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Calorimetric Studies of Ligands Binding to Glutathione *S*-Transferase from the Malarial Parasite *Plasmodium falciparum*

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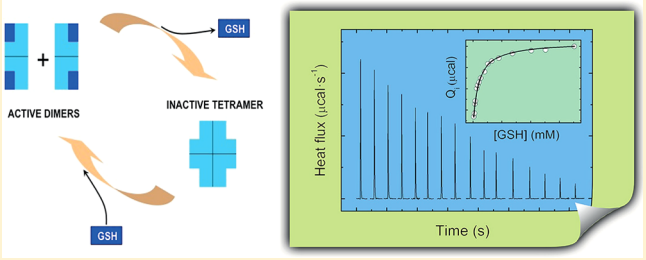
ABSTRACT: Glutathione *S*-transferase, from the malarial parasite *Plasmodium falciparum* (PfGST), exerts a protective role in the organism and is thus considered an interesting target for antimalarial drug development. In contrast to other GSTs, it is present in solution as a tetramer and a dimer in equilibrium, which is induced by glutathione (GSH). These properties prevent a calorimetric titration from being conducted upon binding of ligands to this protein's G-site. Thermodynamic characterization can be an optimal strategy for antimalarial drug development, and isothermal titration calorimetry (ITC) is the only technique that allows the separation of the binding energy into both enthalpic and entropic contributions. This information facilitates an understanding of the changes in the drugs' substituents, improving their affinity and specificity. In this study, we have applied a nontypical ITC procedure, based on the dissociation of the ligand−protein complex, to calorimetrically study the binding of the GSH substrate, and the glutathione sulfonate competitive inhibitor, to dimeric PfGST over a temperature range of 15−37 °C. The optimal experimental conditions for applying this procedure have been optimized by studying the dimer to tetramer conversion using size exclusion chromatography. The binding of these ligands to dimeric PfGST is noncooperative, the affinity of glutathione sulfonate being approximately 2 orders of magnitude higher than that of its natural substrate GSH. The binding of both ligands is enthalpically favorable and entropically unfavorable at all the studied temperatures. These results demonstrate that, although PfGST presents differences when compared to other known GSTs, these ligands bind to its dimeric form with a similar affinity and energetic balance. However, in contrast to that of other GSTs, the binding of GSH to protein, in the absence of the ligand, is slow.



Glutathione *S*-transferases (GSTs, EC 2.5.1.18) make up a family of multifunctional enzymes that catalyze the conjugation of a number of electrophiles with glutathione (GSH). These enzymes are involved in essential biological processes, including cellular metabolism, detoxification, and excretion of a variety of endogenous and exogenous xenobiotic substances.[1,2] Cytosolic isoenzymes are dimeric, grouped into at least 10 gene-independent classes, named alpha, beta, delta, kappa, pi, mu, theta, zeta, omega, and sigma, on the basis of the different amino acid sequences and substrate specificities.[3−6] Each subunit contains a very similar GSH binding site (the G-site) and a second binding site for the hydrophobic cosubstrate (the H-site) with structural differences in the latter that confer some substrate selectivity.[6] Protozoan and metazoan parasite GSTs are of particular interest because these enzymes may be involved in the intracellular detoxification of numerous endogenous and exogenous cytotoxic substances, including chemotherapeutic agents, and thus may play a major role in the development of drug resistance.[7,8] The protozoan parasite *Plasmodium* is responsible for malaria, one of the most important infectious diseases worldwide, affecting and causing the death of several million people annually.[9] One of the reasons for this devastating situation is the emergence of drug resistance to currently affordable antimalarial drugs. GST activity has been identified in the intraerythrocytic stages of the *Plasmodium* species.[10−13] Consequently, great interest is now being focused on the GST from the *Plasmodium falciparum* (PfGST) parasite. This parasite possesses only one GST isoenzyme, which is highly abundant[12,13] and represents a novel and peculiar GST isoenzyme class.[3−7,14] The available data indicate that homodimeric PfGST cannot be assigned to any of the previously known GSTs classes. This isoenzyme is present in solution both as a tetramer and as a dimer,[15,16] with only the dimeric form being enzymatically active. The tetramer−dimer conversion is induced by GSH and is a reversible process.[15,16] Thus, in the absence of GSH, the enzyme is found mainly as a tetramer. The main structural differences, between the determined X-ray crystal structures of PfGST[14,17,18] and other GST structures, seem to be in a particular, extra loop connecting helix α-4 and helix α-5 (residues 113−120) and located in the lower part of the so-called hydrophobic binding pocket (H-site). In this regard, some authors have recently examined the influence of this atypical loop in the dimerization

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process.[16] None of these particular PfGST properties have ever been observed in other members of the GST superfamily. The interest in this enzyme resides in the particular protective role it plays in the parasite against oxidative stress, as well as buffering the detoxification of heme-binding compounds in vivo.[8,12,19] Therefore, this enzyme is considered to be a highly promising target for the development and trials of antimalarial drugs. In this regard, the differences between parasite GSTs and their hosts (such as human GSTs) should be exploited to design PfGST-based specific therapies to prevent potential side effects in human GST activity.

Ligand or drug design involves the optimization of the binding affinity of a lead compound toward a selected target. However, the binding affinity is determined by the Gibbs energy (ΔG), and ΔG is equal to $\Delta H - T\Delta S$. Thus, a good drug−target binding optimization requires knowledge of both the binding thermodynamic data and the favorable and unfavorable contributions. In this regard, isothermal titration calorimetry (ITC) is the only technique that allows one to obtain all of the information mentioned above. Such experimental thermodynamic information can be necessary both in designing computational drug screening methods and in avoiding important side effect consequences (e.g., drug resistance). However, the existence of this atypical change in the aggregation state (dimer:tetramer) is GSH-induced and presumably induced by other ligands with an affinity for the G-site (similar to GSH), preventing complete thermodynamic characterization of binding of the ligand to PfGST. In this scenario, the binding of ligands to the G-site (such as its natural substrate, GSH) of the tetramer PfGST (nonligated state) will be accompanied by different concomitant processes (dimerization and binding), whose individual contribution to global heat is not possible to isolate.

In this work, ITC was used to study the thermodynamics of ligands that bind to the PfGST dimer. As far as we know, no calorimetric study has been performed on this GST isoform. Moreover, here we describe a novel calorimetric strategy for characterizing binding of the ligand, which binds to the G-site of dimeric PfGST. Using this calorimetric procedure, we have studied and characterized the GSH binding and interaction of the competitive inhibitor glutathione sulfonate (GSO_3^-) with this particular GST. The procedure was also checked using the human GST isoenzyme (GST P1-1) and GSO_3^- as a model. The results obtained can contribute to a better understanding of this GST isoform, which can help us to find the most efficient antimalarial drug design.

MATERIALS AND METHODS

Chemicals. GSH, 1-chloro-2,4-dinitrobenzene (CDNB), and GSO_3^- were purchased from Sigma-Aldrich. Centriprep 30 concentrators were from Amicon. Other chemicals were of analytical grade of the highest available purity. All solutions were prepared with distilled and deionized (Milli Q) water.

Protein Expression and Purification. The protein sequence for the wild-type PfGST 3D7 isolate (XP_001348360.1) was used to generate a codon-optimized synthetic gene (GenArt, Invitrogen) for *Escherichia coli* expression. The coding sequence was cloned into prokaryotic expression vector pQE60 (QIAGEN), and the resulting plasmid was then used to transform *E. coli* JM109 cells. A single *E. coli* colony was inoculated in LB culture medium containing 100 μg/mL ampicillin and further incubated for 12−15 h at 37 °C with 100−150 rpm shaking. The overnight culture was diluted 100 times in fresh LB medium with 100 μg/mL ampicillin and grown at 37 °C until the absorbance at 600 nm reached ∼0.6. At this point, isopropyl thio-β-D-galactoside was added to a final concentration of 0.5 mM to induce PfGST expression. After induction for 12 h at 37 °C, cells were centrifuged at 5300*g* for 20 min at 4 °C. The cellular pellet was resuspended in 10 mM sodium phosphate buffer (pH 7.0) containing 10 mM DTT, and lysis was conducted by sonication. The homogenate was centrifuged at 25400*g* for 35 min at 4 °C, and the supernatant was applied directly to a glutathione affinity column. After the PfGST protein had bound to the matrix, it was washed with 100 mM sodium phosphate and 1 mM DTT buffer (pH 7.0) to remove nonspecifically bound proteins. Recombinant PfGST was eluted with 15 mM GSH in 50 mM Tris-HCl and 1 mM DTT (pH 8.0). For storage at −80 °C, the protein was dialyzed against 10 mM GSH in 100 mM sodium phosphate and 0.1 mM EDTA buffer (pH 7.0). Under these conditions, the enzyme was stable for a few weeks. After affinity purification, the enzyme was nearly homogeneous as judged by sodium dodecyl sulfate−polyacrylamide gel electrophoresis. Protein concentrations were determined from absorbance measurements at 280 nm using an extinction coefficient of 5.25×10^4 M^{-1} cm^{-1} for the dimer. The extinction coefficient was calculated from sequence by the method of Gill and von Hippel.[20]

Enzymatic Activity. PfGST activity, using 1 mM GSH and 1 mM CDNB as substrates, was assayed spectrophotometrically on the basis of the extinction coefficient for the enzymatic product (ε = 9600 M^{-1} cm^{-1}) at 340 nm and 25 °C. The reaction mixture was prepared in 0.1 M potassium phosphate buffer (pH 6.5) containing 1.5 μM PfGST and 1 mM GSH. The reaction was initiated by the addition of 1 mM CDNB. One unit of GST activity was defined as the conjugation of 1 μmol of CDNB with GSH per minute at 25 °C.[19] The PfGST samples were routinely assayed both after enzyme purification and as a prerequisite for calorimetric and chromatographic assays. The activity of the samples used was always in the range of 0.7−1.

Size Exclusion Chromatography (SEC). The oligomeric status of PfGST under different experimental conditions was measured by gel filtration chromatography experiments on a Superdex 200 10/300 GL column on an ÄKTA basic fast-performance liquid chromatography (FPLC) system (GE Healthcare Biosciences). Frequently, the column was equilibrated and run with 50 mM sodium phosphate and 10 mM NaCl buffer (pH 7.0) with a flow rate of 0.7 mL/min at 25 °C. For GSH-treated protein samples, the column was equilibrated and run with the buffer described above containing the desired concentrations of GSH. A set of proteins with known molecular masses was used for column calibration: glucose oxidase (160 kDa), serum albumin (67 kDa), ovalbumin (43 kDa), and cytochrome *c* (12 kDa).

Isothermal Titration Calorimetry (ITC). Isothermal titration calorimetry (ITC) experiments were routinely performed in 50 mM sodium phosphate and 10 mM NaCl buffer at pH 7.0 (buffer A). Phosphate buffer was chosen by virtue of its small ionization enthalpy change; hence, the binding enthalpies reported do not reflect the possible contribution due to buffer protonation. ITC measurements were conducted using either a MCS[21] or an ultrasensitive VP-ITC titration microcalorimeter (Microcal Inc., Northampton, MA). The sample preparation and ITC experiments were

conducted as previously described.[22] Raw data for the protein and ligand were collected; the ligand heats of dilution were corrected for, and the data were integrated using Microcal Origin supplied with the instrument. The errors are provided by software from the best fit of the experimental data to the model used and correspond to the standard deviation of the curve fitting. A model of equal and independent sites (noncooperative) was used to fit the experimental data. The data analysis produced three parameters, viz., stoichiometry (n), association constant (K), and standard enthalpy change (ΔH_b), for the binding of the ligand to the protein. Prior analysis of our calorimetric data provided a stoichiometry close to 2 (n = 2). Moreover, this value (two G-sites per dimer) is known.[14,18] Thus, to obtain accurate K and ΔH_b values, we have fixed the stoichiometry (two equal and independent sites for the dimer) in the fitting procedure. Finally, changes in standard free energy $\Delta G°$ and $\Delta S°$ were determined with the equations $\Delta G° = -RT \ln K$ and $T\Delta S° = \Delta H_b - \Delta G°$ (assuming that $\Delta H_b = \Delta H°$), respectively.

RESULTS

Calorimetric Titration of PfGST with GSH. The calorimetric titration of tetrameric PfGST with the GSH substrate at 25 °C is shown in Figure 1. Figure 1A shows an ITC thermogram for a sequential titration of 40 μM PfGST (tetramer) with 5 μL aliquots of GSH (stock concentration of 7 mM) in 50 mM phosphate buffer and 10 mM NaCl (pH 7) (buffer A) at 25 °C. The GSH injections were 5 μL spaced at 8 min intervals. Control experiments involved the same number of 5 μL injections of the substrate solution into the same buffer but with no protein present. Control injections represented the heat of dilution of GSH. As can be observed from Figure 1A, an atypical thermogram was obtained. Briefly, in a typical calorimetric thermogram, the baseline is established at a constant level (the initial baseline) and during the titration the heat generated (or absorbed) in the sample cell for each ligand injection is continuously compensated by the feedback power that nullifies the difference in temperature between sample and reference cells. Thus, following ligand injection, the initial baseline power level is always reached a few minutes after each injection. However, in Figure 1A, the addition of ligand in each injection causes a series of sharp exothermic peaks followed by a slow change in the thermal power reaching a hypothetical baseline with a lower power level. The drop in the baseline indicates that certain particular reactions, with different rates and a negative (exothermic) enthalpy change, are occurring as a consequence of the GSH injection. Moreover, the thermal power generated by the PfGST−GSH interaction decreases with an increase in the number of GSH injections. Furthermore, the differences between the baseline levels of successive injections seem to become smaller as the titration progresses. Similar behavior was also found in enzyme-catalyzed reactions studied when using the ITC multiple-injection method.[23−25] However, in our case, the initial exothermic peak after each injection was larger than the corresponding peak of the control experiment. Consequently, these peaks must have other contributions besides the heat of dilution of GSH. Therefore, the thermogram shown in Figure 1A is indicative of a complex binding reaction with concomitant multiple effects, which seem to be occurring as a consequence of GSH addition, and as a result, any data analysis will include different processes whose separation, into individual contributions, is not feasible. Figure 1B corresponds to a single injection (20 μL) of a great excess of GSH (75 mM) into the calorimetric cell containing a tetrameric enzyme solution similar to that indicated in Figure 1A, at 25 °C. The calorimetric trace shows an initially fast and globally exothermic phase followed by a second phase, also exothermic, corresponding to slow processes.

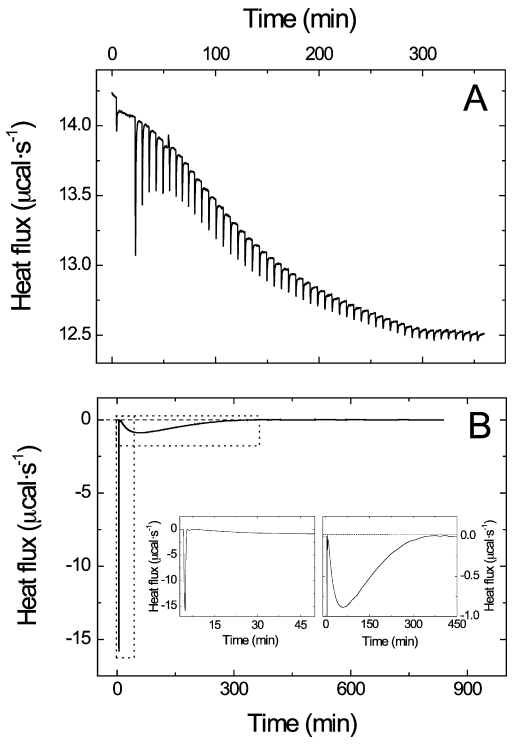

Figure 1. Isothermal titration calorimetry measurements of binding of GSH to tetrameric PfGST at 25 °C. (A) ITC thermogram for the sequential titration of 40 μM PfGST (tetramer) with 5 μL aliquots of GSH (stock concentration of 7 mM) in 50 mM sodium phosphate and 10 mM NaCl (pH 7) (buffer A). (B) Single injection (20 μL) of a great excess of GSH (75 mM) into the calorimetric cell containing a tetrameric enzyme solution similar to that indicated in panel A. The area marked has been enlarged for easy viewing (insets).

The peculiar properties of this GST have been reported compared to those for other known GSTs. In the absence of GSH, this isoenzyme is present mainly as a tetramer. However, in the presence of GSH concentrations of ≥1 mM, the enzyme is a dimer.[15,16] The quaternary structure of PfGST was analyzed by SE-FPLC, before and after the calorimetric experiment. On the Superdex 200 column, the protein showed two peaks in the absence of GSH: one tall peak with a retention time of 19.6 min, which corresponds to the tetramer (80% population), and a short peak with a retention time of 21.7 min, which corresponds to the dimer (20% population). After GSH had been added to the PfGST in the calorimeter experiment (Figure 1A,B), the sample was withdrawn from the calorimetric cell and injected (200 μL) onto the Superdex 200 column. The chromatogram showed a peak corresponding to the dimer. Because the dissociation of the PfGST tetramer, induced by GSH, seems to be a slow process,[16,19] we studied the effect on the oligomeric state of the protein produced by the GSH substrate, as a function of the time, to examine the moment at which the dissociation process occurred in the calorimetric experiment. Figure 2 shows the SEC profiles of the PfGST tetramer after incubation with 700 μM GSH for 0, 30, 60, 150,

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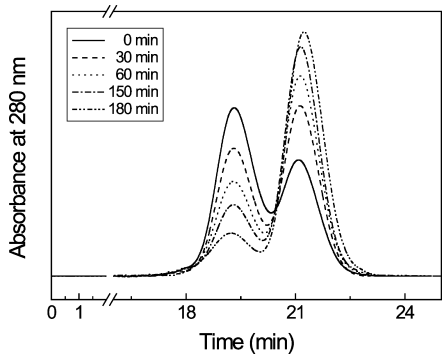

Figure 2. Size exclusion chromatography profiles of a PfGST tetramer after incubation with 700 μM GSH for 0 (—), 30 (---), 60 (···), 150 (—·—), and 180 min (—···—) at 25 °C. The samples were eluted in 50 mM sodium phosphate and 10 mM NaCl (pH 7) (buffer A) containing 700 μM GSH.

and 180 min at 25 °C. A similar experiment was conducted via incubation of the tetramer with 200 μM instead of 700 μM GSH. In both cases, two populations of the protein were observed, one corresponding to the tetramer and the other to the dimer. As the incubation time increased, there was an enhancement of the area under the peak corresponding to the dimer; along with a concomitant decrease in that corresponding to the tetramer. After 360 min, no change was observed. At this point, all the protein was practically dimeric at 700 μM GSH, while at 200 μM GSH, both the dimeric and tetrameric enzyme forms were present (data not shown). These SE-FPLC results help to explain the atypical thermograms obtained before. In this way, the rapid exothermic process can include processes such as the heat of dilution of GSH, contributions of GSH binding heat to tetrameric PfGST, and the small fraction of dimeric PfGST in equilibrium with the tetrameric form. Moreover, the slow process can include the heat of dissociation of the PfGST tetramer into two dimers and the binding heat of a possible change in enzyme saturation by GSH. Because the separation of global heat into different contributions is not feasible, we have proposed an alternative procedure to obtain the thermodynamic parameters characterizing binding of GSH to PfGST.

Calorimetry of Dissociation of the Protein–Ligand Complex. It is widely known that human GST P1-1 is a dimer in the absence and presence of ligands.[6,26] Figure 3A (top panel) shows a typical calorimetric titration of 45 μM GST P1-1 with 2.3 mM GSO_3^- in buffer A (pH 7) at 25 °C. At 5 min intervals, 5 μL of the GSO_3^- solution was injected into the calorimetric cell, giving rise to the characteristic titration effects. The control experiment, involving the same number (50) of ligand solution injections into the same buffer (but with no protein present), was taken as the GSO_3^- dilution. The area under each peak represents the heat for each ligand injection. The bottom panel of Figure 3A shows the plot of the integrated heat after subtraction of the small amount of heat from the control experiment and division by the number of moles of ligand injected versus the molar ratio of GSO_3^- to the protein. The smooth solid line represents the best experimental data fit to two equal and independent sites with a microscopic binding constant (K) and a standard enthalpy change (ΔH_b) of (4.6 ± 0.4) × 10^5 M^{-1} and −15.2 ± 0.4 kcal/mol, respectively (Table 1).

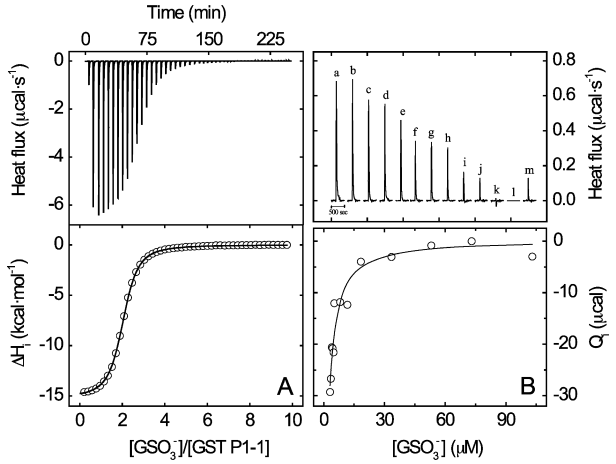

Figure 3. ITC measurements of the interaction of GST P1-1 and GSO_3^- in buffer A (pH 7) at 25 °C. (A) Representative isothermal titration calorimetry measurements of the binding of 2.3 mM GSO_3^- (5 μL injections) to 45 μM GST P1-1 (top). (B) The top panel shows the traces of the calorimetric titration corresponding to the dissociation process of the GST P1-1–GSO_3^- complex. Each calorimetric peak corresponds to an independent experiment in which an aliquot of 10 μL of 200 μM GST P1-1 in buffer A (pH 7) with 0.44 mM GSO_3^- was injected into buffer A, or into different GSO_3^- concentrations in the same buffer [(a) 0, (b) 0.43, (c) 0.86, (d) 1.30, (e) 2.17, (f) 3.47, (g) 5, (h) 8.60, (i) 15.20, (j) 30.40, (k) 50, (l) 70, and (m) 100 μM].

Table 1. Thermodynamic Parameters of the Interaction of GSO_3^- with Human GST P1-1 at pH 7.0 and 25 °C

ITC method	K ($\times 10^{-5}$ M^{-1})	$\Delta G°$ (kcal/mol)	ΔH_b (kcal/mol)	$T\Delta S°$ (kcal/mol)
classical	4.6 ± 0.4	−7.7 ± 0.1	−15.2 ± 0.4	−7.7 ± 0.4
dissociation	5.0 ± 0.9	−7.7 ± 0.1	−15.6 ± 1.3	−7.9 ± 1.3

We have chosen the GST P1-1–GSO_3^- complex to optimize a calorimetric assay for determining the dissociation of a protein–ligand complex. Figure 3B (top panel) shows the traces of the calorimetric titration corresponding to the dissociation process of the GST P1-1–GSO_3^- complex. Each trace (individual calorimetric peak) corresponds to an independent experiment in which an aliquot of 10 μL of 200 μM GST P1-1 in buffer A (pH 7), with 0.44 mM GSO_3^-, was injected either into buffer A or into different GSO_3^- concentrations in the same buffer. Independent control experiments involved 10 μL injections of a 0.44 mM GSO_3^- solution (with no protein present) into the same solution. Control injections represent the heat of dilution of GSO_3^-. The heat of dilution of the ligand–protein complex was measured in an independent experiment, in which an aliquot of 10 μL of 200 μM GST P1-1 in buffer A, with 0.44 mM GSO_3^- (pH 7), was injected into 0.44 mM GSO_3^- in buffer A.

The top panel of Figure 3B shows the raw calorimetric data, denoting the amount of heat produced following the injection of the protein–ligand complex. The positive sign of the measured heat indicates that the enthalpy change was positive. The area under each peak is the heat for that injection. The integrated heats are positive, after subtraction of the heat of dilution of the protein and its corresponding small amount of heat of dilution of the ligand. Thus, the process of protein–ligand complex dissociation under these conditions was endothermic. The bottom panel of Figure 3B shows the plot

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of corrected heat (Q_i) as a function of total ligand concentration in the calorimetric cell. These heats (Q_i) are proportional to the amount of protein−ligand complex dissociated in a particular injection ($Vn[P]_dY$) and the characteristic dissociation enthalpy change (ΔH_d) for the reaction:

$$Q_i = Vn[P]_d \Delta H_d \times \Delta Y \quad (1)$$

where $[P]_d$ is the dimeric protein concentration in the calorimetric cell, n the number of sites in the protein for the ligand (in this case, $n = 2$), V the reaction volume, and ΔY the change in the fraction of protein occupied by the ligand. This equation is equally valid both for dissociation and for binding. Thus, to directly obtain the binding enthalpy change (ΔH_b), Q_i should be expressed with a sign opposite to that obtained for the dissociation heat (in this case, a negative sign).

The quantity ΔY represents the difference in the saturation fraction of the protein before and after the injection, and its functional form depends on the specific binding model. In the case of a dimeric protein with two equal and independent sites, eq 1 becomes

$$Q_i = Vn[P]_d \Delta H_b \left(\frac{K[L]_2}{1 + K[L]_2} - \frac{K[L]_1}{1 + K[L]_1} \right) \quad (2)$$

where K is the characteristic microscopic binding constant and $[L]_1$ and $[L]_2$ are the concentrations of the free ligand in the solution of the protein−ligand complex prior to injection into the calorimeter cell and those present after the injection, respectively. These concentrations must be calculated from the corresponding total ligand concentration by the mass conservation law for the ligand.[27] The thermodynamic binding parameters (ΔH_b and K) were estimated from nonlinear least-squares regression analysis of the experimental data to eq 2, using an algorithm constructed for us using Scientist (Micromath Scientific Software, St. Louis, MO). Values of $(5.0 \pm 0.9) \times 10^5$ M^{-1} and -15.6 ± 1.3 kcal/mol were obtained for the microscopic binding constant and the standard enthalpy change, respectively. The solid line has been drawn using these values (Figure 3B, bottom). The thermodynamic parameter values obtained (ΔH_b and K) from both procedures (binding and dissociation methods) are similar, and the differences are within the experimental error (Table 1). This fact corroborates the validity of the dissociation method as an alternative to the traditional binding method for studying binding processes. Thus, for example, this method could be used if the stability of a protein−ligand complex is greater than the stability of the free protein. In these cases, the elimination of the ligand from the protein−ligand complex can be accompanied by a slow process. Additionally, in the absence of the ligand, the protein might not have the correct conformation to bind the ligand, and in this case, studying the binding is not possible. However, in such cases, the proposed method can indeed be used.

Dimer−Tetramer Equilibrium by SE-FPLC. SE-FPLC was used to examine the optimal experimental conditions for calorimetrically studying the binding of a ligand (for example, GSH) that has an affinity for dimeric PfGST G-sites using the dissociation method for human GST P1-1, described above. For this, a sample of dimeric PfGST (300 μM PfGST, in the presence of 1.2 mM GSH), at 25 °C, was diluted 1:20, 1:60, and 1:120 in buffer A (buffer solution without GSH). Each diluted sample was incubated at 25 °C for 0, 30, 60, 120, and 360 min and then injected and chromatographed on a Superdex 200 column at each time. The SEC profiles contained the two peaks (tetramer and dimer) with different populations, depending on both the dilution and the incubation time. The tetramer:dimer relation was lower to increase the dilution factor (i.e., a lower concentration); the higher the relationship, the greater the incubation time of the diluted sample. Therefore, the quantity of the tetramer generated from dimers (by dilution) is time-dependent and, at each time, also depends on the PfGST concentration. Thus, the rate of conversion from dimer to tetramer is increased when the enzyme concentration is increased (which might be expected for a second-order kinetic). Figure 4 shows the SEC profiles for 1:20, 1:60, and 1:120 dilutions. The samples were injected immediately after each dilution. Clearly, the tetramer:dimer ratio was very low for the 1:120 dilution (2.5 μM dimer). Therefore, the conversion of dimer to tetramer (in the absence of GSH) in PfGST is not too rapid and is highly dependent on the initial dimer concentration. Therefore, with dimeric protein concentrations of <2.5 μM, the tetramer population formed from dimer associations is almost negligible, at least during the time corresponding to calorimetric peak completion (2−3 min).

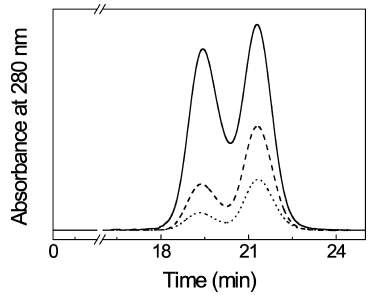

Figure 4. Size exclusion chromatography profiles for 1:20 (—), 1:60 (---), and 1:120 dilutions (···) of a sample of 300 μM dimeric PfGST in the presence of 1.2 mM GSH and buffer A at 25 °C.

Binding of GSH to PfGST Dimer by the Dissociation Method. ITC was used to study binding of GSH to a PfGST dimer in buffer A (pH 7.0) using the dissociation method described for GST P1-1. In this case, in contrast to GST P1-1, the procedure was forced because of the peculiar properties of this enzyme (oligomeric state change) that prevented the use of a conventional titration method. Figure 5 shows, as an example, the different calorimetric peaks corresponding to single, independent injections of 10 μL of dimeric PfGST (200 μM protein and 1.4 mM GSH in the injection syringe) into a buffer solution with GSH concentrations between 0 and 1.4 mM (placed in the calorimetric cell) at 25 °C. The calorimetric signals (peaks) correspond to the absorbed heat (endothermic heat) in the dissociation process of the GSH−PfGST complex inside the calorimetric cell (1.42 mL effective volume). As can be observed, the thermal power (calorimetric signal) increases quickly with each individual injection and the baseline recovery is reached in approximately 2 min. The response time for these calorimetric peaks is similar to those observed for typical protein−ligand binding with no slow process or changes in the aggregation state. Therefore, any possible kinetic process that can be initiated immediately after dissociation of the GSH−PfGST complex is negligible during the time corresponding to the detected thermal signal. Moreover, the experimental conditions, such as the dimeric protein concentration in the

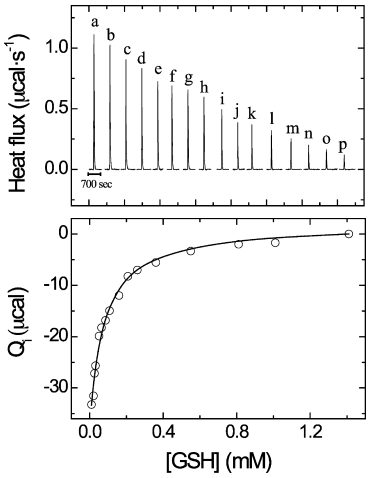

Figure 5. ITC measurements for the interaction of GSH with dimeric PfGST in buffer A (pH 7) using the dissociation method. The top panel shows the different calorimetric peaks corresponding to unique and independent injections of 10 μL of dimeric PfGST (200 μM protein and 1.4 mM GSH located in the injection syringe) into a buffer solution with different GSH concentrations [(a) 0, (b) 11, (c) 16, (d) 22, (e) 41, (f) 54, (g) 76, (h) 98, (i) 150, (j) 198, (k) 250, (l) 350, (m) 540, (n) 800, (o) 1000, and (p) 1400 μM].

calorimetric cell (~1.4 μM PfGST), allow us to discard any slow effect such as a change in the protein's aggregation state and its parallel processes. On the other hand, other experimental conditions such as high dimeric protein concentrations and high temperature are favored to increase the rate of the kinetic tetramerization process initiated after the injection of the GSH–PfGST complex into the calorimetric cell. Figure 6 corresponds to a single injection (25 μL) of a

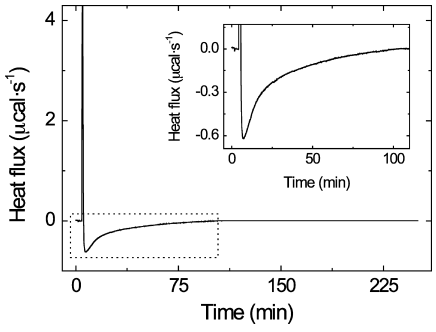

Figure 6. Calorimetric signal (heat flow vs time) corresponding to a single injection (25 μL) of the GSH–PfGST complex (400 μM PfGST and 2 mM GSH) into the calorimetric cell containing buffer A (without GSH) at 45 °C. The area marked has been enlarged for easy viewing (inset).

great excess of the GSH–PfGST complex (400 μM PfGST in the presence of 2 mM GSH) into the calorimetric cell containing buffer A (with no GSH) at 45 °C. Under these conditions, the rapid endothermic signal (complex dissociation) is followed by a slow negative deflection corresponding to the kinetic process derived from the tetramerization process of this enzyme.

Therefore, the enthalpy change of GSH–PfGST binding will have a negative sign (opposite the sign of that of the dissociation process) and, consequently, will be enthalpically favored at 25 °C. Identical calorimetric experiments were conducted at other temperatures in the range of 15–37 °C (Table 2) with a linear dependence of ΔH_b on temperature across the studied range, forming a slope on which the change in heat capacity is calculated.

Binding of GSO_3^- to PfGST by the Displacement Method. As with GSH, we have studied the PfGST–GSO_3^- interaction (at 25 °C) using the dissociation method because conventional titration by ITC of tetrameric PfGST with GSO_3^- also generated anomalous thermograms (data not shown). In addition, this dissociation procedure could be used to investigate the binding of this inhibitor at other temperatures. However, although this procedure provides good results, it is very tedious, and obtaining a complete binding curve requires a large quantity of enzyme, a long period of time, and significant manipulation. For this reason, we have employed the displacement method to assess the thermodynamic binding of the GSO_3^- inhibitor (a strong ligand) to dimeric PfGST in the presence of GSH (a weak ligand). This approach allows us to obtain a complete calorimetric titration in one experiment. Frequently, the displacement method has been used in ITC[28] to determine the complete thermodynamics of binding of strong inhibitors to enzymes with an affinity in either the nanomolar or picomolar range.[29,30] Briefly, this method is based on the fact that the binding properties of a ligand are altered when another competing ligand is present. With this method, the association constant of a high-affinity ligand can be measured indirectly by premixing the protein with a weaker competitive ligand. Nevertheless, it is very important to emphasize that the displacement method can be applied here for GSO_3^- because we have previously characterized GSH binding (a weak ligand) by the dissociation method. Thus, the widespread application of this displacement approach to study any other strong ligand, with PfGST G-site affinity, by calorimetry, will be possible if the thermodynamic parameters of GSH binding (as determined here) have previously been obtained.

It has been reported that GSO_3^- is a strong inhibitor in different GST classes with enhanced affinity, when compared to its substrate GSH.[31,34] Nonetheless, the affinity of this strong inhibitor for other GSTs was not overly high and can thus be directly measured using a conventional method, for example, ITC (in ref 34 and this work). Therefore, here the displacement method could not be used to obtain the affinity and thermodynamics for the interaction of this inhibitor with this enzyme. However, in this work, the objective in applying this displacement method with this inhibitor is different from the traditional use.[28–30] As has been described above, this enzyme is found in dimeric form in the presence of GSH concentrations of >1 mM. In addition, using SE-FPLC on a Superdex S 200 column, we have examined the oligomeric state of this enzyme when this inhibitor is present. PfGST is also a dimer in the presence of GSO_3^- concentrations of >0.4 mM (data not shown), so during all displacement titrations with this inhibitor, and in the presence of ≥1 mM GSH, the oligomeric form of the enzyme is always dimeric and no change in the aggregation state (tetramer–dimer) will occur during the titration as is commonly the case with a slow process (the atypical thermograms in Figure 1). Figure 7 shows a displacement titration of 80 μM PfGST in the presence of 1.4 mM GSH with GSO_3^- at 25 °C. Thermodynamic parameters were obtained using Origin; the parameters obtained for PfGST and GSH (the dissociation method), at the same temperature, were taken as the input data. The

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Table 2. Thermodynamic Parameters of the Interaction of GSH and GSO_3^- with the Dimeric Form of PfGST at Several Temperatures and pH 7.0

ligand	temp (°C)	K ($\times 10^{-4}$ M^{-1})	$\Delta G°$ (kcal/mol)	ΔH_b (kcal/mol)	$T\Delta S°$ (kcal/mol)
GSH	15	2.0 ± 0.7	−5.6 ± 0.2	−8.7 ± 1.0	−3.0 ± 1.1
	20	1.7 ± 0.6	−5.7 ± 0.2	−9.8 ± 1.2	−4.1 ± 1.2
	25	1.4 ± 0.1	−5.6 ± 0.1	−11.8 ± 0.3	−6.1 ± 0.3
	29	0.8 ± 0.4	−5.4 ± 0.3	−13.8 ± 0.9	−8.4 ± 0.9
	34	0.5 ± 0.1	−5.1 ± 0.1	−15.2 ± 0.4	−10.1 ± 0.4
	37	0.3 ± 0.1	−5.0 ± 0.1	−17.5 ± 0.1	−12.5 ± 0.1
GSO_3^-	15	21.5 ± 0.1	−7.0 ± 0.1	−9.8 ± 0.1	−2.7 ± 0.1
	20	37.9 ± 0.1	−7.5 ± 0.1	−11.2 ± 0.1	−3.8 ± 0.1
	25	16.2 ± 0.2	−7.1 ± 0.1	−12.8 ± 0.2	−5.8 ± 0.2
	25[a]	34.2 ± 21.0	−7.5 ± 0.2	−13.3 ± 1.1	−5.7 ± 1.1
	29	23.0 ± 0.1	−7.4 ± 0.1	−14.5 ± 0.1	−7.1 ± 0.1
	34	7.3 ± 0.2	−6.8 ± 0.1	−16.3 ± 0.1	−9.5 ± 0.1
	37	6.5 ± 0.6	−6.8 ± 0.1	−18.2 ± 0.1	−11.4 ± 0.1

[a]Thermodynamic data obtained by the dissociation method.

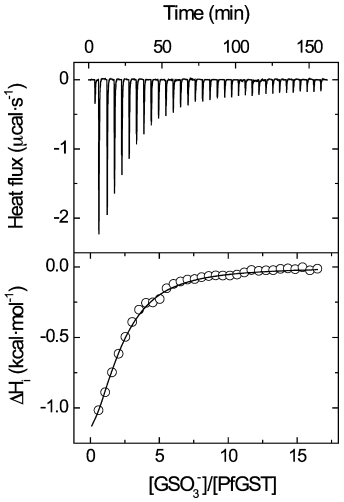

Figure 7. ITC measurements of the interaction of GSO_3^- and dimeric PfGST in buffer A with 1.4 mM GSH (pH 7) at 25 °C. The profile shows a displacement titration of 80 μM PfGST in the presence of 1.4 mM GSH with 12 mM GSO_3^- (5 μL injections) at 25 °C.

thermodynamic parameters obtained via both procedures (the displacement and dissociation methods) were in agreement within the experimental errors. Identical ITC experiments were performed at other temperatures in the range of 15−37 °C. In all cases, the enzyme was placed in the calorimetric cell at a saturated GSH concentration and titrated by successive injections of a GSO_3^- solution. Following this, the thermodynamic parameters for the PfGST−GSO_3^- interaction at each temperature were obtained from a displacement thermogram, always using the corresponding parameters deduced at the same temperature for GSH binding (by the dissociation method) as the input data. The thermodynamic parameters derived from the temperature-dependent titration are listed in Table 2. In all cases, whereas $\Delta G°$ remains practically invariant across the temperature range, the ΔH_b and $\Delta S°$ values are always negative and decreased as the temperature increased, affording a constant $\Delta C_p°$ value across the studied temperature range.

DISCUSSION

Glutathione *S*-transferases (GSTs) make up a superfamily of detoxification enzymes. They possess a wide range of substrate specificities, including endogenous substrates produced in vivo as the breakdown products of macromolecules during periods of oxidative stress.[1] Thus, GSTs play a vital role in protecting tissues against oxidative damage and oxidative stress and/or acting as a buffer for detoxification of heme-binding compounds.[12,13,19] Malarial parasites require an effective antioxidant defense mechanism because they not only have to overcome oxidative attack by the host's phagocytic cells but also have to survive in the pro-oxidant environment of the erythrocyte.[35] Therefore, antioxidant enzymes such as GST are essential for the survival of the parasite by protecting the parasite against oxidative stress. In this regard, some researchers have focused their interest on a novel and particular GST of the malarial parasite *P. falciparum*, PfGST.[7,13,19] This GST cannot be assigned to any of the known GST classes. In solution, this GST presents two oligomeric states, dimer and tetramer, in equilibrium.[15,16,19] The displacement of this equilibrium is GSH-induced. Recently, some authors have found similar behavior in the GST from the parasite *Plasmodium vivax*.[36,37] The different properties of these *Plasmodium* GSTs have been the focus of attention for several researchers who aimed to understand the parasites' biology responsible for malaria. Hence, PfGST is considered a potential target for the development of antimalarial drugs.[7,13,38,39] On the other hand, one aim of drug development is to optimize the binding affinities of inhibitors and ligands for their biological targets. Although different techniques can be applied to determine this affinity, only ITC allows a determination of the binding affinity and the binding enthalpy, simultaneously, providing a complete thermodynamic characterization of the drug. In this work, we have centered our study on the thermodynamic characterization of PfGST with GSH and GSO_3^- by ITC. Because this GST isoform is present in solution in two oligomeric states in equilibrium, the binding of ligands with an affinity for the G-site of this enzyme, such as GSH, induces a displacement of this equilibrium directed to the dimeric form. With this peculiar characteristic, it is very complicated studying ligands binding to dimeric PfGST (the enzymatically active form), for example, by ITC. Thus, anomalous calorimetric thermograms were obtained by a classical titration of tetramer PfGST with GSH. An exhaustive examination, by SE-FPLC, of the samples used in calorimetric titrations showed the presence of the two isoforms (dimer and tetramer) in different proportions during the titration. Therefore, the dissociation of the tetramer into

dimers, induced by addition of GSH into the calorimetric cell, is a slow process with several associated concomitant heat effects whose separation into individual contributions is not possible. Consequently, a classical calorimetric study must be discarded. SE-FPLC was used to examine the optimal experimental conditions for calorimetrically characterizing the binding of ligands (with an affinity for the G-site) to dimeric PfGST by using an ITC nonclassical procedure based on the dissociation of the ligand−protein complex. It has been reported that the tetramerization process of this enzyme, originating from GSH dissociation, occurs more quickly than dimerization.[19] However, we have observed that the rate for the tetramerization process, in contrast to that for dimerization, is dependent on the initial protein concentration. Moreover, we have demonstrated that if the dimer concentration is lower than 2.5 μM, the rate of the tetramerization process greatly decreases and the tetramer population generated from the association of the two dimers (as a consequence of GSH release) is negligible at least during the calorimetric peak time (2−3 min). Furthermore, the rate of the tetramerization process is also temperature-dependent. Thus, high dimer concentrations and high temperatures increase the rate of tetramerization and, therefore, must be avoided when studying this binding process by dissociation of the protein−ligand complex. To check the validity of this dissociation procedure, as an alternative to the classical titration method, we have studied the interaction of human GST P1-1 with GSO_3^-, a strong and competitive inhibitor of GSTs.[31−34,40] The GST P1-1 enzyme was chosen as a model because this isoenzyme is dimeric in the presence and absence of ligands. The thermodynamic parameters obtained (at 25 °C) from a classical titration and those deduced using the dissociation method are in agreement and corroborate the validity of the dissociation method (Table 1). We have applied the dissociation method to study the thermodynamic binding of the GSH substrate to dimeric PfGST at different temperatures in the range of 15−37 °C. Under the optimal experimental conditions (low dimer concentration and temperatures that are not too high), no slow process was observed from calorimetric signals generated after dissociation of the GSH−PfGST complex in the calorimetric cell. In addition, SE-FPLC assays corroborated that the tetramer population was negligible during the calorimetric peak under our experimental conditions. Therefore, the detected endothermic calorimetric signals will be due to only the dissociation of GSH from the PfGST dimer. The thermodynamic parameters indicate that binding of GSH to dimeric PfGST is enthalpically favored and entropically unfavored at all the studied temperatures. Moreover, the GSH affinity, expressed as dissociation ($K_d \sim 70$ μM at 25 °C), is similar to that deduced from early kinetic studies ($K_m \sim 108$ μM[18]). The data in Table 2 reveal that, whereas ΔG° remains practically invariant with changes in temperature, both ΔH_b and ΔS° decrease with an increase in temperature. Hence, the binding ΔG° is contributed exclusively by a favorable ΔH_b (negative values), and the van der Waals interactions and hydrogen bonding will probably be the main contributions to those ΔH_b negative values. ΔH_b depends linearly on temperature in the range of 15−37 °C. A ΔC_p° of -390 ± 30 cal K^{-1} mol^{-1} was obtained from the slope of ΔH_b versus temperature. A similar thermodynamic profile was found for binding of GSH to SjGST,[41] human GST P1-1,[42] and human GST A1-1.[34] These results indicate that, although this malarial parasite GST seems to have certain particular differences compared to other known GSTs, binding of GSH to the dimeric form is very similar to that of other GSTs.

On the other hand, the dissociation procedure was also used to study the binding of the GSO_3^- inhibitor to dimeric PfGST in buffer A at pH 7 and 25 °C. The thermodynamic parameters obtained were similar, within experimental error, to those deduced by the displacement method (Table 1). Hence, the displacement approach was used to determine all thermodynamic parameters for binding of GSO_3^- to PfGST across a temperature range of 15−37 °C. The application of the displacement method for studying the PfGST−GSO_3^- interaction by ITC is valid for four reasons. (i) PfGST is a dimer in the presence of GSO_3^- (similar to GSH), and thus, no changes in the aggregation state are possible during the complete titration. (ii) The GSO_3^- ligand is a competitive GSH inhibitor. (iii) The affinity of this inhibitor is approximately 2 orders of magnitude higher than that for GSH. (iv) The binding enthalpies of the weak ligand (GSH) and the strong ligand (GSO_3^-) at each temperature are sufficiently different to distinguish the measured heat signals. Nevertheless, if the binding enthalpies were equal, the heat of binding would not be measurable because the thermal effect associated with the dissociation of the weak ligand would completely compensate for the thermal effect associated with the binding of the strong ligand. Like binding of GSH, binding of GSO_3^- to dimeric PfGST was also enthalpically favored and entropically unfavored at all studied temperatures, the main difference being the higher affinity of this inhibitor compared with that of glutathione (approximately 2 orders of magnitude higher); this higher GSO_3^- affinity at all studied temperatures is a consequence of both a more favorable enthalpy change and a less unfavorable entropy change compared to those of GSH binding. The moderate binding affinity of GSO_3^- ($K_d = 6$ μM) for PfGST has also been found in other GSTs such as porcine class pi GST[31] and human GST A1-1.[34] A negative value of the heat capacity change, like that of GSH, was obtained ($\Delta C_p^\circ = -360 \pm 20$ cal K^{-1} mol^{-1}) from the slope of ΔH_b versus temperature. This value is similar to that deduced for GSH binding, which also suggests an analogous binding mode, and only small differences in the binding processes can be found. Negative values of ΔC_p, similar to those deduced here for these ligands and PfGST, are common in binding studies with no large conformational changes.[42−46] On the other hand, the binding of these two ligands to PfGST is noncooperative, consistent with previous findings for similar ligands in other GSTs.[34,41,42,44,45,47]

Early studies showed that the active enzymatic structure of PfGST is a homodimer[12] that adopts a folding similar to that of other GSTs. Thus, the active site is also located in the cleft between the two domains of each monomer and is composed of two binding sites: the G-site, which binds reduced glutathione, and the more variable H-site, which can accommodate a variety of substrates. X-ray crystallographic analysis of the PfGST−*S*-hexylglutathione complex[14,17,18] revealed a similar binding mode for GS-moiety compared to liganded GSTs from the pi[48] and mu classes.[49] The main difference between the different structures is the binding mode of the conjugated moiety, which stretches out in different directions from the H-site. On the other hand, the comparison of liganded and native PfGST structures reveals a similar overall tertiary structure, but the crystal packing of the molecules is different. In the absence of ligands, two biological dimers form a tetramer; these homodimers are interlocked with each other

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by the 113−120 loop of each monomer.[14] In the determined structures of PfGST with *S*-hexylglutathione, some differences were found, all related to a possible rearrangement of loop 113−120 on the *S*-hexylglutathione binding.[17,18] This rearrangement of H-site residues shows that the shape of the H-site is very flexible and that large compounds can be bound. These data provide valuable hints about interactions of the ligand with H-site residues, which can currently be exploited for the design of more potent inhibitors. Because the glutathione binding sites (G-sites) in GSTs, including PfGST, are highly conserved, a similar thermodynamic profile is expected between different GSTs, in agreement with the data deduced via ITC for both GSH and GSO_3^- binding.

CONCLUSIONS

GST from the malarial parasite *P. falciparum* represents a novel and peculiar GST isoform with structural characteristics that are not present in the other known GSTs. This enzyme is the only GST present in this parasite and plays a particular protective role against oxidative stress and buffering the detoxification of heme-binding compounds. This isoenzyme is present in solution in two oligomeric states (tetramer and dimer) in equilibrium. The binding of ligands with affinity for the G-site of this enzyme, such as GSH, induces a displacement of this equilibrium directed to the dimeric form. These different PfGST properties have been the focus of several researchers who aim to understand the part of the parasites' biology responsible for malaria. Hence, PfGST is considered as a potential target for the development of antimalarial drugs. The presence of two oligomeric states in solution makes it very complicated to calorimetrically study binding of the ligand to the dimeric form of PfGST (the active form). We have thermodynamically characterized the binding of its natural substrate, GSH, and a strong competitive inhibitor (glutathione sulfonate) to dimeric PfGST at different temperatures. An ITC nonclassical strategy, based on the dissociation of the ligand−protein complex, was proposed for the study of the affinity of the ligands binding to the G-site of this peculiar GST. The binding of these ligands is enthalpically favored and entropically unfavored at all the studied temperatures, the main difference being the higher affinity of the GSO_3^- inhibitor compared to that of glutathione. This higher GSO_3^- affinity at all studied temperatures is a consequence of more favorable enthalpic and entropic changes compared to those for GSH binding. These results demonstrate that although PfGST presents differences compared to other known GSTs, the mode of binding of ligands to the G-site is similar. However, in contrast to that of other GSTs, the binding of GSH to this isoenzyme in the absence of ligands is slow. This nonclassical ITC procedure can be combined with the displacement method to obtain the thermodynamic parameters for the binding of other competitive ligands with higher affinities than glutathione.

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Notes

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ABBREVIATIONS

GST, glutathione *S*-transferase; PfGST, glutathione *S*-transferase from *P. falciparum*; ITC, isothermal titration calorimetry; GSH, reduced glutathione; CDNB, 1-chloro-2,4-dinitrobenzene; GSO_3^-, glutathione sulfonate; SEC, size exclusion chromatography.

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