4.1 INTRODUCTION
Glutathione S-transferases (GSTs) are dimeric cytosolic proteins with subunits of molecular mass 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 (Salinas and Wong, 1999; Sheehan et al., 2001). On the basis of amino acid sequence similarity, immunological cross reactivity and substrate specificity, the GSTs have been grouped into at least 13 classes (Sheehan et al., 2001; Tripathi et al., 2007). 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 (Dirr et al., 1994; Lian, 1998; Wilce and Parker, 1994). Each subunit of the dimeric GST consists of two domains; the N-terminal Trx 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 consists of 2 sites— a conserved GSH binding G-site and an H-site where a variety of substrate 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 thiolate anion to the hydrophobic substrate possessing an electrophilic centre, product formation and finally product release (Mannervik and Danielson, 1988; Salinas and Wong, 1999).

The presence of a single isoform of GST in Plasmodium spp. underlines its functional importance and drug target characteristics (Deponte and Becker, 2005; Harwaldt et al., 2002; Hiller et al., 2006). 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 (Deponte and Becker, 2005; Na et al., 2007). The crystal structure of PiGST shows distinct uniqueness
*Plasmodium vivax* Glutathione S-transferase among other GSTs as it possess prominent structural difference in the active site region in having more solvent-accessible H-site compared with any other GSTs, where large spectrum of molecules including inhibitors can bind (Burmeister et al., 2003; Fritz-Wolf et al., 2003; Hiller et al., 2006; Liebau et al., 2005; Perbandt et al., 2004). Thus, structurally, *Plasmodium* GSTs have not been classified to any of the previously known GST classes (Deponte and Becker, 2005; Fritz-Wolf et al., 2003). Our recent work showed that changes in the oligomeric status via GSH binding regulates the functional activity of *Pg*GST (Tripathi et al., 2007). This observation is unique for *Pg*GST as no other GST has been reported to show similar characteristics till 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 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 (Alves et al., 2006; Dirr and Reinemer, 1991; Erhardt and Dirr, 1995; Kaplan et al., 1997; Tripathi et al., 2007; Wallace et al., 1998) to a three-state unfolding mechanism (Aceto et al., 1992; Hornby et al., 2000; Sacchetta et al., 1999; Wallace et al., 1998; Wang et al., 2008) to even more complex mechanism of unfolding (Abdalla and Hamed, 2006; Sacchetta et al., 1993; Stevens et al., 1998). 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 a structural probe) along with the functional studies. Our data indicates that the unfolding pathway of *Pv*GST is three-state with stabilization of an inactive dimeric intermediate. The *Pv*GST exhibit marked difference in unfolding pathway from *Pg*GST. Salts inhibit the functional activity of the protein by quenching the nucleophilicity of the enzyme-bound thiolate anion (GS\(^-\)). Our results also demonstrate that the regulation of the functional activity of *Plasmodium* GSTs, through reversible dimer-tetramer transition is an exclusive feature of the *Plasmodium* genera (Tripathi et al., 2009).
4.2 MATERIALS AND METHODS

4.2.1 Materials
Most of the chemicals used in the study were purchased from Sigma–Aldrich Chemical Company, St. Louis, MO, USA, and were of the highest purity available. IPTG was purchased from USB Corporation, Ohio, USA. All chromatographic columns and molecular weight marker for gel filtration chromatography were purchased from GE Healthcare Biosciences, Piscataway, USA, with the exception of Ni–NTA agarose, which was from Qiagen. Bacterial culture media was purchased from Himedia laboratories, Mumbai, India and Centricon filters were from Millipore. All the buffers were prepared in Milli Q™ water of 18.2 \( \Omega \) resistivity and filtered with 0.22 mm filters (Millipore). The pH of solutions was maintained using pH meter with glass electrode (EUTECH Instruments pH 510) calibrated with pH standard buffers obtained from Sigma Chemical Co, St. Louis, MO, USA.

4.2.2 Transformation of rPvGST plasmid into E. coli
To study the expression of recombinant PrGST, plasmid constructs containing PrGST gene in pQE vector was transformed in E. coli strain M15. In a vial of competent M15 cells, 5 \( \mu \)L of plasmid was pipetted and kept on ice. Heat shock was given at 42°C for 90 secs. 800 \( \mu \)L of LB broth was then pipetted in the vial and kept in incubator for growth. After 45 mins it was centrifuged at 7000 rpm for 2 mins and then plated in LB–Amp–Kan plate. The plate was kept at 37°C for overnight growth.

4.2.3 Protein overexpression and purification
Recombinant PrGST was overexpressed in E. coli M15 cells and purified as described earlier (Na et al., 2007). In short, single colony from transformed plates was inoculated in 5 ml of LB broth containing 100 \( \mu \)g/mL ampicillin and 50 \( \mu \)g/mL kanamycin. Cells were grown for 4–5 h at 37°C with shaking at 160 rpm. Subsequently, two 5 ml LB broth tubes containing above–mentioned antibiotics were inoculated with 1\% (v/v) of 4–5 h grown culture and incubated at 37°C with shaking at 160 rpm. Cultures were grown until OD\(_{600}\) reached 0.5–0.6, at this stage culture was induced with 1 mM IPTG and other un–induced culture served
as control. After 4 h of induction with IPTG, both cultures were harvested and pelleted by centrifugation at 7000 rpm for 10 min at 4°C. The pellet was then resuspended in lysis buffer in a volume 1/50 of culture volume. The dissolved cells were lysed by sonication with pulse–rest cycle (60 cycles; 20 s pulse at 15% amplitude with 10 sec interval after each pulse). The lysate was centrifuged at 12,000 rpm for 20 min at 4°C and the supernatant was collected. All further steps were performed under cold conditions. Ni–NTA agarose matrix (3 mL) was equilibrated with equilibration buffer and the supernatant was poured on the column and was allowed to bind slowly. Non–specifically bound, contaminating proteins were removed by washing with equilibration buffer containing 60 mM imidazole. Recombinant protein was eluted with 10 ml of elution buffer (equilibration buffer containing 200 mM imidazole). The protein was dialyzed against 100 mM potassium phosphate buffer, pH 8.0 containing 1 mM EDTA. Two different buffer systems were used– containing or not containing 2 mM GSH.

4.2.4 Protein quantification
Protein concentration was determined by Bradford method (Bradford, 1976) using BSA as a standard.

4.2.5 Computational analysis
Three–dimensional modelling was performed using the Protein Homology/analogY Recognition Engine (Phyre), a protein fold recognition server (Bennett-Lovsey et al., 2008). Subsequent analyses, visualization and preparation of 3D Figures were performed using the PyMOL software.

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

4.2.7 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 sec. The assay was initiated upon addition of 0.5 mM CDNB to a 1 mL quartz cuvette containing dimeric GST in 100 mM potassium phosphate buffer (pH 8.0) having 1 mM EDTA and 2 mM GSH. 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).

4.2.8 GdnHCl and Urea Denaturation
The equilibrium denaturation experiments with GdnHCl/Urea were performed in 100 mM potassium phosphate buffer (pH 8.0) containing 1 mM EDTA and 2 mM GSH. The defined volumes of GdnHCl/Urea solution were added to PvGST samples (final protein concentration 2 and 8 μM) to obtain an increasing denaturant concentration. 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.

4.2.9 Far–UV CD Measurements
Far-ultraviolet 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 mentioned protein
concentrations in the above buffer 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°C to 75°C, taking the readings at 222 nm. The data was corrected for the baseline contribution of the buffer at all conditions.

4.2.10 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.

4.2.11 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.

4.2.12 Glutaraldehyde cross-linking
About 20 μg of protein was incubated with 5 μL of 1:25 dilution (w/v) of 25% glutaraldehyde in a 100 μL reaction volume in 100 mM potassium phosphate buffer (pH 8.0) containing 1mM EDTA and 2 mM GSH for 30 min. The reaction was stopped by addition of 10 μL of 1.0 M Tris–HCl, pH 8.0 in the reaction mixture and the molecular mass of the cross–linked products was determined by 10% SDS–PAGE. In case of sample containing GdnHCl, protein was incubated with 2 M GdnHCl for 2 h and then cross–linked.
4.3 RESULTS

4.3.1 Structural features of \( P_v \text{GST} \)

Similar to the other members of the GST superfamily, \( P_v \text{GST} \) adopts the canonical GST fold. The tertiary structure and topology of \( P_v \text{GST} \) was predicted using the protein–fold recognition algorithm PHYRE. \( P_v \text{GST} \) monomer consists of two non-identical domains (Figure 4.1). Domain I (residues 3–87, N–terminal) is a Trx–like domain and consists of four stranded \( \beta \)-sheets flanked by three \( \alpha \)-helices, while the domain II (residues 89–205, C–terminal) is primarily an \( \alpha \)-helical domain which contains the tryptophan residue at position 131. The indole ring of tryptophan is projected towards the core of the protein molecule.

![Molecular model of \( P_v \text{GST} \)](image)

**Figure 4.1—Molecular model of \( P_v \text{GST} \)**

Structure of a monomer of the \( P_v \text{GST} \) homodimer. The 3–D model was made using protein threading program PHYRE. The N–terminal domain is coloured in blue, while the C–terminal domain in pink colour. The tryptophan residue is projected towards the inner core in the C–terminal domain.

4.3.2 Purification and oligomeric status of \( P_v \text{GST} \)

Recombinant \( P_v \text{GST} \) was overexpressed and purified to homogeneity using Ni–NTA chromatography (Figure 4.2). The protein had the expected molecular mass of about 25 kDa and a purity of more than 98%.
Figure 4. 2– Overexpression of PvGST in *E. coli* and purification of the recombinant protein over Ni–NTA agarose
SDS–PAGE analysis of cell lysate showing overexpression of *PvGST* and the purified protein. Lanes 1–4 represent molecular weight markers, supernatant of un–induced culture lysate, supernatant of induced culture lysate and purified protein, respectively.

Figure 4. 3– Oligomeric status of *PvGST*
(A) Molecular weight and oligomeric status of *PvGST* were determined on Superdex™200 10/300 GL column at pH 8.0 and 25°C. The red and black lines represent *PvGST* in buffer containing and not containing 2 mM GSH, respectively. The curves have been displaced on the Y–axis for presentation. The column calibration curve was same as in Figure 3.2 B. (B) SDS–PAGE profile of glutaraldehyde crosslinked *PvGST* protein samples. Lanes 1–3 represent uncross–linked native *PvGST*, glutaraldehyde cross–linked dimeric *PvGST* and tetrameric *PvGST* protein samples, respectively.
The oligomeric status of \( P \text{v} \text{GST} \) in the presence and absence of GSH was determined by SEC and the curves plotted in Figure 4.3A. In presence of 2 mM GSH, gel filtration of the recombinant \( P \text{v} \text{GST} \) protein on Superdex S-200 column showed a single peak with an elution volume of 15.3 mL corresponding to the dimeric protein, while in the absence of GSH the protein existed predominantly as tetramer with an elution volume of 14.3 mL, though around 8% of the total population of protein was also found to be present presumably as octamer. This observation was further confirmed by the glutaraldehyde cross-linking of \( P \text{v} \text{GST} \) with or without GSH. The results showed the cross-linked protein bands of molecular masses of about 50 and 100 kDa, respectively corresponding to the dimer and tetramer of the enzyme (Figure 4.3B).

### 4.3.3 Functional activity regulation of \( P \text{v} \text{GST} \)

The enzymatic activity analyses of \( P \text{v} \text{GST} \) tetramer with increasing concentration of GSH was performed similarly to that of \( P \text{z} \text{GST} \) (Tripathi et al., 2007) and plotted in Figure 4.4. An exponential increase in the enzymatic activity of \( P \text{v} \text{GST} \) was observed with increasing GSH concentration till about 0.7 mM. Further increase in GSH concentration did not lead to increase in activity, indicating that \( P \text{v} \text{GST} \) also undergoes enzymatic activity regulation through tetramer–dimer transition similar to that of \( P \text{z} \text{GST} \) (Tripathi et al., 2007).

![Figure 4.4](image_url)

**Figure 4.4—Enzymatic activity analyses of \( P \text{v} \text{GST} \)**

Enzymatic activity of tetrameric \( P \text{v} \text{GST} \) with increasing concentrations of GSH.
4.3.4 GdnHCl–induced alteration in molecular characteristics of \( \text{PvGST} \)

Native dimeric \( \text{PvGST} \) exhibits the peculiar CD spectrum of \( \alpha/\beta \)-proteins (Figure 4.5A). The Far–UV CD spectrum was analyzed for the secondary structure content using K2D software (Andrade et al., 1993). The percentage of secondary structure was found to be \( \alpha \)-helix 61%, \( \beta \)-sheet 9%, and random coil 30%. The results are in good agreement with the secondary structure prediction based on the homology with the crystal structure of \( \text{PvGST} \) (Fritz-Wolf et al., 2003; Hiller et al., 2006; Perbandt et al., 2004).

The spectral changes associated with the unfolding of \( \text{PvGST} \) were studied by intrinsic tryptophan fluorescence and Far–UV CD spectroscopy. The change in ellipticity at 222 nm of \( \text{PvGST} \) samples as a function of increasing concentration of GdnHCl showed a three–state unfolding transition with stabilization of an intermediate at around 2 M GdnHCl, and almost complete unfolding of the protein was observed at concentration of GdnHCl above 4 M (Figure 4.5B). The stabilization of an intermediate was further confirmed by ANS binding studies. Figure 4.5C shows the results of ANS binding to \( \text{PvGST} \) during GdnHCl–induced denaturation. It was observed that maximum binding of ANS occurred within the first unfolding transition, suggesting increased exposure of the hydrophobic surface area in the intermediate.

The intrinsic tryptophan fluorescence for the native state of the \( \text{PvGST} \) protein was characterized by a \( \lambda_{\text{max}} \) of 331 nm (Figure 4.5D). This indicates that the single tryptophan residue present at the position 131 in the primary amino acid sequence resides in the hydrophobic environment, complementing the results of molecular modelling studies. Figure 4.5B shows the dependence of tryptophan fluorescence on GdnHCl concentration. At 4 M GdnHCl, a \( \lambda_{\text{max}} \) of 351 nm was observed, indicating the complete exposure of the tryptophan residue to the aqueous solvent. A biphasic behavior was obtained which is similar to the profile observed for Far–UV CD. The position of the equilibrium unfolding transition curve measured by intrinsic fluorescence and Far–UV CD were coincident and indicates the stabilization of an intermediate during GdnHCl denaturation. Figure 4.5E shows the GdnHCl–induced denaturation curves obtained through various structural probes.
Figure 4.5– GdnHCl–induced unfolding
(A) Far-UV CD curve. (B) GdnHCl-induced unfolding followed by monitoring CD ellipticity at 222 nm (■) for the secondary structure and tryptophan fluorescence (○) for the tertiary structure. (C) ANS binding curve for GdnHCl denaturation. (D) Tryptophan
fluorescence spectrum. (E) Comparison of various structural probes for GdnHCl–induced denaturation of PrGST monitored by Far-UV CD (○), tryptophan fluorescence (○), and ANS binding (●). (F) Effect of protein concentration on the GdnHCl–induced unfolding. The concentration of PrGST was 1 µM (○) and 8 µM (●) and denaturation was monitored by Far-UV CD. The data were plotted as a percent fraction folded. The percent fraction unfolded (F_u) was calculated from the equation F_u = 100 × (F_observable - F_n) / (F_u - F_n), where F_observable is the observed value of the signal at a given denaturant concentration and F_n and F_u are the values of the native and unfolded protein respectively. (G) SEC profile of PrGST with increasing GdnHCl concentration. The solid, dashed, and dotted lines represent PrGST protein at pre–transition, transition, and post–transition regions of the GdnHCl denaturation curve. The curves have been displaced on the Y-axis for presentation. (H) SDS–PAGE gel of PrGST. Lanes 1–3 represent uncrosslinked PrGST, crosslinked PrGST at 0 M GdnHCl, and crosslinked PrGST at 2 M GdnHCl concentration, respectively.

In order to determine at what point of the unfolding process, dissociation of the dimer takes place, the protein concentration dependence of the unfolding transitions was studied. It was observed that at increased enzyme concentration the second transition of the unfolding curve shifts to a higher GdnHCl concentration, while the first transition remains unaltered (Figure 4.5F). This indicates that the PrGST partially unfolds to a dimeric intermediate during the first transition and dissociation and unfolding occurs simultaneously during the second transition. The dimeric nature of the unfolding intermediate was further confirmed by SEC and chemical cross–linking. The column profiles of PrGST at different GdnHCl concentrations are plotted in Figure 4.5G. In the pre–transition and post–transition regions of the denaturation curve, a single species corresponding to the native dimer and unfolded monomer, respectively was observed, while in the transition region partially unfolded dimer with no peak of the monomer protein was perceived, indicating that the intermediate formed within the pre–transition and transition region was clearly dimeric, while the protein existed as unfolded monomer in the post–transition region. The glutaraldehyde cross–linking of PrGST also showed the presence of a cross–linked protein band of a molecular mass of about 52 kDa corresponding to the dimeric enzyme at 2 M GdnHCl concentration (Figure 4.5H).
4.3.5 Salt-induced modulation of the enzymatic activity of *Pv*GST

We have studied the effect of salt on the structural and functional properties of *Pv*GST. Figure 4.6 shows the effect of various salts on the enzyme activity and secondary structure of *Pv*GST. The enzyme activity of *Pv*GST decreased consistently with increasing salt concentration (Figure 4.6A). The relative decrease at the given concentration is in the order Na$_2$SO$_4$ < NaCl < MgSO$_4$ < MgCl$_2$. No loss of Far–UV CD signal was observed with any of the salt used (Figure 4.6B). To confirm that if the inactivation of enzyme is due to dissociation of the subunits, functional activity analyses were performed at various protein concentrations with different salts. At enzyme concentration ranging from 0.5 µM to 5 µM the inactivation curve did not change significantly (data not shown). Thus, inactivation of the enzyme is not coincident with dissociation of the protein and is only due to effect of these salts on the protein. It should be noted that the structural changes observed by Far–UV CD or tryptophan fluorescence occurred at denaturant concentration higher than that which causes enzyme inactivation.

![Figure 4.6](image)

**Figure 4.6**– Effect of salt on the enzymatic activity of *Pv*GST

(A) Salt–induced inhibition of enzymatic activity of *Pv*GST. The protein concentration was kept as 2 µM. (B) Far–UV CD data at 222 nm for *Pv*GST with different salts used.

4.3.6 Urea–induced alteration in molecular characteristics of *Pv*GST

To confirm that the three-state equilibrium unfolding of *Pv*GST is not due to modulation of ionic interactions in enzyme by GdnHCl, urea induced unfolding
studies with and without NaCl were carried out. The results confirmed the non-cooperative nature of the enzyme.

Figure 4. Urea–induced unfolding
(A) Urea–induced unfolding followed by monitoring CD ellipticity at 222 nm (■) tryptophan fluorescence (□). (B) ANS binding curve in urea denaturation. (C) Comparison of various structural probes for urea–induced denaturation of PvGST monitored by Far–UV CD(□), tryptophan fluorescence (○), and ANS binding(□). (D) Effect of protein concentration on the Urea–induced unfolding. The concentration of PvGST was 1 μM (○) and 8 μM (■) and denaturation was monitored by Far–UV CD. (E) Urea–induced
transition curves of PvGST recorded by Far-UV CD in the absence (○) and presence of 200 mM NaCl (Δ). The GdnHCl–induced unfolding curve (□) is plotted alongside for comparison. The percent fraction unfolded (F_u) was calculated the same way as that of GdnHCl-induced denaturation (Figure 4.7D).

The change in Far-UV CD ellipticity at 222 nm of PvGST samples as a function of urea also showed a three-state unfolding transition as that of the GdnHCl, with stabilization of an intermediate at around 5 M urea, and at urea concentration above 8.5 M, the protein was completely unfolded (Figure 4.7A). The ANS binding experiment also confirmed the presence of an intermediate at the transition region (Figure 4.7B). Figure 4.7A also shows the biphasic tryptophan fluorescence transition curve with increasing urea concentrations which is similar to that of loss of secondary structure as observed by Far-UV CD. The urea–induced denaturation curves obtained through various structural probes is plotted in Figure 4.7C. The protein concentration dependence of the urea–induced unfolding transitions showed that at increased enzyme concentration the second transition of the unfolding curve shifts to a higher urea concentration, while the first transition remains unaltered (Figure 4.7D). Taking together all the above results, it can be concluded that the unfolding of PvGST is a three–state process with stabilization of an intermediate and that the unfolding is independent of the denaturant used. We also investigated the urea–induced unfolding of PvGST in the presence of NaCl, to determine if the presence of salt affects the unfolding of PvGST. The urea–induced unfolding curves of PvGST in the absence and presence of 200 mM NaCl are shown in Figure 4.7E. It can be inferred from the curves that the presence of NaCl causes the denaturation curve to shift towards a lower urea concentration.

4.3.7 Irreversible thermal unfolding of PvGST

To determine the thermal stability of PvGST, temperature–induced unfolding transition was studied by monitoring the Far-UV CD signal at 222 nm as a function of increasing temperature (Figure 4.8). A thermal denaturation curve with a T_m of about 62°C was observed for dimeric PvGST. Unfortunately, the protein was found to precipitate at the end of the experiment at high
temperature. Due to this the T<sub>m</sub> was found to shift with change in scan rate, and exact T<sub>m</sub> value associated with the thermal denaturation could not be determined. Thus the irreversible nature of the thermal transitions prevented further thermodynamic analysis of the data.

Figure 4. 8– Thermal unfolding of <i>PvGST</i>
Effect of temperature on the CD ellipticity of <i>PvGST</i> was studied at 222 nm. The red and blue lines represent 2 μM <i>PvGST</i> samples at scanned at scanning rates of 1.0°C/min, and 0.5°C/min, respectively. A linear extrapolation of the baselines in the pre- and post–transition region was used to determine the fraction of folded protein within the transition region by assuming a two–state mechanism of unfolding. The data have been presented in percent fraction folded. The percent fraction unfolded (F<sub>u</sub>) was calculated from the equation F<sub>u</sub> = 100 × (F<sub>obs</sub> - F<sub>n</sub>) / (F<sub>u</sub> - F<sub>n</sub>), where F<sub>obs</sub> is the observed value of the signal at a given denaturant concentration and F<sub>n</sub> and F<sub>u</sub> are the values of the native and unfolded protein respectively.

4.4 DISCUSSION
Despite the fact that the overall topological architecture of the GST family proteins is highly conserved, the unfolding property vary extensively from two–state to three–state to even multi–state behavior, strengthening the basic notion of protein folding that the amino acid sequence holds the key for the protein to adopt its native structural fold. The complexity of GST folding is so high that even the members of the same class demonstrate completely different folding pattern. Along with its <i>falciparum</i> counterpart, <i>PvGST</i> has not been assigned to any of the existing GST class due to major structural and functional


Plasmodium vivax Glutathione S-transferase
differences and is identified as a potential target for malaria therapy (Fritz-Wolf et al., 2003; Hiller et al., 2006; Na et al., 2007). Our present studies reveal that the unfolding property of PvGST is peculiar and completely different from PzGST by the presence of an inactive dimeric intermediate during the unfolding pathway. This dimeric intermediate further unfolds and dissociates during the second transition. No monomeric intermediate is stabilized during the unfolding process suggesting that the intersubunit interactions play an important role in the stability of PvGST. Salts inhibit the enzymatic activity of PvGST by decreasing the nucleophilicity of enzyme bound GSH. In extension with our previous paper (Tripathi et al., 2007), we conclude that the regulation of GST activity via reversible dimer-tetramer transition is a peculiar feature of Plasmodium genera.

We have performed the solvent-induced denaturation studies of PvGST to understand the unfolding pathway and identify the structural intermediate stabilized during the process. The CD data at 222 nm and tryptophan fluorescence emission maxima at increasing GdnHCl concentrations profiles were super imposable and showed that both GdnHCl- and urea-induced unfolding of PvGST is consistent with a three-state mechanism, with stabilization of an inactive intermediate. The increase in ANS fluorescence intensity in the transition region of the denaturation curves confirms the presence of an intermediate. Typically, the hydrophobic dye 8-anilino-1-naphthalenesulfonic acid (ANS) does not bind to the folded or unfolded protein, but its fluorescence intensity increases upon binding to the partially structured folding intermediates (Matulis et al., 1999; Matulis and Lovrien, 1998; Semisotnov et al., 1991).

According to law of mass action– the molecularity of a reaction is dependent on the protein concentration. The unimolecular reaction (and therefore the probes monitoring these reactions) should be protein concentration independent while the bimolecular reaction should be dependent on the protein concentration. This dependence has been used as a diagnostic tool for distinguishing the molecularity of the reaction and thereby dissociation of the subunits of the proteins. The protein concentration dependent denaturation studies showed that the first transition is protein concentration independent, while the second transition is

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protein concentration dependent thereby indicating that the subunits dissociate occurs along with unfolding during the second transition.

The enzymatic inactivation of \( Pv\)GST under salts precedes conformational and structural changes. The aromatic nucleophilic substitution reaction of GST activity follows an addition–elimination mechanism. The nucleophilicity of the thiolate anion of GSH is enhanced by the Tyr9 of the G–site, where the SH group of the Cys moiety of GSH is stabilized. In the presence of hydrated salts, the nucleophilicity of the GS\(^{-}\) anion is quenched and the activity of the enzyme is decreased. The analysis of these results as a function of ion class according to Hofmeister’s series suggests that the loss of activity is higher as the tendency to accumulate water molecules increases and the ionic radii decreases (Zhang and Cremer, 2006).

To mimic the screening effect of charge–charge interactions exerted by GdnHCl, we preformed urea–induced unfolding of \( Pv\)GST in the presence of NaCl. The presence of Na\(^{+}\) and Cl\(^{-}\) ions cause a general screening effect of favourable electrostatic interactions among charged groups on the protein surface. The results from this experiment indicate that (a) the conformational stability of \( Pv\)GST depends on the ionic strength of the solution, indicating that electrostatic interactions play an important role, (b) the presence of salt does not alter the unfolding pathway of \( Pv\)GST, and (c) the denaturing action of GdnHCl cannot be mimicked by the combined action of urea and NaCl. Taken together all the above results it can be concluded that no compact, folded monomeric intermediate is formed during the unfolding process of dimeric \( Pv\)GST, thereby suggesting that the intersubunit interactions play an important role in the stability of the protein.

In the chapter III we have established that the \( P. falciparum\) GST undergoes a reversible dimer–tetramer transition depending on the concentration of GSH. In the absence of GSH, \( P\)GST dimers self–associate to form homotetramers and this change in oligomeric status is associated with the loss of enzymatic activity (Tripathi et al., 2007). Gel filtration and enzymatic activity profile of the recombinant \( Pv\)GST protein showed similar results to that of \( P\)GST (Tripathi et al., 2007). These results collectively suggest that the \( Pv\)GST shows similar GSH dependent regulation of oligomeric status and functional activity as
*P*GST. Thus, we conclude that the regulation of enzymatic activity through dimer–tetramer transition is an exclusive feature of the *Plasmodium* genera and is exhibited by the species of *Plasmodium*. This phenomenon is unique as no such regulation is observed for any of the classes of GST. Consequently, this behavior can be exploited for better understanding the biology of malarial parasites and development of chemotherapeutic strategies.

### 4.4.1 Comparative analysis of *Pv*GST and *Pf*GST unfolding

The unfolding of *Pv*GST was found to be completely different from *Pf*GST (Tripathi et al., 2007) in being non-cooperative, though the proteins share sequence similarity of approximately 85% (Figure 4.9).

![Sequence alignment of *Pv*GST, *Pf*GST and human GST](image)

**Figure 4.9** - Sequence alignment of *Pv*GST, *Pf*GST and human GST

The sequences of *Plasmodium falciparum* glutathione S-transferase (*Pf*GST, Swiss-Prot acc. no. Q8MU52), *Plasmodium vivax* glutathione S-transferase (*Pv*GST, Swiss-Prot acc. no. Q0ZS46), human glutathione S-transferase (hGST, Swiss-Prot acc. no. P09488), were aligned with ClustalW.

Investigation of the literature revealed that this fact is a common phenomenon of GST unfolding as different GSTs from a same class have been reported to unfold via different pathways. Table II summarizes the unfolding pathways of dimeric GSTs from various classes. The pi class GSTPI-I from
Porcine lung unfolds cooperatively without any unfolding intermediate (Dirr and Reinemer, 1991; Erhardt and Dirr, 1995), while GSTPI-I from human placenta and *Bufo bufo* unfolds non-cooperatively (Aceto et al., 1992; Sacchetta et al., 1999) with stabilization of an intermediate. Similarly the mu-class GST from *Schistosoma japonicum* unfolds cooperatively (Kaplan et al., 1997), while GSTM1-1 and GSTM2-2 from rat unfolds through an unfolding intermediate (Hornby et al., 2000).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Class</th>
<th>Organism</th>
<th>Unfolding pathway</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSTPI-1</td>
<td>π</td>
<td>Porcine</td>
<td>D → U</td>
<td>Dirr and Reinemer,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1991; Sacchetta et al., 1993</td>
</tr>
<tr>
<td>GST</td>
<td>π</td>
<td><em>H. sapiens</em></td>
<td>D → M → U</td>
<td>Aceto et al., 1992</td>
</tr>
<tr>
<td>GSTBI-I</td>
<td>β</td>
<td><em>P. mirabilis</em></td>
<td>D ↔ D* ↔ M → U</td>
<td>Sacchetta et al., 1993</td>
</tr>
<tr>
<td>SGST</td>
<td>μ</td>
<td><em>S. japonicum</em></td>
<td>D → U</td>
<td>Kaplan et al., 1997</td>
</tr>
<tr>
<td>GSTSI-I</td>
<td>σ</td>
<td>Squid</td>
<td>D ↔ D* ↔ M → U</td>
<td>Stevens et al., 1998</td>
</tr>
<tr>
<td>GSTAI-I</td>
<td>α</td>
<td><em>H. sapiens</em></td>
<td>D → U</td>
<td>Wallace et al., 1998</td>
</tr>
<tr>
<td>GSTPI-1</td>
<td>π</td>
<td><em>B. bufo</em></td>
<td>D ↔ D* ↔ M → U</td>
<td>Sacchetta et al., 1999</td>
</tr>
<tr>
<td>GSTM1-1</td>
<td>μ</td>
<td><em>R. rattus</em></td>
<td>D → M → U</td>
<td>Hornby et al., 2000</td>
</tr>
<tr>
<td>GSTM2-2</td>
<td>μ</td>
<td><em>R. rattus</em></td>
<td>D → M → U</td>
<td>Hornby et al., 2000</td>
</tr>
<tr>
<td>PGST3</td>
<td>σ</td>
<td><em>P. acuta</em></td>
<td>D ↔ D* ↔ D** ↔ M → U</td>
<td>Abdalla and Hamed, 2006</td>
</tr>
<tr>
<td>PGST</td>
<td>-</td>
<td><em>P. falciparum</em></td>
<td>D → U</td>
<td>Tripathi et al., 2007</td>
</tr>
<tr>
<td>EcGST</td>
<td>β</td>
<td><em>E. coli</em></td>
<td>D ↔ D* ↔ M → U</td>
<td>Wang et al., 2008</td>
</tr>
<tr>
<td>P-GST</td>
<td>-</td>
<td><em>P. vivax</em></td>
<td>D ↔ D* ↔ U</td>
<td>Tripathi et al., 2009</td>
</tr>
</tbody>
</table>
Such heterogeneity in protein unfolding pathway is not restricted only to GST family but is also reported for various other proteins including intracellular lipid-binding proteins (Gunasekaran et al., 2001; Ropson et al., 2008), globins and c-type lysozymes (Gunasekaran et al., 2001). Opportunity is there to dissect out and characterize this unique phenomenon as it will provide deeper knowledge into the question that why the proteins of a same class which are structurally, functionally and evolutionary closely related unfold via different pathways. Few recent studies suggest that only a few residues of the hydrophobic core which make a specific set of native-like contacts are involved in determining the overall architecture of a protein (Dalessio et al., 2005), nevertheless vivid description is lacking and genuine effort is required to illustrate this fact.