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A monomer form of the glutathione *S*-transferase Y7F mutant from *Schistosoma japonicum* at acidic pH☆

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Abstract

Dissociation and unfolding of homodimeric glutathione *S*-transferase Y7F mutant from *Schistosoma japonicum* (SjGST-Y7F) were investigated at equilibrium using urea as denaturant. The conserved residue Tyr7 plays a central role in the catalytic mechanism and the mutation Tyr–Phe yields an inactive enzyme that is able to bind the substrate GSH with a higher binding constant than the wild type enzyme. Mutant SjGST-Y7F is a dimer at pH 6 or higher and a stable monomer at pH 5 that binds GSH (K value of $1.2 \times 10^5 \pm 6.4 \times 10^3\ M^{-1}$ at pH 6.5 and $6.3 \times 10^4 \pm 1.25 \times 10^3\ M^{-1}$ at pH 5). The stability of the SjGST-Y7F mutant was studied by urea induced unfolding techniques ($\Delta G_W = 13.86 \pm 0.63$ kcal mol^{-1} at pH 6.5 and $\Delta G_W = 11.22 \pm 0.25$ kcal mol^{-1} at pH 5) and the monomeric form characterized by means of size exclusion chromatography, fluorescence, and electrophoretic techniques.

Keywords: Size exclusion chromatography; Fluorescence; Folding; Isothermal titration calorimetry

Glutathione *S*-transferases (GST, EC 2.5.1.18) are a family of multifunctional dimeric enzymes which are involved in the detoxification of harmful physiological and xenobiotic compounds. They have been classified into at least 13 different classes based on their amino acid sequence identities, immunological properties, and substrate specificities. 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%) [1]. Each GST monomer has two distinct binding sites: one called G site, which binds glutathione and is well conserved, and another that binds hydrophobic substrates called H site. Variability in the structure of the H site largely accounts for the wide range of substrate specificities of the GSTs.

Despite the high level of sequence similarity and identical fold topology, different GST isoenzymes give different folding pathways. Unfolding of GST in the presence of urea has been reported for many GSTs and different models have been proposed. Some of these models proposed a three-state mechanism involving the dimeric native state, stable but inactive monomers and unfolded monomers [2]. Alternatively other authors proposed a two state model, with a native folded dimer and an inactive unfolded monomer [3].

The sequence and structural homology of *Schistosoma japonicum* GST (SjGST) are more similar to those of the μ class, although it exhibits a mixture of α, μ, and π type biochemical properties [1]. SjGST crystal structure has been determined for the apoenzyme form [4] and for complexes with either the physiological substrate glutathione (GSH) [5] or the non-substrate ligands [6]. Tyr7 (or equivalent in other GST sequences) is one of the well-conserved residues at the G active site and it has been proposed to play a central role in the catalytic mechanism of GSTs by stabilizing the thiolate anion of glutathione and enhancing the nucleophilicity of the thiol anion versus the protonated thiol. Moreover, a role

☆ *Abbreviations:* SjGST, *Schistosoma japonicum* glutathione *S*-transferase; SjGST-Y7F, *Schistosoma japonicum* glutathione *S*-transferase tyrosine 7 to phenylalanine mutant; GSH, reduced glutathione; CDNB, 1-chloro-2,4-dinitrobenzene; SEC, size exclusion chromatography; ITC, isothermal titration calorimetry; ANS, 8-anilino-1-naphthalenesulphonate.

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for this amino acid at the interaction between G and H sites through a conformational change upon binding at the G site has been proposed [7]. In this study we report the changes in stability and aggregation state that result in Tyr7 to Phe mutation in order to investigate the role of this amino acid in the aggregation equilibrium and stability of the dimeric SjGST.

Experimental

Materials. 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). Site-directed mutagenesis, expression, and purification are described at Andujar et al. [8].

Protein concentration and enzyme assays. SjGST and SjGST-Y7F were measured spectrophotometrically at 278 nm using extinction coefficients 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 enzyme monomer, as calculated by the Gill–von Hippel method [9]. Enzyme activity towards 1-chloro-2,4-dinitrobenzene (CDNB) was assayed at 25 °C by the Habig–Jakoby method [10].

SDS–PAGE and cross-linking experiments. Electrophoresis in SDS–PAGE was conducted according to the Laemmli method [11], using a 4% (w/v) stacking gel at a constant current of 20 mA for 30 min and a 15% (w/v) separating gel at a constant current of 50 mA for 2 h. The gel was stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 in 40% (v/v) methanol with 7% (v/v) acetic acid for 1 h at room temperature and destained with 40% (v/v) methanol with 7% (v/v) acetic acid for 12 h. The molecular mass of purified protein was estimated by comparison with electrophoretic migration of standard molecular mass (LMW-SDS Marker Kit, Amersham Biosciences, Barcelona).

Glutaraldehyde cross-linking was used to evaluate the quaternary structural changes with the pH. Experiments were conducted as described elsewhere [12]: at 25 °C and pH 5.5 and 6.5 using 28 μM SjGST-Y7F in 20 mM acetate buffer, DTT 2 mM, EDTA 1 mM at pH 5 or 10 mM phosphate buffer, 50 mM NaCl, and 1 mM DDT at pH 6.5. Cross-linking was carried out adding 20 mM glutaraldehyde. The cross-linking reaction was stopped after 2 min by the addition of 0.1 M borate buffer (pH 8.9) and $NaBH_4$ to a final concentration of 0.25 M. After dialysis against 10 mM phosphate buffer, pH 6.5, the samples were diluted with an equal volume of SDS–PAGE sample buffer (62.5 mM Tris–HCl buffer, 0.75 M of 2-ME, 2% SDS, and 10% glycerol, pH 6.5), heated for 10 min at 100 °C, and then subjected to SDS–PAGE as described above.

SEC–HPLC. Size exclusion chromatography–HPLC (SEC–HPLC) analysis was performed in an HPLC System (Breeze HPLC System, Waters, Barcelona) using a Superdex 200 HR 10/30 column equilibrated with the same buffers used for ITC analysis. 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); ovalbumin (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).

Fluorescence. Fluorescence emission spectra were measured at 25 °C in a Perkin–Elmer LS55 spectrofluorimeter. The temperature of the cuvette holder was controlled using a circulating thermostated water bath. Intrinsic fluorescence of the protein was measured recording the emission spectra using an excitation wavelength of 280 or 295 nm to selectively excite tryptophans. Excitation bandwidth was 2.5 nm and emission bandwidth was 5 or 10 nm.

Unfolding studies with urea as denaturant were performed at pH 5 and 6.5. Protein concentration was 0.5–3.8 μM and the concentration range of denaturant agent was 0–8 M. In order to attain equilibrium, samples were left to stand for 24 h at 25 °C before being measured. Structural changes were monitored by tryptophan fluorescence. The profiles of fluorescence intensity versus denaturant concentration were analysed according to a two-state denaturation model proposed by Ibarra-Molero and Sanchez-Ruiz [13]. The denaturation Gibbs energy in water (ΔG_W) was calculated from the $C_{1/2}$ and $m_{1/2}$ values using the equation: $\Delta G_W = m_{1/2}C_{1/2}$, for the monomeric form or the equation, or $\Delta G_W = m_{1/2}C_{1/2} + RT\ln[\text{SjGST}]$, for the dimeric form [14].

ANS binding to the enzyme was used to probe structural changes in the enzyme conducted at different pH between 4.5 and 6.5. Unbound ANS emission spectra show a maximum at 530 nm that is blue shifted upon binding of the dye to the protein. Different protein concentrations were used (1.4–10 μM). The binding of ANS was monitored by excitation at 380 nm and emission was measured at the maximum wavelength at each pH (495–480) nm.

Isothermal titration calorimetry. Isothermal titration calorimetry (ITC) experiments were conducted at 25 °C as described at Andujar et al. [8]. 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. The titration was performed in 2 mM DTT, 1 mM EDTA, and 20 mM acetate or 20 mM Mes at pH 5, 25 °C. The enzyme concentration was 47.22–50.38 μM and was titrated by addition of 10 μl of two solutions with concentrations of GSH 1.77 and 1.89 mM for pH 5.

Results

Effect of pH on the aggregation state of SjGST-Y7F

SEC–HPLC experiments have shown that SjGST-Y7F is a monomer at pHs between 5 and 5.5 and a dimer in the pH range 6–7.5 (Fig. 1). Cross-linking

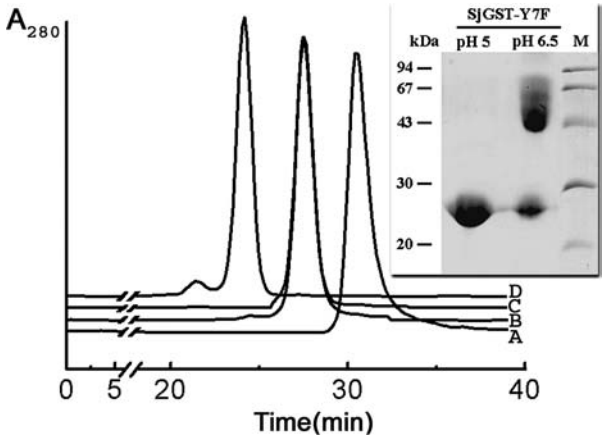

Fig. 1. Size exclusion chromatography–HPLC of SjGST and SjGST-Y7F. Size exclusion chromatography–HPLC was performed with 40 μM of each protein, while absorbance was monitored at 280 nm. All experiments were carried out at room temperature and a flow rate of 0.5 ml min^{-1}. (A) SjGST-Y7F at pH 5; (B) SjGST-Y7F at pH 6.5; (C) SjGST at pH 6.5; and (D) SjGST-Y7F at pH 8. Inset: SDS–PAGE of SjGST and SjGST-Y7F at pH 5 and pH 6.5 cross-linked by glutaraldehyde. Cross-linked proteins were separated by SDS–PAGE and stained with Coomassie brilliant blue.

experiments support these results (Fig. 1). The aggregation state does not change under GSH addition at saturating conditions. The same results were obtained from cross-linking experiments conducted at pH 5 and 6.5, as is shown in Fig. 1. SEC–HPLC experiments at pH above 8 result in shorter elution times that correlate with the presence of aggregates in SjGST-Y7F as observed for SjGST by other authors [15].

Urea unfolding SjGST-Y7F

In order to study the structural changes in SjGST-Y7F after addition of urea, fluorescence experiments were performed. Each subunit of SjGST has four tryptophan residues at positions 8, 41, 201, and 206. Trp8 is placed next to Tyr7 that is implied at the substrate binding at the active site of the enzyme. This makes the tryptophan fluorescence a very sensitive probe to monitor conformational changes at the active site. The SjGST-Y7F lacks the water molecule ligated by hydrogen bond to the tyrosine hydroxyl group. This makes the G site more hydrophobic and the maximum emission wavelength shows a blue shift from 342 to 338.5 nm as compared to the wild type SjGST.

Whole denatured GST shows maximum emission wavelength red shift and an increase of about 44% of protein fluorescence intensity. At pH 6.5 the midpoint of the unfolding transition is shifted to higher concentration of urea as protein concentration is increased and $\Delta G_W = 13.86 \pm 0.63$ kcal mol^{-1} and $m_{1/2} = 6.05 \pm 0.80$ mol^{-1}/mol of urea (by monomer of enzyme). At pH 5, the urea unfolding experiments conducted with SjGST-Y7F do not show protein concentration dependence in $C_{1/2}$ (Fig. 2). ΔG_W and $m_{1/2}$ values at pH 5 are shown in Table 1.

Binding of the dye ANS to SjGST-Y7F shows a blue shift of emission maximum from 500–495 to 480 nm (Fig. 3). At 6 or higher the emission maximum was 490 nm, meanwhile at pH 5 the maximum of emission was 480 nm at all the range of concentrations studied.

Binding of GSH to the monomeric SjGST-Y7F

As aggregation state of SjGST-Y7F depends on the pH, GSH binding to SjGST-Y7F was studied at different pH by means of ITC and fluorescence techniques [8]. Results from ITC experiments at pH 5 do not fit well a two non-interacting binding site model as corresponding to the SjGST dimer. Fitting of the experimental results to a one-site model is summarized in Table 2 and Fig. 4. The best fit of the experimental data to a model of one site gives a microscopic association constant K of $6.33 \times 10^4 \pm 1.25 \times 10^3$ M^{-1}. K values obtained by fluorescence experiments were equal within experimental error to those obtained by ITC experiments (results not shown).

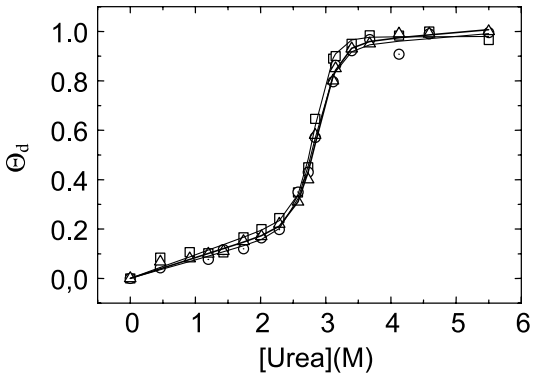

Fig. 2. Urea unfolding curves for SjGST-Y7F. Fraction unfolded (Θ_d) was calculated at different urea concentrations in 20 mM acetate, 2 mM DTT, and 1 mM EDTA, pH 5, 25 °C. The concentrations of SjGST-Y7F were 0.52 μM (■), 1.5 μM (○), and 3.8 μM (△).

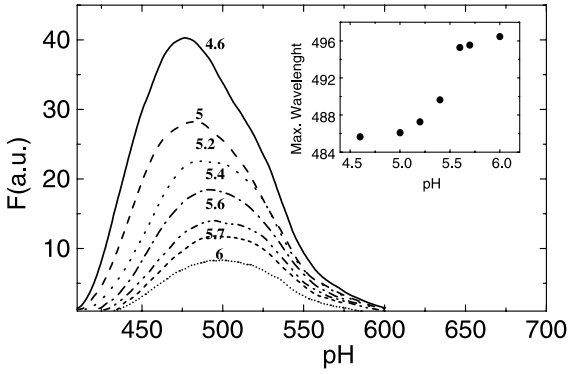

Fig. 3. ANS binding to SjGST-Y7F. Emission fluorescence spectra of ANS were monitored by excitation at 380 nm by addition of 200 μM ANS at each sample of SjGST-Y7F at 1.4 μM in acetate 20 mM, 2 mM DTT, and 1 mM EDTA equilibrated at pH between 6 and 4.6, maximun displacement as a function of the pH is represented at the inset.

Table 1
Thermodynamics parameters of SJGST-Y7F unfolding in urea (per monomer mol)

[SjGST-Y7F] (μM)	pH 5		
	$m_{1/2}$ (kcal mol^{-1} M^{-1})	$C_{1/2}$ (M)	ΔG_W (kcal mol^{-1})
0.52	3.80 ± 0.30	2.89 ± 0.04	10.95 ± 0.01
1.50	3.80 ± 0.40	2.92 ± 0.03	11.20 ± 0.01
3.80	3.90 ± 0.60	2.99 ± 0.03	11.50 ± 0.02

Table 2
GSH binding to SjGST-Y7F at pH 5

Buffer	pH	ΔH_{ion} (kcal mol^{-1})	ΔH_{tot} (kcal mol^{-1})	ΔH_b (kcal mol^{-1})	n
Acetate	5	0.12	−7.86	−7.90	0.53
Mes	5	3.72	−5.93	−7.90	0.53

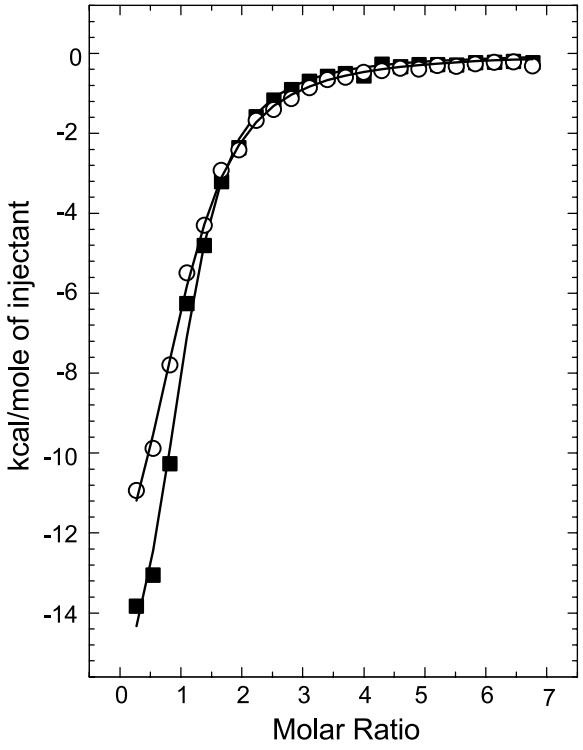

Fig. 4. GSH binding to SjGST-Y7F. The titration was performed in 2 mM DTT, 1 mM EDTA, 20 mM acetate (■), and 20 mM Mes (○), pH 5, 25 °C. The enzyme concentration was 50.38 and 47.22 μM, respectively, and was titrated by addition of 10 μl injections of two GSH at 1.89 (■) and 1.77 mM (○). The continuous line is the theoretical curve generated using a non-cooperative model fitted to a single site.

Discussion

Unfolding studies at pH 5 and 6.5 of SjGST-Y7F with urea as denaturant agent showed a monophasic unfolding curve. At pH 6.5 the midpoint of the unfolding transition is shifted to higher urea concentration as protein concentration is increased. It has been described to be characteristic of dimeric enzymes. The ΔG_W values obtained by monomer of enzyme are in accordance with those reported by Kaplan et al. [15] for the wild type dimer in urea at pH 6.5 and Yassin et al. [16] for the wild type and Y7F mutant dimer. No $C_{1/2}$ dependence was found at pH 5 and lower $C_{1/2}$ and ΔG_W values were obtained in accordance with a lower stability as can been expected from a monomeric protein [14]. Moreover, SEC and cross-linking experiments support a stable monomer at acidic pH. The monomer is able to bind GSH with lower affinity than the dimer without change in the aggregation state as it was confirmed by SEC in the presence of 2 mM GSH.

ANS bound to SjGST-Y7F shows a blue shift displacement with an enhancement of fluorescence intensity as the pH changes from 6 to 4.6. Binding of the dye occurs at the dimer interface and unfolded GST does not bind ANS. This makes ANS an excellent probe to monitor changes at the packing of hydrophobic cores in protein which undergoes structural changes and has been broadly used to study the presence of monomeric intermediates at the urea/GdmCl unfolding of several GSTs. The monomeric intermediate shows a loser packing displaying enhanced binding of ANS and a blue shift of emission maximum from 500–495 to 480 nm [17]. Our results agree with those reported in the literature to demonstrate the existence of a stable monomer intermediate at the unfolding of σ and μ class GSTs (2,17-18).

Equilibrium folding experiments conducted with mutants of the μ class GSTs where phenylalanine 56 was substituted by several polar amino acids showed the presence of monomeric intermediates that were catalytically inactive [2]. Phe56 is one of the major hydrophobic contacts from domain I (terminal region) of one subunit into a hydrophobic pocket in domain II (carboxyl terminal region) of the second subunit. This interaction has been described as a "lock-and-key motif" that is also present at SjGST, where the phenylalanine residue is the Phe52. This aromatic residue is placed next to a Glu51 that is forming a salt bridge with Arg136 (2.88 Å distance) from the other subunit. The role of this salt bridge in the dimer stability at SjGST-Y7F has not been studied but results from Hornby et al. [2] with class μ glutathione transferases show that the mutation of the Phe56 to Arg result in a mutant with a high fraction of folded monomer in solution in equilibrium with the dimer. Meanwhile, Phe56 to Glu mutation yields the only mutant that shows a constant fraction of dimer, consistent with a more stable dimer mutant. Glu51 at SjGST protonation at acidic pH can disrupt the salt bridge between Glu51 and Arg136 promoting dissociation of the dimer.

SjGST-Y7F shows similar properties to tyrosine to phenylalanine mutants in other GTSs class. However, it is the first time that a stable monomeric form of SjGST has been described at acidic pH. Moreover, ITC experiments at pH 5 show the ability of the monomeric SjGST-Y7F to bind GSH. These results agree with those of Hornby et al. [2] that showed the presence of a monomeric GST F56R mutant at 2 mM glutathione

sulphate concentration in SEC experiments at low protein concentrations.

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