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Structural, functional and unfolding characteristics of glutathione S-transferase of *Plasmodium vivax*

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ABSTRACT

Glutathione S-transferases (GSTs) of *Plasmodium* parasites are potential targets for antimalarial drug and vaccine development. We investigated the equilibrium unfolding, functional activity regulation and stability characteristics of the unique GST of *Plasmodium vivax* (*Pv*GST). Despite high sequence, structural, functional, and evolutionary similarity, the unfolding behavior of *Pv*GST was significantly different from *Plasmodium falciparum* GST (*Pf*GST). The unfolding pathway of *Pv*GST was non-cooperative with stabilization of an inactive dimeric intermediate. The absence of any compact, folded monomeric intermediate during the unfolding transition suggests that inter-subunit interactions play an important role in stabilizing the protein. Presence of salts effectively inhibited *Pv*GST enzymatic activity by quenching the nucleophilicity of the thiolate anion of GSH. Based on the present findings, together with our previous studies on *Pf*GST, we propose that the regulation of GST enzymatic activity through a dimer–tetramer transition via GSH binding is an exclusive feature of *Plasmodium*.

Introduction

Glutathione S-transferases (GSTs[1]) are dimeric cytosolic proteins with a subunit molecular mass of about 25 kDa. They are multifunctional enzymes which mediate the conjugation of toxic electrophilic compounds to GSH, thereby playing an important role in cellular detoxification [1,2]. On the basis of amino acid sequence similarity, immunological cross reactivity and substrate specificity, the GSTs have been grouped into at least 13 classes [2,3]. Interestingly, the sequence similarity between the classes is low, but the global architecture of the proteins is highly similar. The three-dimensional structure of GST isoenzymes from various classes reveals that they have a conserved overall topology [4–6]. Each subunit of the dimeric GST consists of two domains; the N-terminal thioredoxin-like domain linked via a loop to the C-terminal domain which is mostly α-helical. The N-terminal domain contains the key structural determinants for the recognition of GSH, whereas the C-terminal domain provides the structural elements associated with the second substrate specificity. The active site is located along the interface between the two domains with each domain contributing essential residues for functional activity and consisting of 2 sites—a conserved GSH binding G-site and an H-site where a variety of substrates can bind. The catalytic mechanism proceeds through GSH binding to the enzyme, activation of GSH by promoting and stabilizing the thiolate anion group (GS^-), nucleophilic attack by the GS^- to the hydrophobic substrate possessing an electrophilic centre, product formation and finally product release [1,7].

The presence of a single isoform of GST in *Plasmodium* spp. underlines its functional importance and drug target characteristics [8–10]. The GST from *Plasmodium vivax* shares almost 85% sequence identity with *Plasmodium falciparum* GST and thus both the proteins show high structural and functional similarity [9,11]. The crystal structure of *Pf*GST shows distinct uniqueness among other GSTs as it possesses prominent structural differences in the active site region with a more solvent-accessible H-site where a large spectrum of molecules including inhibitors can bind [8,12–15]. Thus, structurally, *Plasmodium* GSTs have not been classified in any of the previously known GST classes [9,12]. Our recent work showed that changes in the oligomeric status via GSH binding regulates the functional activity of *Pf*GST [3]. This observation is unique for *Pf*GST as no other GST has been reported to show similar characteristics to date.

Understanding the folding/unfolding and assembly of parasite enzymes is of importance in understanding the oligomerization process, the influence of quaternary structure and subunit

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[1] *Abbreviations used:* GST(s), glutathione S-transferase(s); *Pv*GST, *Plasmodium vivax* glutathione S-transferase; *Pf*GST, *Plasmodium falciparum* glutathione S-transferase; SEC, size exclusion chromatography; GdnHCl, guanidine hydrochloride; GSH, reduced glutathione.

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association on protein stability, and in in vitro designing of molecules that can obstruct assembly of the subunits. Different unfolding pathways have been reported for GSTs, ranging from a two-state [3,16–20] to a three-state unfolding mechanism [18,21–24] to even more complex mechanisms of unfolding [25–27]. In the present study we have investigated the structural and functional changes during solvent-induced denaturation of *Pv*GST under equilibrium conditions by monitoring enzyme activity (as a functional probe) and spectroscopic studies (as structural probe) along with the functional studies. Our data imply that the unfolding pathway of *Pv*GST is three-state with stabilization of an inactive dimeric intermediate. The *Pv*GST exhibits a marked difference in the unfolding pathway from that of *Pf*GST as in the latter case the unfolding was found to be two-state [3]. Salts inhibit the functional activity of the protein by quenching the nucleophilicity of the enzyme-bound thiolate anion (GS^-). Our results also suggest that the regulation of the functional activity of *Plasmodium* GSTs through reversible dimer–tetramer transition is an exclusive feature of the *Plasmodium* genus.

Materials and methods

Materials

All chemicals used in the study were purchased from Sigma–Aldrich Chemical Co., USA, and were of the highest purity available. Superdex™ 200 10/300 GL column was purchased from GE Healthcare Biosciences, USA, while Ni-NTA was from Qiagen. Recombinant *Pv*GST was prepared and dialyzed against 100 mM potassium phosphate buffer, pH 8.0, containing 1 mM EDTA with or without 2 mM GSH (hereafter called as buffer A and B, respectively) [3,11], unless otherwise mentioned.

Computational analysis

Three-dimensional modeling was performed using the Protein Homology/analogY Recognition Engine (Phyre), a protein-fold recognition server [28]. Subsequent analysis, visualization and preparation of 3D figure was performed using the PyMOL software.

Size exclusion chromatography

The oligomeric status of *Pv*GST in different conformational states was measured using the SEC. Experiments were carried out with a Superdex™ 200 10/300 GL column (V_o = 8.2 mL) using an ÄKTA-FPLC (GE Health Care Biosciences). The column was pre-equilibrated with the buffer A and 500 μL of the sample was injected into the column at a flow rate of 0.3 mL/min. The eluent was detected on-line by absorbance at 280 nm. In the case of GdnHCl-denatured protein, the column was equilibrated with defined concentration of GdnHCl dissolved in buffer A and the protein was incubated for 2 h in GdnHCl before injecting into the column. A set of globular proteins with known molecular weights was used for column calibration: Glucose oxidase (160 kDa), albumin (66 kDa), ovalbumin (43 kDa), ribonuclease A (13.7 kDa) and aprotinin (6.5 kDa).

Enzymatic activity

The catalytic activity was determined spectroscopically using an UV-1650PC, UV–Visible spectrophotometer (Schimadzu, Japan) with the chromogenic substrate 1-chloro-2,4-dinitrobenzene. Experiments were carried out by measuring the absorbance increase at 340 nm at 25 °C over 60 s. The assay was initiated upon addition of 0.5 mM CDNB to a 1 mL quartz cuvette containing dimeric GST in buffer A. The GST-catalyzed formation of CDNB-GSH produces a dinitrophenyl thioether which can be detected at 340 nm. For salt dependent activity measurements, the phosphate buffer was replaced with Hepes buffer and was incubated with the increasing concentrations of salts for 2 h before the measurements were made. Analogous control experiments were done and the baseline was subtracted. All data were fitted using Origin 7.0 server software (Northampton, MA, USA).

GdnHCl and urea denaturation

The equilibrium denaturation experiments with GdnHCl/Urea were performed in buffer A. The defined volumes of GdnHCl/Urea solution were added to *Pv*GST samples (final protein concentration 2 and 8 μM) to obtain an increasing denaturant concentration. Time-dependent changes in the structural parameters of the protein with increasing GdnHCl/urea concentrations were monitored to standardize the incubation time required to achieve equilibrium under these conditions. Under all the conditions studied, the changes occurred within a maximum of 2 h with no further alterations in the values obtained up to 24 h (data not shown) suggesting that a minimum time of about 2 h is sufficient for achieving equilibrium under any of the denaturing conditions studied. Furthermore, under all the experimental conditions the unfolding/refolding curves were fully reversible. The mixtures were incubated for 2 h at 25 °C. The denaturation reactions were studied by measuring the intrinsic tryptophan fluorescence and the Far-UV CD.

Far-UV CD measurements

Far-UV circular dichroism measurements were carried out in a JASCO J-810 spectropolarimeter, equipped with a peltier-type temperature controller and a thermostated cell holder, interfaced with a thermostatic bath. Spectra were recorded in a 1 mm path length quartz cuvette at the aforementioned protein concentrations in buffer A with or without GdnHCl/urea. Five consecutive scans were accumulated and the average spectra stored. Thermal denaturation experiments were performed by increasing the temperature from 50 to 75 °C, taking the readings at 222 nm. Unfortunately, the protein was found to precipitate at the end of the experiment at high temperature. Due to this irreversible nature of the thermal transitions, further thermodynamic analysis of the data could not be performed. All data were corrected for the baseline contribution of the buffer at all conditions.

Fluorescence spectroscopy

The intrinsic fluorescence spectra were measured using a Perkin–Elmer LS50B luminescence spectrometer in a 5 mm path length quartz cell. 2.0 μM protein was used for the studies. The samples were excited at 285 nm and the emission spectra were recorded in the wavelength range of 300–400 nm. The excitation and emission slits were kept at 8 and 6 nm, respectively, and the data were recorded at 25 °C. The final spectra were the average of three scans, and every spectrum was corrected by subtraction of the corresponding blank sample without protein.

ANS fluorescence

ANS was added to 2 μM protein samples with increasing concentration of GdnHCl/urea to get a final ANS concentration of 10 μM and kept for 2 h before measuring the fluorescence. The excitation wavelength was 350 nm, and the emission spectra were recorded between 400 and 500 nm. The values were normalized by subtracting the base line recorded for the probe alone under similar conditions as used for the experiments.