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Thermodynamics of glutathione binding to the tyrosine 7 to phenylalanine mutant of glutathione *S*-transferase from *Schistosoma japonicum*

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Abstract

The binding of glutathione (GSH) to the tyrosine 7 to phenylalanine mutant of *Schistosoma japonicum* glutathione *S*-transferase (SjGST-Y7F) has been studied by isothermal titration calorimetry (ITC). At pH 6.5 and 25 °C this mutant shows a higher affinity for glutathione than wild type enzyme despite an almost complete loss of activity in the presence of 1-chloro-2,4-dinitrobenzene (CDNB) as second substrate. The enthalpy change upon binding of GSH is more negative for the mutant than for the wild type GST (SjGST). Changes in accessible solvent areas (ASA) have been calculated based on enthalpy and heat capacity changes. ASA values indicated the burial of apolar surfaces of protein and ligand upon binding. A more negative ΔC_p value has been obtained for the mutant enzyme, suggesting a more hydrophobic interaction, as may be expected from the change of a tyrosine residue to phenylalanine.

Keywords: Microcalorimetry; Fluorescence; Site-direct mutagenesis

1. Introduction

Glutathione *S*-transferases (GST, EC 2.5.1.18) are a family of multifunctional enzymes that catalyse the nucleophilic attack of the thiol group of glutathione on electrophilic centres in a second substrate [1,2]. The enzyme molecule is composed of two subunits, each containing a functional active site. The dimeric structure of GSTs plays an important role in stabilising the tertiary structure [3], and it is also a requirement for the formation of a non-substrate binding site at the dimer interface. Enzymes of this family of dimeric proteins can be grouped into a variety of species-independent gene classes, categorised according to sequence similarity and subcellular distribution. Cytosolic GSTs are designated as alpha, mu, pi, theta and sigma. Evolutionary relationships of the isoenzymes suggest that alpha, mu and pi class GSTs evolved from a theta-gene duplication [4]. Members within any class exhibit similar monomer size and high amino acid sequence identity (60–80%), while inter-gene class similarity is considerably lower (about 25–35%) [5]. Homodimers or heterodimers can be obtained within a given gene class but not between different gene class GSTs. This fact suggests that there are specific structural requirements for subunit–subunit interactions in each class.

Schistosoma japonicum GST (SjGST) crystal structure has been determined for the apoenzyme form [6], and for complexes with either the physiological substrate glutathione (GSH) [7] or the non-substrate ligand and major anti-schistosomal drug praziquantel [6]. Structural differences in the xenobiotic substrate binding site of SjGST suggest that it belongs to a novel class of GSTs. The overall folding at the binding site of different GSTs is well conserved, with 12 conserved residues. One of these amino acids is Tyr^7 (SjGST sequence). This amino acid has been postulated to play a central role in the catalytic mechanism of GSTs by stabilising the thiolate anion of glutathione and enhancing the nucleophilicity of the thiol anion versus the protonated thiol. Recently a new role has been reported for

Abbreviations: SjGST, *Schistosoma japonicum* glutathione *S*-transferase; SjGST-Y7F, *Schistosoma japonicum* glutathione *S*-transferase tyrosine 7 to phenylalanine mutant; CDNB, 1-chloro-2,4-dinitrobenzene; SEC, size exclusion chromatography; ITC, isothermal titration calorimetry

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equivalent Tyr^{9} in alpha class GSTs inducing a conformational change at the C-terminal helix [8,9].

In this article, we study the binding of GSH to the mutant Tyr^{7} to Phe of *S. japonicum* GST (SjGST-Y7F). Our results show that this mutation dramatically reduces the activity towards 1-chloro-2,4-dinitrobenzene (CDNB), whereas affinity for the substrate GSH increases. This result has been supported by isothermal tritation calorimetry and fluorescence measurements.

2. Materials and methods

All chemicals were of analytical grade and were obtained either from Sigma Aldrich Quimica (Madrid, Spain) or Merck Farma y Quimica (Barcelona, Spain). GSH Sepharose 4B and Superdex 200HR 10/30 column were obtained from Amersham Biosciences (Barcelona, Spain).

2.1. Site-directed mutagenesis, expression and purification

Plasmid pGEX-2T was used as template in the site-directed mutagenesis experiment. The Tyr^{7-5} (5′-CTATACTAGGTTTTTGGAAAATTAA-3′) and Tyr^{7-3} (5′-TTAATTTTCCAAAAACCTAGTATAG-3′) oligonucleotides were used to change the TAT codon in the glutathione *S*-transferase sequence to TTT, so that the tyrosine amino acid was replaced by phenylalanine (SjGST-Y7F). In the mutagenesis reaction, 30 ng of plasmid template was mixed with reaction buffer (50 mM Tris–HCl, 15 mM ammonium sulphate pH 9.3, 2.5 mM $MgCl_2$ and 0.1% (v/v) Tween 20%), 0.2 mM each of dATP, dCTP, dGTP and dTTP, 600 nM of each oligonucleotide and 2.5 units of AccuTaq LA DNA Polymerase Mix (Sigma Aldrich Quimica) supplemented with water to a total volume of 50 μl. The amplification parameters were as follows: an initial DNA denaturation at 94 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min and extension at 68 °C for 10 min with 10 s of auto-extension for cycles 16–30. Half a microliter (5 U) of *Dpn*I restriction enzyme (Sigma Aldrich Quimica) was added to the PCR product and incubated at 37 °C for 2 h. The ligation product was used to transform competent *E. coli* cells JM109 [10] by the Ca^{2+}-coprecipitation technique [11]. The new mutated plasmid was confirmed by DNA sequencing and named pJMC40. In order to increase the biomass of recombinant cells, the plasmid pJMC40 was transformed in *E. coli* BL21 [12]. The expression and purification of SjGST and SjGST-Y7F were carried out as previously described [13].

The concentration of both SjGST and SjGST-Y7F was measured spectrophotometrically at 278 nm, using the extinction coefficient of $3.5 \times 10^4 \pm 1.3 \times 10^3\ M^{-1}\ cm^{-1}$ for SjGST and $3.56 \times 10^4 \pm 2.2 \times 10^3\ M^{-1}\ cm^{-1}$ for SjGST-Y7F monomer, as calculated by the Gill and von Hippel method [14].

2.2. Enzyme assays

Enzyme activity towards CDNB was assayed at 25 °C by the Habig and Jakoby method [15]. For kinetic determination of the enzyme, CDNB concentration was held constant at 1 mM for SjGST assay and 2 mM for SjGST-Y7F assay, while GSH concentration was varied from 0.05 to 1.25 mM.

2.3. Size exclusion chromatography-HPLC (SEC-HPLC)

Size exclusion chromatography-HPLC (SEC-HPLC) analysis was performed in an HPLC System (Breeze HPLC System, Waters, Barcelona) using a Superdex 200HR 10/30 column equilibrated with the same buffers used for ITC analysis (Table 1). Samples of 40 μM SjGST and SjGST-Y7F were dialysed for 24 h in each buffer before injecting. Protein elution was monitored at 280 nm. Molecular mass determination was performed using protein standards: β-amylase (200 kDa); alcohol dehydrogenase (148 kDa); ovalbumine (44 kDa); and ribonuclease A (14 kDa). In order to check the aggregation state after GSH binding, SEC-HPLC experiments were performed in each buffer in the presence of saturating concentration of GSH (2 mM).

2.4. Fluorescence

Fluorescence emission spectra were measured at 25 °C in a Perkin-Elmer LS55 spectrofluorimeter. Samples of SjGST and SjGST-Y7F (3 ml in a 10 mm pathlength cuvette) were excited at 295 nm in order to obtain the tryptophan fluorescence spectra. The binding of GSH to the enzyme was monitored using the decrease of fluorescence emission of the tryptophan at 335 nm. Excitation bandwidth was 2.5 nm and emission bandwidth was 10 nm. Spectra were collected immediately after adding the GSH. Fluorescence measurements were corrected for dilution.

Table 1
GSH binding to SjGST-Y7F at different pHs

Buffer	pH	ΔH_{ioniz} (kJ mol^{-1})	ΔH_{tot} (kJ mol^{-1})	ΔH_b (kJ mol^{-1})
Pipes	6	11.45	−31.01	−29.30
Mes	6	15.55	−31.64	−29.30
Phosphate	6.5	5.10	−27.72	−27.29
Mes	6.5	15.55	−31.89	−27.29
Aces	7	31.39	−33.06	−27.71
Phosphate	7	5.10	−28.51	−27.71
Phosphate	7.5	5.10	−31.94	−31.85
Mops	7.5	21.82	−32.23	−31.85
Mops	8	21.82	−28.51	−32.19
Hepes	8	20.98	−28.67	−32.19

Buffer composition was 20 mM of the buffer indicated in the table plus 2 mM DTT and 1 mM EDTA.

It was assumed that the dimeric protein (experiment to pH 6.5) has two equal and independent sites for the substrate GSH, with a characteristic microscopic association constant, K. The saturation fraction, Y, can be expressed as:

$$Y = \frac{K[\mathrm{GSH}]}{1 + K[\mathrm{GSH}]} \quad (1)$$

where [GSH] is the free concentration of glutathione and can be expressed as:

$$[\mathrm{GSH}] = [\mathrm{GSH}]_\mathrm{T} - 2Y[\mathrm{SjGST}] \quad (2)$$

where $[\mathrm{GSH}]_\mathrm{T}$ is the total concentration of glutathione and [SjGST] is the SjGST-Y7F concentration. Using Eqs. (1) and (2) the free concentration of glutathione after each addition can be determined as:

$$[\mathrm{GSH}] = \frac{\sqrt{(1 - K[\mathrm{GSH}]_\mathrm{T} + 2K[\mathrm{SjGST}])^2 + 4K[\mathrm{GSH}]_\mathrm{T}} + K[\mathrm{GSH}]_\mathrm{T} - 2K[\mathrm{SjGST}] - 1}{2K} \quad (3)$$

Moreover, the saturation fraction, Y, can be calculated as:

$$Y = \frac{\Delta F_\mathrm{corr}}{\Delta F_\mathrm{corr}^\mathrm{max}} = \frac{F(\mathrm{GSH}) - F(0)}{F(\infty) - F(0)} \quad (4)$$

where $F(0)$, $F(\mathrm{GSH})$ and $F(\infty)$ are 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. (1)–(3), the association constant K and $F(\infty)$ can be calculated from the experimental values.

2.5. Isothermal titration calorimetry (ITC)

Titrations were performed using the MCS high-sensitive microcalorimeter manufactured by Microcal Inc. (Microcal, Northhampton, MA). This instrument has been described elsewhere [16]. The reference cell was filled with water, and the instrument was calibrated using standard electrical pulses. A circulating water bath was used to stabilise the temperature. The instrument was allowed to equilibrate overnight. SjGST-Y7F was dialysed extensively against ITC buffer prior to all titrations. The enzyme was loaded into the sample cell of the calorimeter (volume = 1.38 ml). GSH, in the same buffer, was placed in a 250 μl syringe at a concentration at least 35–40 times that of the SjGST-Y7F.

The system was allowed to equilibrate and a stable baseline was recorded before initiating an automated titration. The titration experiment consisted of 23 injections of 10 μl each into the sample cell. The injections were carried out at 4-min intervals. The sample cell was stirred at 400 rpm. Dilution experiments were performed by identical injections of GSH into the cell containing only buffer. The thermal effect of protein dilution was negligible in all cases. The peaks of the obtained thermograms were integrated using the ORIGIN software (Microcal, Inc.) supplied with the instrument.

Two different buffers were used at each pH in order to measure the contribution of protonation heat of the buffer from the protons released or taken up under the binding process.

3. Results and discussion

SEC-HPLC experiments have shown that SjGST is a dimer at pHs ranging from 6.0 to 8.0. The aggregation state does not change under GSH addition at saturating conditions. Experiments at pH above 8 result in shorter elution times that correlate with the presence of aggregates in SjGST and SjGST-Y7F. Kaplan et al. [17] showed that in the absence of dithiothreitol (DTT) or other reducing agents, SjGST becomes oxidised and results in a mixture of native protein with 160 kDa and larger aggregates. SjGST has four cysteine residues per unit, none of which are involved in intra-subunit disulphide bonds [6,7]. Cysteine free amino acid has a pK of 8.3 for the thiol group, and as the four cysteines are exposed to the solvent the thiol pK_as at these cysteines were not expected to change too much. It is, therefore, plausible that at pHs above 8 thiolate anion becomes more easily oxidised, yielding the 160 kDa aggregate.

The binding of GSH to SjGST-Y7F was studied by means of fluorescence and ITC. Fig. 1 shows the decrease of tryptophan fluorescence as a function of substrate concentration at 25 °C and pH 6.5 in 20 mM sodium phosphate, 2 mM of DTT and 1 mM of EDTA. The data were fitted to a model of two equal and non-interacting sites. The good-fit of the experimental curve is evidence of the absence of co-operativity in the binding of GSH to SjGST-Y7F and yielded the K value of $5.2 \times 10^4 \pm 4 \times 10^3\ \mathrm{M}^{-1}$.

A typical ITC profile for the binding of GSH to SjGST-Y7F is shown in Fig. 2, which corresponds to the raw and integrated data for the titration of 20.39 μM SjGST-Y7F with 23 aliquots (10 μl each) of GSH (stock solution 1.32 mM) in 20 mM phosphate buffer, 2 mM of DTT and 1 mM of EDTA at pH 6.5 and 25 °C. The bottom

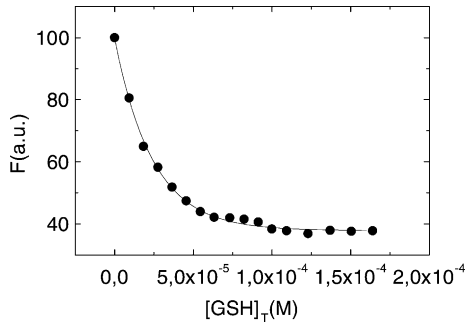

Fig. 1. GSH binding to SjGST-Y7F. Titration was performed in 20 mM Mes, 2 mM DTT, 1 mM EDTA pH 6.5 and 25 °C. Enzyme concentration was 0.18 μM and was titrated by addition of 3 μl of two solutions with concentrations of GSH 9.23 and 14 mM for pH 5 and 7.44 and 11.16 mM for pH 6.5. The continuous line is the theoretical curve generated using a non-cooperative model.

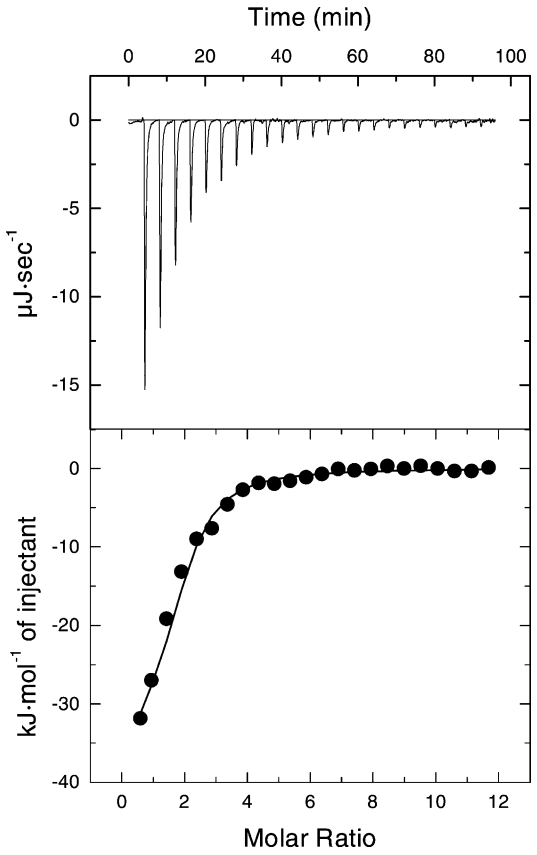

Fig. 2. Calorimetric titration of the binding of GSH to SjGST-Y7F in 20 mM phosphate, 2 mM DTT, 1 mM EDTA pH 6.5 and 30 °C. The experiment consisted of 23 injections of 10 μl each of a 1.32 mM stock solution of GSH. GSH was injected into a sample cell (volume = 1.38 ml) containing 20.39 μM of SjGST-Y7F. Injections were at 4-min intervals.

panel in this figure shows 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 a model of two equal and independent sites (one per monomer) with a microscopic association constant (K), and the standard enthalpy change (ΔH_{obs}) of $1.2 \times 10^5 \pm 6.4 \times 10^3\,M^{-1}$ and $-27.72 \pm 0.79\,kJ\,mol^{-1}$, respectively.

Standard enthalpy changes measured from a single ITC experiment include heat that is due to buffer ionization. This happens whenever binding is coupled to protonisation, as is often the case for biological macromolecules [18,19]. The enthalpy contribution due to the protonation–deprotonation of the buffer must be subtracted from the enthalpy change observed in order to obtain the binding enthalpy of the complex form. To determine the number of protons released or taken upon complex formation, ITC experiments were carried out using two different buffers at each pH. The data obtained were analysed using the following relationship:

$$\Delta H_{obs} = \Delta H_b + n\Delta H_{ioniz} \quad (5)$$

where n is the number of protons exchanged with the buffer media to stabilize the complex, ΔH_{ioniz} is the ionization enthalpy of the buffer, and ΔH_b is the binding enthalpy. At pH 6.5 and 25 °C the thermodynamical parameters ΔG°, ΔH_b, and ΔS° for the binding of GSH to SjGST-Y7F were $-28.2\,kJ\,mol^{-1}$, $-28.7\,kJ\,mol^{-1}$, and $1.7\,J\,K^{-1}\,mol^{-1}$, respectively. Therefore, binding at this pH and temperature is exothermically and entropically favoured. If we compare our results with those previously reported for the binding of GSH to wild type SjGST, it can be seen that enthalpy change is more negative for the binding of GSH to SjGST-Y7F than to wild type SjGST ($\Delta H_b = -23.8\,kJ\,mol^{-1}$), while ΔS° is higher [13]. Crystallographic studies of the complex GSH-SjGST [7] reveal that sulphur in GSH is found as thiolate and forms a hydrogen bond to Tyr^7 hydroxyl group. This hydrogen bond would not be present in the complex between GSH and SjGST-Y7F. Thus, one would expect a less negative enthalpy change for the GSH-SjGST-Y7F complex than for the GSH-SjGST complex. In this context, Connelly et al. [20] have studied the thermodynamics of the binding of tacrolimus and rapamycin (two macrocyclic compounds) to the protein FKBP-12 to probe the energetic contributions of protein–ligand hydrogen bonds formed in the binding reactions. They observed that on changing Tyr^{82} to Phe in FKBP-12 protein–ligand hydrogen bond interactions in the complexes with the two ligands were abolished, and this also led to a large apparent enthalpic stabilisation of the binding. Crystallographic data revealed that two water molecules bound to Tyr^{82} hydroxyl group, through hydrogen bonds, in wild type unliganded FKBP-12 were displaced upon formation of the protein–ligand complexes, and this would explain the less negative binding enthalpy for the wild type protein. Similarly, in unliganded SjGST a water molecule forms a hydrogen bond with Tyr^7 that is displaced upon the formation of the SjGST-GSH complex (Fig. 3). This water

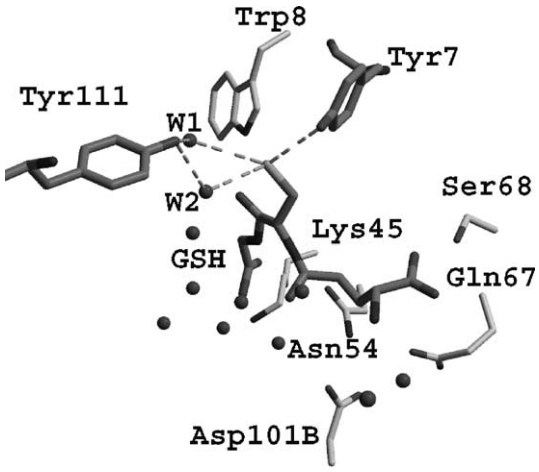

Fig. 3. GSH binding site. Hydrogen bond and salt bridge contacts of wild type SjGST (PDB code 1GNE [7]) at the GSH binding site. All amino acid at the binding site belongs to chain A except Asp101B, which belongs to chain B. The thiol group is forming a hydrogen bond with the Tyr^7 hydroxyl group. In the absence of GSH this position is occupied by one water molecule (PDB code 1GTA [6]). Two water molecules (W1 and W2) are at hydrogen bond distance from the thiol group and Tyr^{111}.

molecule would not be present in Y7F mutant. The balance between the number of hydrogen bonds before and after substrate binding would account for the more favourable enthalpy change of binding for the Y7F mutant. This more negative enthalpy change, together with the more favourable entropy change, is responsible of the higher negative value of ΔG_b and would explain the enhanced affinity of the substrate GSH to SjGST-Y7F at pH 6.5 and 25 °C.

The enthalpy of binding and the number of protons exchanged were also determined at different pHs ranging from 6.0 to 8.0 (Table 1). At pH lower than 7.5 the number of protons exchanged is negative, which means that a protein or ligand residue should decrease its pK_a value as a consequence of binding and release protons. At this point it is interesting to consider that a common property of all known classes of GST is the lowering of pK_a of the thiol group of bound GSH from 9.0 to a value between 6.0 and 6.9. Potentiometric measurements have revealed that the thiol proton of GSH is released quantitatively to the buffer upon binding at pH lower than 7 [21]. This thiol group could be responsible for the negative number of protons exchanged. At pH higher than 7.5 protons are taken up upon binding, which means that a protein or ligand residue increases its pK_a. Tyr^{111} in the active site of SjGST is related via a hydrogen bonding network to the thiol of GSH and to Tyr^7 [7] and could play a role in the deprotonation of GSH (Fig. 3). An increase in the pK_a of this Tyr residue in SjGST-Y7F upon binding would lead to a positive number of protons exchanged. It has been suggested that equivalent Tyr residues participate in the catalytic mechanisms in both the pi [22,23] and mu [24,25] enzymes.

ΔH_b calculated by Eq. (5) has contributions from the ionization heat of groups of the protein or the ligand, and in this sense it can be considered the sum of two terms: intrinsic enthalpy, which does not depend on the ionization of the groups, and a term which is pH dependent. At pH 7.5 the number of protons exchanged is approximately zero, and therefore the intrinsic binding enthalpy of GSH to SjGST-Y7F at 25 °C is $-31.9\,kJ\,mol^{-1}$ [26].

We have studied the effect of temperature on the thermodynamic parameters of the binding of GSH to SjGST-Y7F at pH 6.5 analysing the experiments performed in different buffers at temperatures ranging from 15 to 30 °C. Data obtained, corrected by the buffer ionization heat, are collected in Table 2. It can be seen that the values of ΔG° are practically independent of temperature, indicating the occurrence of enthalpy entropy compensation at this pH. This effect seems an almost inevitable property of perturbation of any system comprising multiple, weak intermolecular forces [27]. ΔH_b decreases linearly with temperature, and from the slope of the graphical representation of ΔH_b versus temperature a value of ΔC_p of $-1.28\,kJ\,K^{-1}\,mol^{-1}$ is obtained. Experimental work and theoretical considerations indicate that heat capacity changes involved in protein–ligand binding originate from changes in the degree of surface hydration in the free and complex molecules and to a lesser extent from changes in molecular vibrations [28–30]. In the association of a protein to a ligand a substantial fraction of polar and non-polar surface is buried and some semi-empirical methods have been developed to calculate ΔC_p from the molecular surface buried in the complex. Freire et al. have suggested the following equations for ΔC_p and ΔH_{60} (enthalpy change at 60 °C):

$$\Delta C_p = 1.88 \times \Delta ASA_{ap} - 1.09 \times \Delta ASA_p \quad (6)$$

$$\Delta H^\circ_{60} = -35.3 \times \Delta ASA_{ap} + 131 \times \Delta ASA_p \quad (7)$$

where ΔC_p, ΔH_{60} and ΔASA are in $J\,K^{-1}\,mol^{-1}$, $J\,mol^{-1}$ and $Å^2$ units, respectively [29,31]. ΔASA_{ap} and ΔASA_p represent the changes in non-polar and polar areas exposed to the solvent (accessible surface area) that take place upon protein–ligand binding. The temperature of 60 °C in the expression is the mean value of the denaturation temperatures of the model proteins used in the analysis. Using ΔH_{60} calculated assuming a constant ΔC_p and the experimental heat capacity change, the changes in accessible surface areas are $\Delta ASA_{ap} = -1191.46\,Å^2$ and $\Delta ASA_p = -881.37\,Å^2$. It can be seen that a reduction of solvent access to non-polar surfaces accompanies protein–ligand binding, as is expected for a negative ΔC_p [32]. If we compare our results with those obtained for wild type SjGST, a more negative value of ΔC_p is obtained for the mutant (-1.28 versus $-0.99\,kJ\,K^{-1}\,mol^{-1}$) at 25 °C and pH 6.5, which suggests a higher and significant hydrophobic component in the SjGST-Y7F-GSH interaction. One would expect the change of Tyr^7 to Phe at the active site to increase its hydrophobicity.

The entropy change was calculated from the ΔH_b, and the value of ΔG° was calculated from the microscopic binding constant at each temperature ($\Delta G^\circ = -RT\ln K = \Delta H_b - T\Delta S^\circ$). ΔS° values are positive at temperatures 289.25, 293.55 and 298.25 K and negative at a temperature 303.24 K. Therefore, binding is entropically and enthalpically driven in the temperature range of 289.25–298.25 K.

Our results indicate that changing Tyr^7 to Phe in SjGST results in an almost complete loss of activity towards CDNB substrate, but mutant protein has a higher affinity to GSH than wild type at pH 6.5 and 25 °C. Calorimetric studies at various pHs show that the pK_a values of two groups of protein or ligand are changed upon binding. The negative number of protons exchanged at pH below 7 agrees with the loss of a proton from the thiol group of GSH. The negative ΔC_p value suggests changes in hydrophobic and

Table 2
Thermodynamic parameters of GSH binding to SjGST-Y7F at pH 6.5

T (K)	ΔH ($kJ\,mol^{-1}$)	ΔG° ($kJ\,mol^{-1}$)	$T\Delta S^\circ$ ($kJ\,mol^{-1}$)
289.25	−17.18 ± 0.71	−28.05 ± 0.67	10.83 ± 0.67
293.55	−22.03 ± 0.50	−28.13 ± 0.75	6.10 ± 0.54
298.25	−28.72 ± 0.84	−28.22 ± 0.63	0.50 ± 0.16
303.24	−34.90 ± 0.54	−28.13 ± 0.71	−6.77 ± 0.54

hydrophilic areas buried upon GSH binding, with liberation of water molecules from both the protein and the ligand. The presence of the hydroxyl group of Tyr^7 in SjGST does not seem fundamental for the binding of GSH, but it would give the thiolate group the right orientation for the attack on the electrophilic residue in the second substrate [33]. This may explain the drastic decrease in the k_{cat} for this mutant ($1.4 \times 10^{-3} \pm 3.4 \times 10^{-4}$ and $1.9 \times 10^{-5} \pm 6.5 \times 10^{-6}$ for SjGST and SjGST-Y7F, respectively). Our results agree with those published by other authors for equivalent Tyr mutants [8,9,34].

4. Conclusions

The binding of GSH to SjGST-Y7F was studied by means of fluorescence and ITC. Mutation at the active site amino acid Tyr^7 results in an almost complete loss of activity towards CDNB substrate, but mutant protein shows a higher affinity to GSH than wild type at pH 6.5 and 25 °C. At the studied temperature range the binding is entropically and enthalpically driven. The more negative value of ΔC_p obtained for the mutant suggests a higher and significant hydrophobic contribution to the SjGST-Y7F-GSH interaction. At pHs lower than 7.5, the binding process is accompanied by protons release and at higher pHs protons are taken up. A change in the Tyr^{111} pK_a has been proposed to contribute to the positive number of protons exchanged at pHs higher than 7.5.

Acknowledgements

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