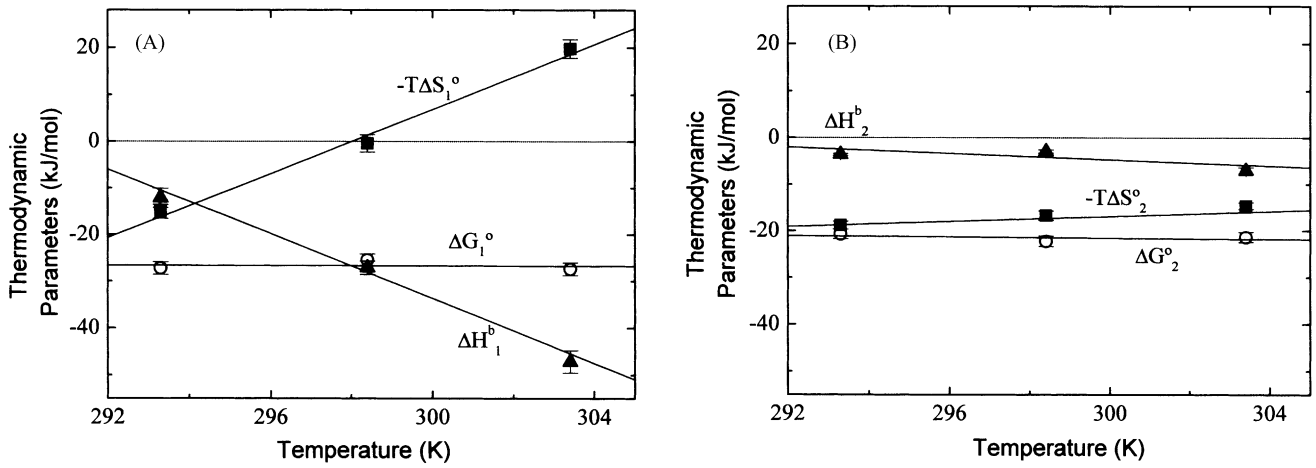

Fig. 4. Temperature dependence of ΔH^b (▲), $-T\Delta S^\circ$ (■), and ΔG° (○) for the binding of GSH to the first site (panel A) and the second (panel B) of Y6F mutant. $\Delta C_{\mathrm{p}1}^\circ$ and $\Delta C_{\mathrm{p}2}^\circ$ were obtained by the slope of a linear regression to ΔH_1^b and ΔH_2^b data, respectively.

In that case, several factors may contribute to the experimental thermodynamic parameters (Table 2): a net formation of hydrogen bonds and electrostatic interactions; changes in van der Waals interactions between specific groups; and changes in the hydration of ligand and protein groups.

The temperature dependence of ΔH_1^b reveals a large negative ΔC_{p1}° for the first site, $-3.45 \pm 0.63\,kJ\,K^{-1}\,mol^{-1}$, obtained from the slope of a linear-regression analysis of the ΔH_1^b versus T data. Furthermore, we obtained a ΔC_{p2}° equal to $-0.33 \pm 0.05\,kJ\,K^{-1}\,mol^{-1}$ from the slope of ΔH_2^b versus T. In the temperature range studied, both ΔC_{p1}° and ΔC_{p2}° were found to be linear. Therefore, a global ΔC_p° ($\Delta C_{p1}^\circ + \Delta C_{p2}^\circ$) of $-3.78\,kJ\,K^{-1}\,mol^{-1}$ is obtained, which is more negative than that of the wild-type enzyme ($\sim -1.01\,kJ\,K^{-1}\,mol^{-1}$) [16].

Possible reasons that can contribute to this large ΔC_p° might be: (1) conformational changes in protein or substrate; (2) changes in ionization; (3) changes in the water network in the binding site and (4) release of water from the hydrophobic surface upon binding. Recently, the change in the hydrogen bonding network from cooperativity ordered in the complex to floating in aqueous solution was proposed as a fifth reason [32]. Traditionally, a large negative ΔC_p° is taken as a sign of hydrophobic interactions [33–35], i.e. the release of water from hydrophobic surfaces upon binding, although large changes in heat capacity could also arise due to changes in the hydrogen bonding network [32]. If we assume the main contribution to global ΔC_p° ($\Delta C_{p1}^\circ + \Delta C_{p2}^\circ$) is due to conformational changes in the active sites and to hydrophobic effects, i.e. to significant changes in the polar and apolar surfaces accessible to the solvent, ΔC_p°, could be expressed as a function of the contributions of buried non-polar and polar surface areas. For protein folding reactions and protein–protein interactions, both the binding enthalpy and the change in heat capacity were successfully predicted by using the empirical formulas:

$$\Delta C_p = \Delta C_{p,np} + \Delta C_{p,p} = 1.88\,\Delta ASA_{np} - 1.09\,\Delta ASA_p \quad (9)$$

$$\Delta H(60) = -35.28\,\Delta ASA_{np} + 131.25\,\Delta ASA_p \quad (10)$$

where ΔASA_{np} and ΔASA_p are, respectively, the changes in non-polar and polar areas accessible to the solvent (in $Å^2$), $\Delta H(60)$ is the enthalpy change at 60 °C, being that, the temperature taken as the reference, because it is approximately the average denaturation temperature of the model proteins used in the analysis [36]. In both equations, ΔC_p and $\Delta H(60)$ are expressed in $J\,K^{-1}\,mol^{-1}$ and $J\,mol^{-1}$, respectively.

From our experiments, the value of total ΔH^b ($\Delta H_1^b + \Delta H_2^b$) for the binding of GSH to Y6F at 25.2 °C was obtained. The value for 60 °C can be calculated simply from $\Delta H(60) = \Delta H^b(25.3) + \Delta C_p$ (60–25.3). The ΔASA_{np} and ΔASA_p values were -3205 and $-2060\,Å^2$, respectively. The structure of this mutant with GSH has not been solved yet. However, the structure of the analogous complex GSH–Y6F for the isoenzyme 3–3 of glutathione S-transferase is known [7]. We could use the structural data of this analogous mutant to estimate the changes in accessible surface areas. The surface area calculations indicate that a total of $2112\,Å^2$ of apolar surface area and $935\,Å^2$ of polar surface area are buried upon binding ($\Delta ASA_{np} = -2112\,Å^2$; $\Delta ASA_p = -935\,Å^2$). Solvent accessible surface areas were calculated using the program NACCESS [37], using a probe radius of 1.4 Å and a slice width of 0.25 Å. This program allows us to calculate the accessible surface areas according to the algorithm-defined by Lee and Richards [38]. The predicted value of ΔC_p° ($-2.96\,kJ\,K^{-1}\,mol^{-1}$) from Eq. (9) is lower than the experimental value ($-3.78\,kJ\,K^{-1}\,mol^{-1}$). The difference of $\sim 0.82\,kJ\,K^{-1}\,mol^{-1}$ is not too high, compared with the large capacity change and might be justified by, (1) structural differences between the Y6F mutant of *S. japonicum* and Y6F mutant of isoenzyme 3–3; or/and (2) small differences on the binding of GSH to both transferases. Moreover, we must also consider the possibility that the discrepancy between observed and calculated ΔC_p° values can be due to flaws in the theoretical analysis of our experimental results. ΔC_p° is a parameter intimately linked to the reorganization of hydration shells upon association. Thus, functional group contributions to ΔC_p° may not be additive and would depend strongly on the local chemical environment [39]. On the other hand, the parametrization of Spolar and co-workers and Freire and Murphy, which work relatively well in predicting protein folding parameters, is not always applicable to protein–ligand interactions [39,40]. Nevertheless, we can use this correlation to explain the binding process. According to Eq. (9), a capacity change higher (by about $\sim 0.82\,kJ\,K^{-1}\,mol^{-1}$) than expected might be explained in terms of apolar surface burial, as burial of polar surface would lead to an increase in heat capacity (note the signs of the coefficients ΔASA_{np} and ΔASA_p in Eq. (9)). The amount of non-polar surface involved appeared too large to be accounted for in 'rigid body' association [41]. Hence, our results suggest that conformational changes in the environment of the active sites were coupled to binding, justifying the accessible surface area values calculated.

3.4. Conclusions

The mutation of the Tyr^6 by Phe in GST of *S. japonicum* generates a mutant with low activity, compared to the wild-type enzyme. However, it retains the ability to bind GSH. The binding of GSH to Y6F mutant exhibits a negative cooperativity at pH 6.5, which can be justified postulating a conformational change during the binding. As a consequence of this cooperativity a different number of protons exchanged is obtained by the first and second site. On

the other hand, the binding is enthalpically driven over the temperature range studied. Moreover, at temperatures below 25 °C, the binding is also favored entropically, similarly to the wild-type enzyme. The enthalpy change of binding is strongly temperature-dependent for the first site, arising from a large negative ΔC_p° of binding, whereas this is practically constant with the temperature for the second site. The global ΔC_p° suggests changes in the apolar surfaces accessible to the solvent.

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