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Chemical and Acidic Denaturation of a Homodimeric Glutathione Transferase mu Class from *Rhipicephalus (Boophilus) annulatus*

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ABSTRACT

The equilibrium unfolding of a mu class glutathione transferase from *Rhipicephalus (Boophilus) annulatus* (BaGSTM) has been performed using guanidinium chloride (GdmCl), urea and acid denaturation to investigate the unfolding intermediates. Protein transitions were monitored by intrinsic fluorescence and 8-anilino-1-naphthalenesulfonate (ANS) binding. The results indicate that unfolding of BaGSTM using GdmCl (0-4.0 M) is a multi-step process, i.e., at least two intermediates coexist in equilibrium. The first intermediate, a partially dissociated dimer, exists at low GdmCl concentration (less than 1.5 M). This intermediate undergoes dissociation into two monomers at GdmCl concentration between 1.5 and 2.0 M. The monomeric intermediate started to be completely unfolded at higher GdmCl concentrations (> 2.0 M). Unfolding using urea (0-8.0 M) and acid-induced structures as well as the ANS fluorescence in presence of different concentrations of GdmCl or urea confirmed that the unfolding is a multi-step process. The formation of a molten globule state (a monomeric intermediate) at pH less than 3.8 was suggested by the strong enhancement of fluorescence of ANS and protein concentration dependent.

Key Words: Glutathione transferase, *Boophilus annulatus*, fluorescence, folding, unfolding, subunit interactions, intermediates.

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INTRODUCTION

Cytosolic glutathione transferases (GST; EC 2.5.1.18) comprise a family of multifunctional dimeric proteins, which catalyze conjugation of glutathione to a large variety of endogenous and exogenous electrophilic compounds *(Mannervik and Danielson, 1988 and Armstrong, 1997)*. The structure and functions of mammalian and non-mammalian cytosolic glutathione transferases (GSTs) have been the subject of numerous investigations and the multitude of forms so far characterized can be grouped into several species-independent gene classes, namely, alpha, mu, pi, theta, sigma, zeta and Omega *(Board et al., 2000 and Sheehan et al., 2001)*. GST isoenzymes belonging to the same class show 60-70% identity in their primary structure, whereas GST isoenzymes belonging to different classes have less than 30% sequence identity. All cytosolic GSTs are homo- or heterodimers of subunits whose molecular masses are in the 23- to 27-kDa range *(Mannervik and Danielson, 1988 and Armstrong, 1997)*. According to X-ray crystallographic analyses, alpha, mu, pi, theta and sigma GST monomers show an overall similar structural organization, i.e., two domains joined by a short linker of six or seven amino acid residues. Domain I (GSH-binding site), which is mainly located in the N-terminal region of the protein, is an α/β structure. GST domain II provides most of the hydrophobic binding sites (H-site) and it is formed by α–helices *(Sinning et al., 1993, Wilce et al., 1995 and Ji et al., 1995)*. Cytosolic GSTs can be grouped according to the features at their subunit interfaces. The classes alpha mu/pi group have a prominent hydrophobic lock-and-key type interaction at each end of the subunit interface, the key being a phenylalanine/tyrosine side chain in domain I of one subunit and the lock being a hydrophobic pocket in domain II of the other subunit. On the other hand, GSTs in the class sigma/theta group lack this interaction and their subunit interface are more hydrophilic *(Ji et al.,1995 and Stevens et al., 1998)*. The cattle tick *Rhipicephalus (Boophilus) annulatus* GST (*Ba*GSTM) is a member of mu class, the overall sequence homology shared between *Ba*GSTM and mammalian mu class is at least 53% and the degree of similarity is much higher at the N-terminus than the C-terminus among GSTs

which is common in GST families *(Shahein et al., 2008)*. Proteins "folded" in a certain structural conformational state provide the function necessary to participate in biological processes. The conformational states of a protein consist of an ensemble of stable folded states and a subset ensemble of conformationally "unfolded" or unstable states where a protein is no longer functionally active and is more labile to aggregation processes *(Ptitsyn, 1995 a, b and Staniforth et al., 1998)*. The analysis of unfolding and refolding intermediates should give useful information for our understanding of the protein folding mechanism. Here, we explored the unfolding of *Ba*GSTM in presence of different concentrations of guanidinium chloride and urea as well as at acidic pH condition. It seems that the unfolding of *Ba*GSTM is a multi-step process and low pH conditions induce the formation of a state with some properties similar to molten-globule intermediate.

MATERIALS AND METHODS

Expression of Recombinant *Ba*GSTM:

*Ba*GSTM was expressed as described by *Shahein, et al. (2008)* with a slight modification. E. coli BL21, using the prokaryotic expression vector pET3b. Clones were grown in 5 ml LB media at 37°C overnight. This culture was used for inoculation of 500 ml LB medium in 2-L Erlenmeyer flask and placed in a rotary shaker operated at 150 rpm at 37°C. At an OD600 of 0.5, isopropyl-β-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM. The culture was grown for ~20 h and the bacteria were collected by centrifugation for 10 min at 7000×*g* and re-suspended in lysis buffer, containing 20 mM potassium phosphate, pH 7.0, 2 mM EDTA and 1 mg/ml chicken egg-white lysozyme. After sonication (4×20 seconds), the soluble fraction was obtained by centrifugation at 30000×*g* for 20 min. This process was repeated once and the pooled lysate was centrifuged at 30000× *g* for 45 min.

Preparation of GSH-Sepharose and Enzyme Purification:

Glutathione was coupled to epoxy-activated Sepharose 6B according to *Simons* and *Vander Jagt (1977)*. The enzyme was purified from *E. coli* lysate in one step by GSH-Sepharose affinity matrix. Unbound material was washed using 20 mM potassium phosphate buffer, pH 7.0 containing 150 mM NaCl. Enzyme was eluted from the column by 50 mM Tris-HCl buffer, pH 9.5 containing 25 mM GSH. The homogeneity was checked using sodium dodecyl sulphate (SDS-PAGE) (12%) with subsequent commassie blue staining according to the method of *Laemmli (1970)*. The protein was concentrated by lypholyzation after dialysis. Gel filtration was used to remove GSH if present.

Glutathione Transferase (GST) Assay:

The GST activity was determined spectrophotometrically with 1-chloro-2,4- dinitrobenzene as substrate by monitoring the change in absorbance due to thioether formation at 340 nm at 25°C as described by *Habig et al. (1974)*. One unit of transferase activity is defined as the amount of enzyme which catalyzes the formation of 1 µmol of thioether per min and the specific activity is expressed as µmol/min/mg protein.

Protein Determination:

Protein was determined according to the method described by *Bradford (1976)* with bovine serum albumin as a standard.

Unfolding Monitored by Intrinsic Fluorescence:

Measurements of the intrinsic fluorescence of the proteins were performed at room temperature using Jasco FP-777 SPF-500 spectrofluorometer (Japan). The excitation and emission band width were both set to 5 nm. Fluorescence was measured by excitation of all aromatic residues at 280 nm. Spectra at wavelengths 300-360 nm were collected at a scan speed of 60 nm/min. Equilibrium unfolding studies of *Ba*GSTM was measured after the protein samples (20 µg/ml) were incubated at room temperature in 20 mM potassium phosphate buffer, pH 7.0 containing 1 mM EDTA/1 mM dithiothreitol (buffer A) in the presence of different concentrations of urea (0-8 M) or GdmCl (0-4 M) for 1 h before spectral recordings.

Acid Induced Unfolding Monitored by Fluorescence:

Acid inactivation was carried out at room temperature by equilibrating the protein (20 µg/ml) in 0.02 M citrate-phosphate buffer (pH 1.2 to 7.0) for 60 min. Fluorescence was measured by excitation of all aromatic residues at 280 nm. Spectra at wavelengths 300-400 nm were collected at a scan speed of 60nm/min.

Unfolding Monitored by 8-anilino-1- Naphthalenesul-Fonate Fluorescence:

The binding of 8-anilino-1-naphthalenesulfonate (ANS) to the *Ba*GSTM was studied using fluorescence at room temperature as a function of different pH (1.2 to 7.0), or at different concentrations of GdmCl or urea. 100 µM of ANS was added to protein samples (20 µg/ml) previously incubated for 1 h at different pH or in buffer A in the presence of different concentrations of GdmCl (0-4M) and urea (0-8M). The reaction mixture was excited at 380 nm and fluorescence intensity was measured at 480 nm. All intensities were corrected for blank.

RESULTS

Expression and Purification:

*Ba*GSTM was purified in a single chromatographic step using affinity chromatography on GSH-Sepharose. All enzymes were eluted using 20 mM GSH. The enzyme represented approximately 20% of total soluble protein in the bacterial lysate. The homogeneity was checked using SDS-PAGE (Figure 1). The specific activity of the enzyme was about120 µmol/min/mg protein.

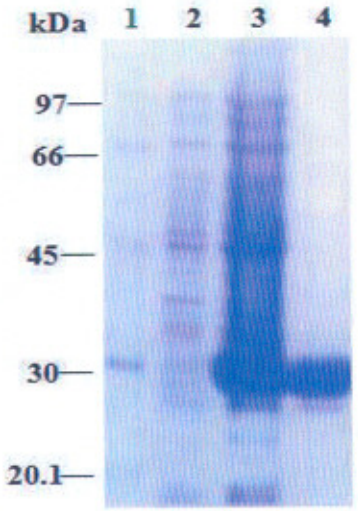


Figure 1: 12 % SDS-PAGE of expressed and purified recombinant GST from the cattle tick *B. annulatus*. Lane (1) molecular weight marker, lane (2) bacterial lysate of induced BL21/pET30b (empty vector), lane (3) bacterial lysate of induced BL21/pET30b with recombinant GST and lane (4) purified GST.

Unfolding Transition as a Function of GdmCl Concentration Monitored by Intrinsic Fluorescence:

Class mu GST from *Boophilus annulatus* contains four tryptophan residues (Trp 7, Trp 45, Trp 110 and Trp 214) and ten tyrosine residues *(Shahein et al., 2008)*. The enzyme in its native state exhibits an emission spectrum with a maximum at 329 nm (excitation 280 nm). This feature is a characteristic of tryptophan residues partially buried in the protein matrix (Figure 2 A). As shown in figure 2, compared with the native dimer, the fluorescence intensity increased with a slight red shift of λmax as the GdmCl concentration was increased. The intensity reached a maximum at approximately 1.45 M (partially unfolded dimer or nonnative dimeric intermediate). At GdmCl concentration between 1.5 M and 1.9 M there was no change in the fluorescence intensity or in λmax. The nonnative dimeric intermediate undergoes dissociation into monomeric intermediate at these GdmCl concentrations. Increasing the GdmCl concentration leads to another increase in the fluorescence intensity with a red shift of λmax and the intensity reached a maximum at approximately 2.4 M. This might be due to the formation of a partially unfolded monomer. After this concentration, the intensity started to decrease with a red shift of λmax and at 4.0 M GdmCl, a λmax of 352 nm occurred, indicating the complete exposure of the tryptophan residues to the aqueous solvent which is consistence with the complete unfolding of the protein (unfolded monomer). From these results, at least two transition states between the native dimer and unfolded monomer could be identified for *Ba*GSTM.

Unfolding Transition as a Function of Urea Concentration Monitored by Intrinsic Fluorescence:

As shown in Figure 3, there was an increase in the fluorescence intensity as the concentration of urea increased from 0 to 1.75 M without any detectable shift of λmax. The intensity of fluorescence was increased by 50% at 1.75 M urea concentration compared with the native state of the protein indicating a partial exposure of the fluorophore (first phase). Increasing the concentration of urea, between 2.0 M and 3.0 M, resulted in a slight red shift (by 3 nm) of the emission maximum (second phase). Whereas, the increase in fluorescence intensity decreased as the concentration of urea was increased. The fluorescence intensity at the end of the first phase was higher than that at the end of the second phase. This indicating the movement of the fluorophore back into a more hydrophobic environment. At higher urea concentration, (between 3.25 and 4.5 M) the fluorescence intensity started to increase again with a shift of λmax (10 nm red shift). The fluorescence intensity was increased by three fold at 4.5 M urea compared to that of the native protein (third phase). Addition of higher concentrations of urea (5.0-7.0 M) did not change the intensity of the fluorescence significantly compared to that at 4.5 M urea but progressively shifted the λmax to 347 nm. At 8.0 M urea, the fluorescence intensity was decreased again with a shift of λmax to 352 nm indicating the complete unfolding of the protein. The present results indicate that three intermediates could be identified between the native dimer and unfolded monomer during the unfolding of *Ba*GSTM.

pH-induced Unfolding Monitored by Fluorescence:

To investigate the behaviour of the protein in an acidic environment, analyses of fluorescence emission spectra of *Ba*GSTM in solutions at different pH values were performed. The position of the emission maximum of a protein>s fluorescence spectrum, upon excitation at 280 nm, was highly sensitive to the environment around its tryptophanyl and tyrosyl residues. The acid denaturation of *Ba*GSTM, as followed by the intrinsic fluorescence changes, was characterized by the presence of at least three transition states (Figure 4). At pH 7.0, the fluorescence spectrum of the protein at 0.4 µM concentration showed an emission maximum at 329 nm. The λmax of the protein fluorescence

did not changed by lower the pH to 5.8. However, at pH between 5.8 and 4.6 the fluorescence intensity was characterized by a blue shift of the emission maximum. The maximum blue shift was 326 nm at pH 5.0, suggesting that the tryptophan and tyrosine residues become more buried in a hydrophobic environment and the protein was partially unfolded to compact structure. At pH between 4.6 and 3.8 the fluorescence intensity was increased as the pH decreased but the emission maximum was the same as that of the protein at pH 7.0 (partially dissociated dimer). A red shift of λmax to 332 was observed at pH 3.4 with more than one and half fold increase in fluorescence intensity compared with that at pH 7.0. By lowering the pH to 3.0-2.6, the intensity of the fluorescence was increased by 3.5 fold with a red shift of the emission maximum to 335 nm. This indicates the exposure of tryptophan residues to a more hydrophilic environment and the dimeric protein might be dissociated into two monomers.

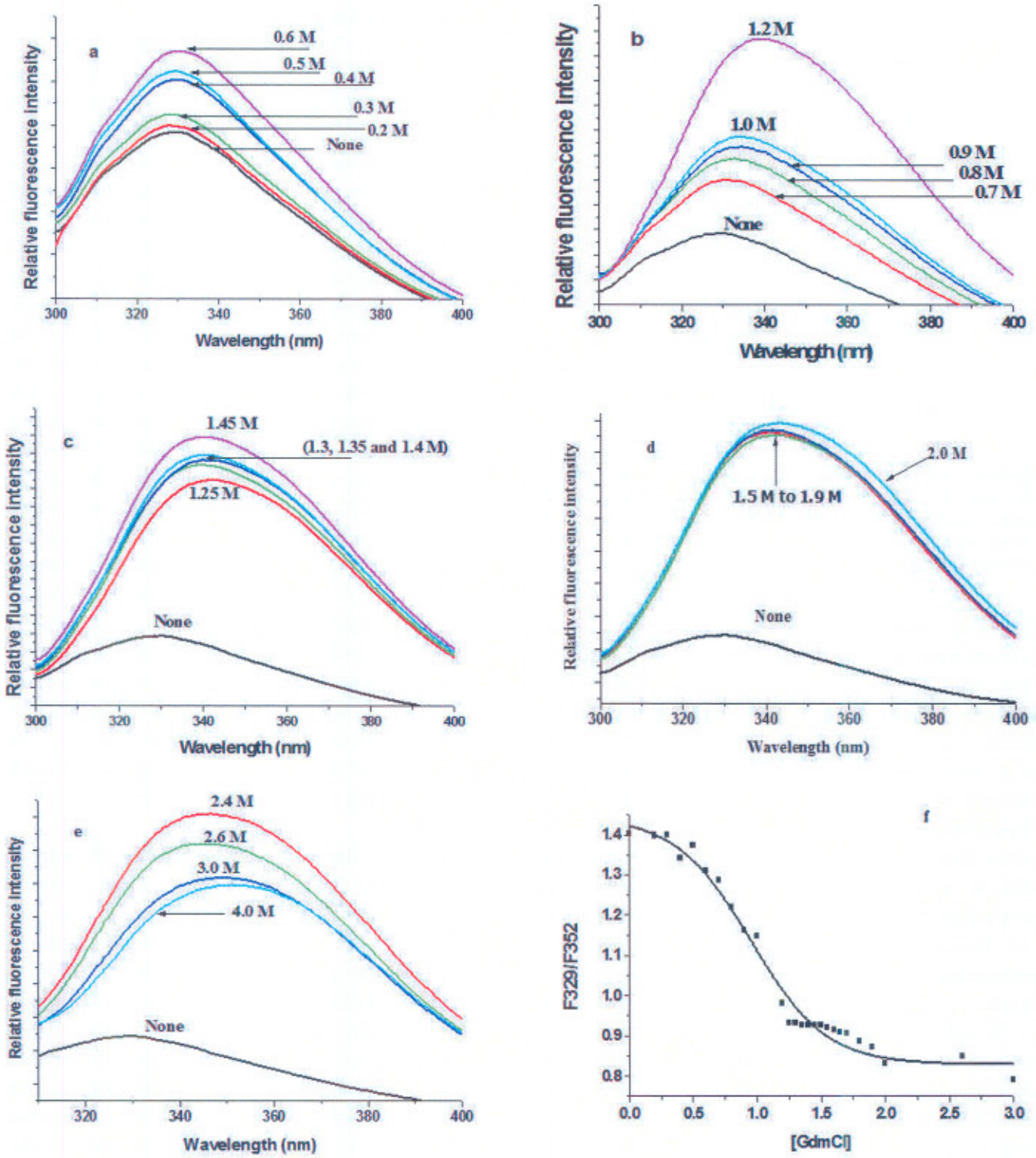


Figure 2: Fluorescence–emission spectra of BaGSTM (20 µg/ml) equilibrated in buffer A at different GdmCl concentrations ranging from 0 to 4.0 M at room temperature. Excitation was done at 280 nm and fluorescence was recorded from 300 to 400 nm (a to e). Unfolding was expressed as the ratio of fluorescence at 329 nm to the fluorescence at 352 nm (f).

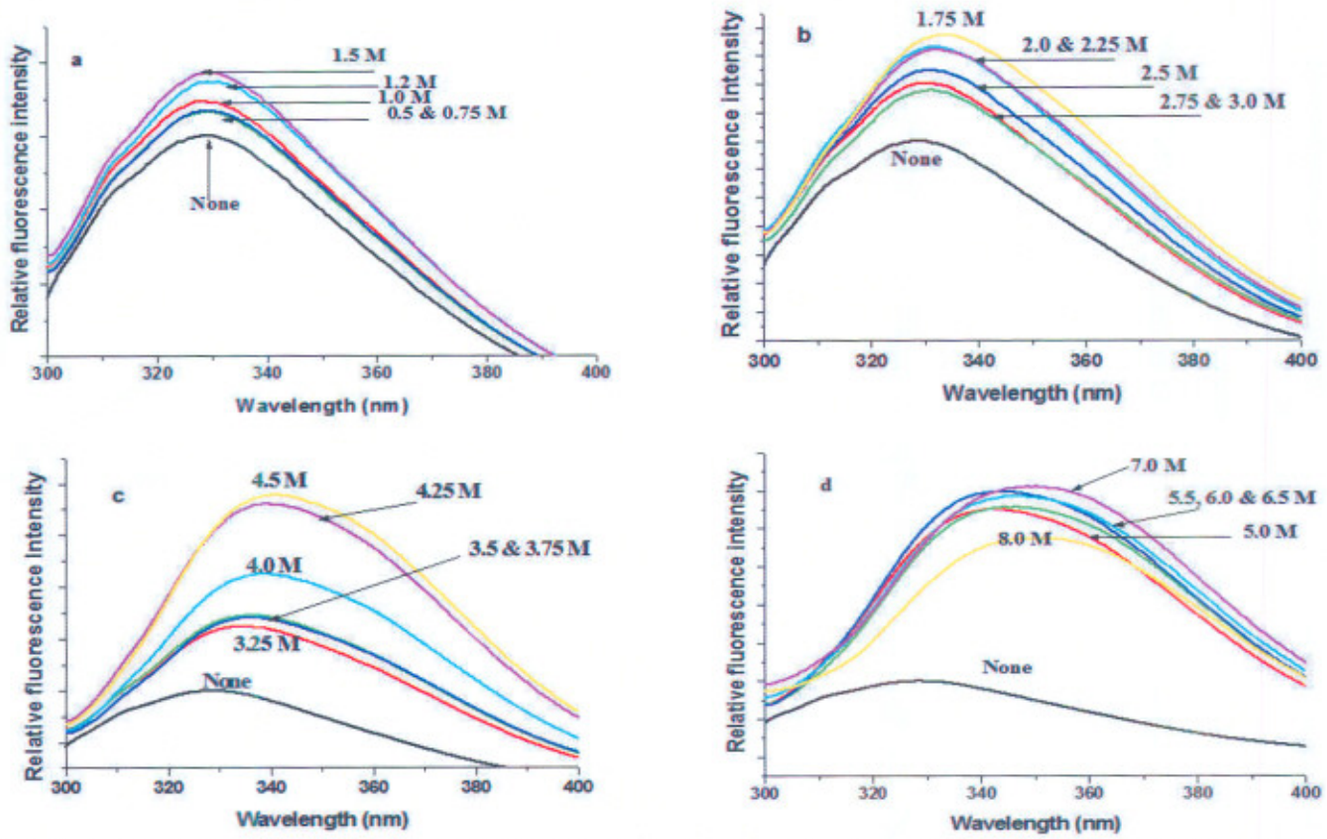


Figure 3: Urea-induced equilibrium unfolding for BaGSTM monitored by fluorescence. The protein (20µg/ml) was equilibrated in buffer A in the presence of the indicated concentration of urea at room temperature. Excitation was done at 280 nm and fluorescence was recorded from 300 to 400 nm.

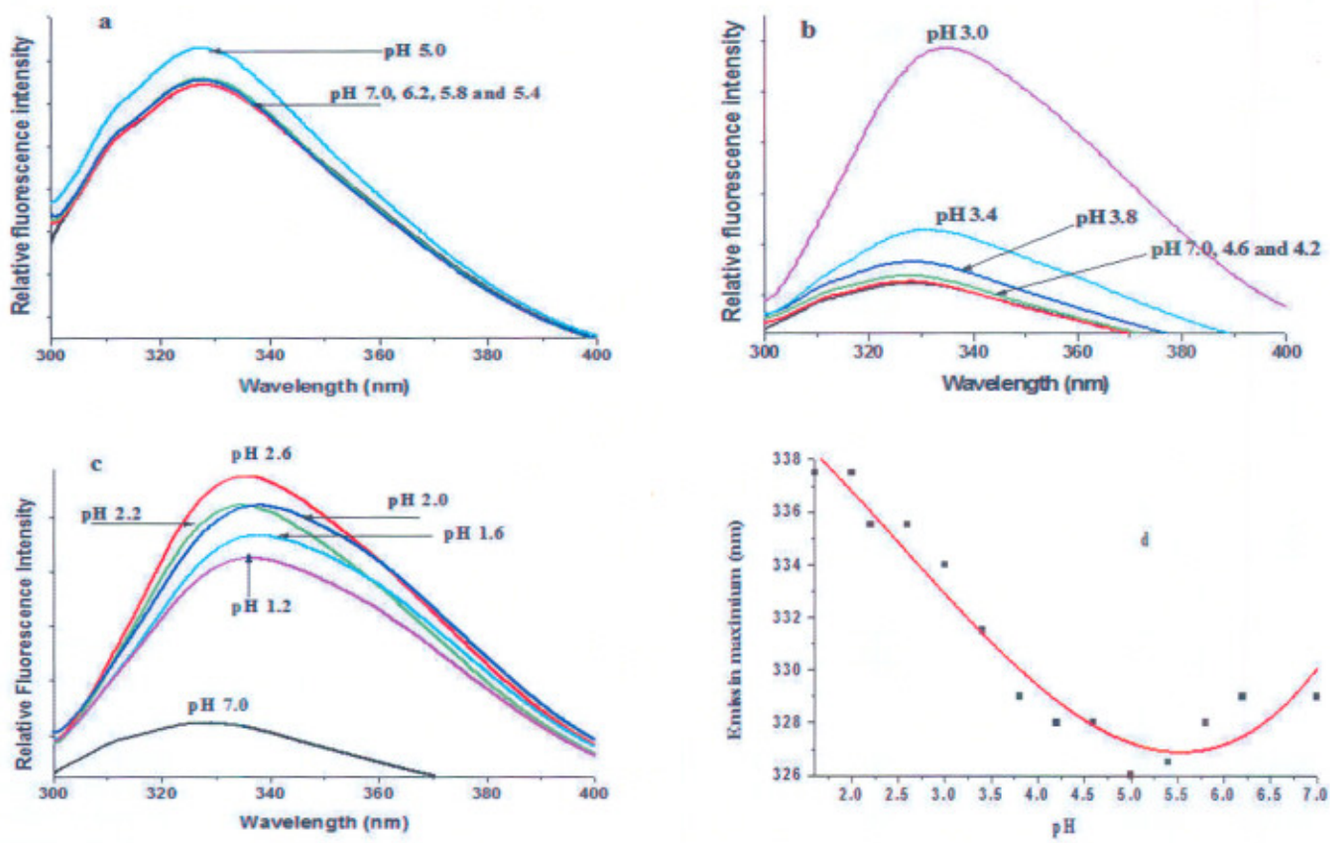
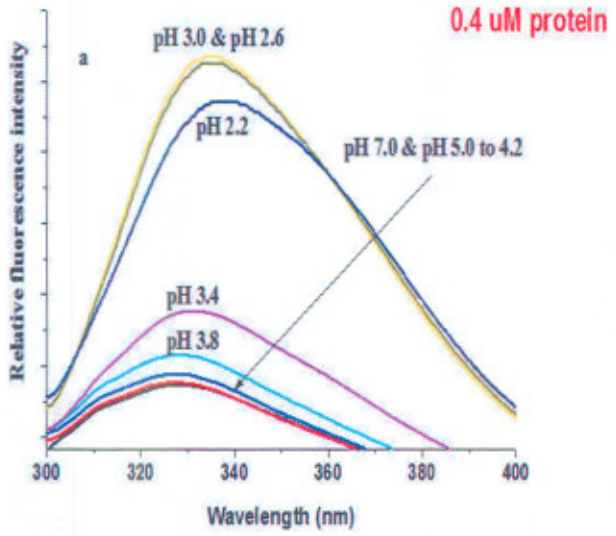
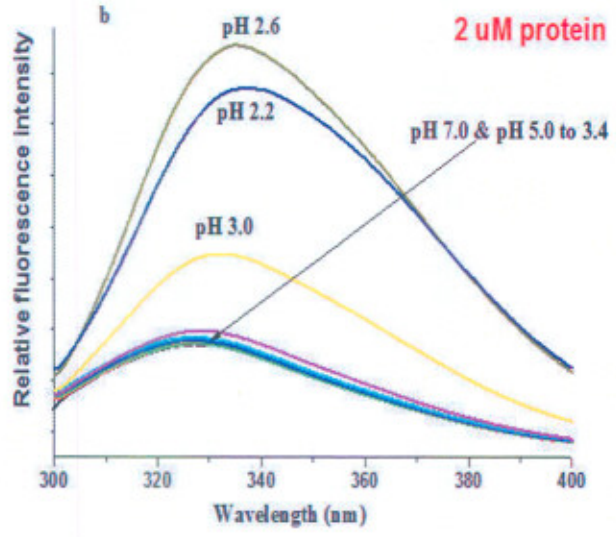


Figure 4: pH-induced equilibrium unfolding for BaGSTM monitored by fluorescence. The protein (20µg/ml) was equilibrated at room temperature in 0.02 M citrate-phosphate buffer at pH from 7.0 to 1.2 for 1 h before measurement. Excitation was done at 280 nm and fluorescence was recorded from 300 to 400 nm (a-c). Emission maxima were determined using the same excitation wavelength (d).





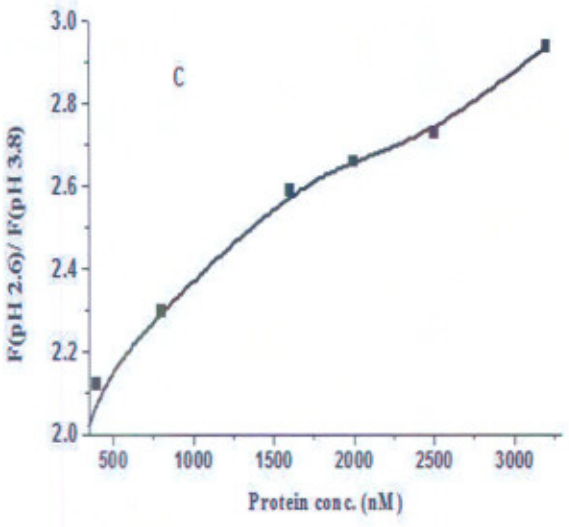
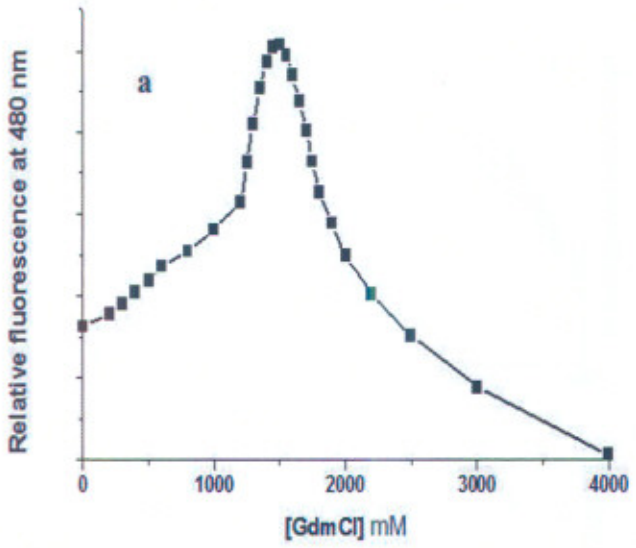
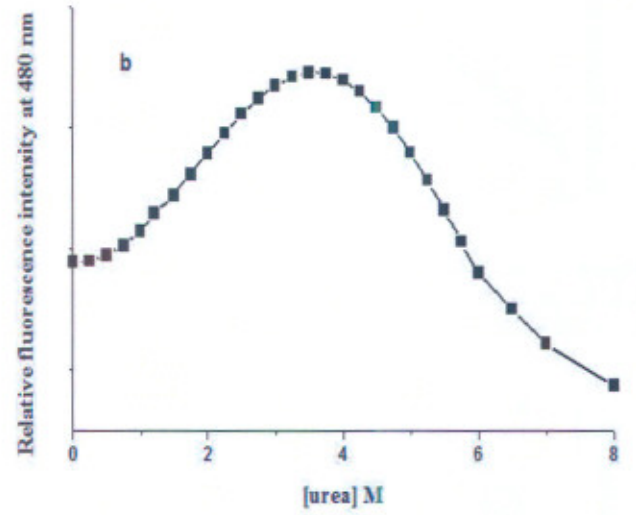


Figure 5: Effect of BaGSTM protein concentration on acid-induced unfolding. The indicated protein concentration was incubated at different pH at room temperature for 1 hour in 0.02 M citrate buffer. Excitation was done at 280 nm and fluorescence was recorded from 300 to 400 nm (a and b). Unfolding was expressed as the ratio of maximum fluorescence intensity at pH 2.6 to the maximum fluorescence at pH 3.8 (c) at different protein concentrations.





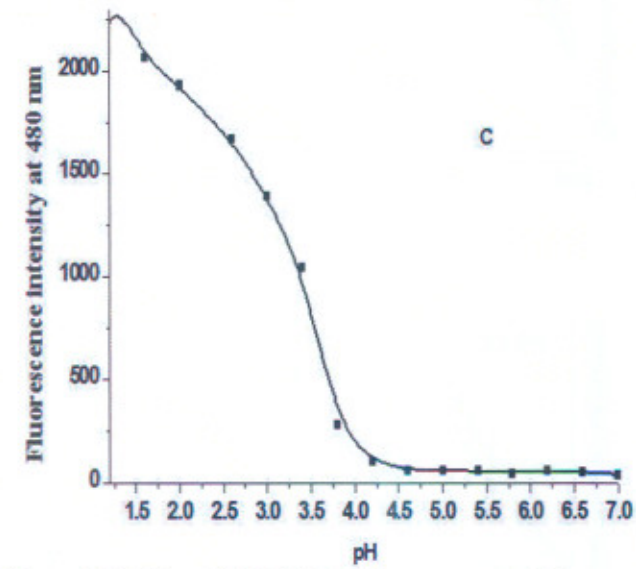


Figure 6: Variation of ANS binding fluorescence at 480 nm as a function of GdmCl concentrations (a) or urea (b) or as a function of pH (c). The proteins (20 µg/ml) were equilibrated in buffer A at room temperature in the presence of the indicated concentration of denaturant or pH. 100 µM ANS was added to the solution. Excitation was done at 380 nm and emission at 480 nm.

Effect of Protein Concentration on pH-Induced Unfolding Monitored by Fluorescence:

The protein concentration dependence of the unfolding of *Ba*GSTM was monitored by fluorescence at different pH's. The concentration of the protein was 0.2-and 2μM, respectively (Figures 5 A,B). The result showed that there was protein concentration dependent step on the pH-induced unfolding of *Ba*GSTM at low pH. This result was confirmed by measuring the relative fluorescence intensity at two pH values (2.6 and 3.8) and at protein concentrations ranging from 0.2 to 3.2 μM (Figure 5 C). The relative fluorescence intensity (F (pH2.6)/F(pH3.8)) was increased as the protein concentration increased. These results indicate that at low pH the dimeric protein dissociates into compact monomers displaying enhanced hydrophobicity.

Unfolding Monitored by ANS Bonding Fluorescence:

ANS binding to the enzyme was used to probe structural changes in the enzyme conducted at different GdmCl and urea concentrations. Unbound ANS emission spectra showed a maximum at 530 nm that was blue shifted upon binding of the dye to the protein. Binding of ANS to *Ba*GSTM as a function of GdmCl concentration showed one peak centered at 1.5 M and one peak as a function of urea concentrations centred at 3.5 M (Figures 6 A and B). ANS binding fluorescence of *Ba*GSTM as a function of pH did not show any transition peak. However, the fluorescence intensity was increased as the pH decreased. The fluorescence intensity about 2000 fold higher at pH 2.0 compared with that at neutral pH (Figure 6 C).

DISCUSSION

The nature of protein folding mechanisms and the manner in which the compact native state is achieved are still not well understood. From a wide range of experiments, it is now evident that specific pathways of folding are involved, at least for many proteins. At equilibrium, most monomeric and many oligomeric proteins display essentially a two-state pathway upon folding/unfolding, for which thermodynamically stable folding intermediates do not exist. Other mechanisms result in the formation of stable intermediates. These monomeric intermediates sometimes have preserved tertiary structure or appear as molten globules *(Eftink et al., 1994, Ptitsyn, 1995 a and b)*. For proteins composed of subunits, the intermediates are either partially folded oligomeric states or monomeric states. Full understanding of the protein folding process requires the identification and characterization of all intermediate steps, which are often very transient and detected by kinetic studies only. In these cases, some properties of the intermediates can be inferred, but little structural information can be derived from this approach. It is known that mild denaturing conditions, such as moderately high temperature or low pH, promote partially unfolded states that are similar to those observed at moderate concentrations of guanidinium chloride *(Ptitsyn, 1995a)*. Therefore studies have been performed on these partially folded states *(Ausili et al., 2005)*. Some of these more or less stable intermediates, called "molten globules"are characterized by a largely conserved secondary structure but loss of tertiary structure and due to the presence of a loosely packed hydrophobic core, binding of ANS is often observed *(Kuwajima 1989 and Arai and Kuwajima 2000)*. Clear evidence of acidic pH induced stable folding intermediates has been obtained with some lipocalins, such as beta-lactoglobulin *(Ikeguchi et al. 1997 and D'Alfonso et al., 2002)*, retinol binding protein *(Bychkova et al., 1998)* and hGSTP1-1 *(Dragani et al., 2000)*.

Electrostatic interactions between charged residues on the surface of a protein play an important role in conferring stability to its folded structure. Change of pH alters the ionization state of these residues, causing intramolecular charge repulsion and possible disruption of salt bridges that can lead to destabilization of the native protein conformation *(Goto et al., 1990 a)*. pH is an important factor determining protein structure and function. Most proteins are stable and active at physiological pH and show varying degrees of denaturation in acid medium. However, as the acid concentration increases, a number of these proteins revert back to a compact conformation containing substantial secondary structure that resemble the folding intermediates known as molten globules *(Goto et al., 1990 b and Fink, 1995)*. Study of the structural stability of a protein as a function of pH thus helps understand the thermodynamic or kinetic intermediates in its folding pathway and identifies the electrostatic interactions important for the stability of its folded state *(Pace et al., 2000 and Sheinerman and Honig, 2002)*. Because of the non-identity of the different transitions monitored, the acid denaturation of *Ba*GSTPM does not appear to be a simple two-step transition, rather a multi-step process during which several intermediates coexist in equilibrium. In particular, the pH-dependent fluorescence transition of *Ba*GSTPM is clearly characterized by many distinct steps. The blue shift of λmax from 329 to 326 nm at pH 5.0 with an increase in the fluorescence intensity indicates the formation of a new type of structure in which the environment of the trypthophanyl residues is more hydrophobic. It has been proposed that the molten globule represents a common intermediate of the acid denaturation of many monomeric proteins *(Ptitsyn, 1995 a, Fink 1995 and Abdalla et al., 2002)*.

GSTs are crystallized as dimers, but in solution class mu GST from rat its Asp97 mutant enzymes undergo reversible association and dissociation, the extent of which depends on

protein concentration. Addition of 3 M potassium bromide to buffer solutions containing the wild-type rGSTM1-1 has generated monomers (GSTM1) *(Hearne and Colman, 2006 a and b)*. A monomeric species of a human GSTpi has been constructed by introducing 10 site specific mutations. This drastically changed enzyme was structurally stable, but retained no activity *(Abdalla et al., 2002)*.

ANS is a negatively charged hydrophobic fluorescent molecule, largely used to check the presence of compact partially folded intermediates. In fact, its very low fluorescence quantum yield in polar environment is strongly increased in non polar solvents *(Turner and Brand, 1968)*. Therefore, the binding of this molecule to partially folded proteins, containing clusters of hydrophobic side chains accessible to solvent, is often observed in the presence of molten globules *(Arai and Kuwajima, 2000)*. At neutral pH, the fluorescence of ANS in the presence of *Ba*GSTM is perfectly super imposable to that of ANS alone. At pH less than 3.8, ANS binds to *Ba*GSTM showed a blue shift displacement with an enhancement of fluorescence intensity. Binding of the dye occurs at the dimer interface and unfolded GST does not bind ANS. This makes ANS an excellent probe to monitor changes at the packing of hydrophobic cores in protein which undergoes structural changes and has been broadly used to study the presence of monomeric intermediates at the urea/GdmCl unfolding of several GSTs *(Bico et al., 1995, Erhardt and Dirr, 1995, Abdalla et al., 2002 and Andujar-Sanchez et al., 2004)*. ANS was also used to detect the presence of folding intermediates with hydrophobic patches such as the molten globule in penicillin G acylase *(Lindsay and Pain, 1990)*, apomyoglobin *(De Young et al., 1993)* and GST from the fresh water snail *Physa acuta (Abdalla and Hamed, 2006)*. The present unfolding results demonstrate that the probes used (intrinsic fluorescence, excitation of tryptophan and tyrosine and ANS binding fluorescence) are differentially sensitive to various conformational states of *Ba*GSTM. The presence of multiple nonsuperimposable transitions for this enzyme indicates that the two states unfolding mechanism is not applicable and it is highly suggestive of the existence of at least two well-populated stable intermediates. The present results agree with those reported in the literature demonstrating the existence of stable intermediates at the unfolding of GSTs *(Aceto et al., 1992, Stevens et al., 1998, Sacchetta et al., 1999, Andujar-Sanchez et al., 2004 and Abdalla and Hamed, 2006)*. However, only one transition intermediate was detected using ANS binding fluorescence for *Ba*GSTM in presence of different concentrations of GdmCl or urea. This result is similar to that observed for sigma class GST in the presence of urea *(Stevens et al., 1998)*.

Although, the intrinsic fluorescence of acid denaturation of *Ba*GSTM is more complex, a multi-step process during which several intermediates coexist in equilibrium was observed. A similar multi-step process was also found for acid induced unfolding of hGSTP1-1 and *Physa acuta* GST3 *(Dragani et al., 2000 and Abdalla and Hamed, 2006)*. Acid denaturation is useful to verify whether the unfolding intermediates observed during the chemical denaturation (urea and GdmCl) of this enzyme are really due to a multi-step process rather than to unspecific effects of the chemical denaturant used *(Dragani et al., 2000)*. Dimeric proteins such as trp aporepressor, aspartate aminotransferase, MM-creatine kinase, aminoacylase and yeast prion protein determinant Ure2 also yield unfolded intermediates *(Eftink et al., 1994, Donate et al., 1998, Leydier et al., 1998, Bai et al., 1999 and Perrett et al., 1999)*.

Based on the present study, the multistate denaturation pathway of the native *Ba*GSTM dimer to a denatured monomer may include nonnative dimeric, native monomeric, or nonnative monomeric intermediates. These intermediates forms may be detected either by a characteristic spectroscopic signature or by the influence of protein concentration on the denaturation curves, the effect of protein concentration on the changes of fluorescence intensity was clearly observed.

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