Characterization of proteolyzed GST and study of complementary fragments

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3.1 Introduction

A formidable, long outstanding problem in molecular biology pertains to elucidating the principles underlying protein folding. An understanding of this aspect of protein molecules is of considerable importance given the keen interest in designing rational molecules for both academic investigations as well as industrial purposes. Attempts have been made towards understanding the mechanistics of the process utilizing small protein molecules or domains. Given the hierarchy of structure in a protein molecule, domains are known to be independent functional units with an inherent ability to fold autonomously. Therefore, studies of these independently folding units would serve as models for understanding the folding mechanism of larger, more complex molecules (Anfinsen and Scheraga, 1975; Kim and Baldwin, 1990). Fragment complementarity studies have been used as a tool to gain insights into the folding mechanism. Investigations have been performed on some proteins using this approach in order to study the complexation of fragments to form a fully folded native-like structure, therefore showing that the largely, disordered fragments contain information pertaining to the generation of the native-like three-dimensional structure (Sancho and Fersht, 1992). Following the pioneering investigations on RNaseA and staphylococcal nuclease (Richards and Vithayathil, 1959; Taniuchi et al., 1967), a number of complementary fragment systems have been used to study the basic principles underlying the folding mechanism and dynamics. Several methods, including limited proteolysis have been used to generate complementary fragments of a given molecule. Limit digest has also been extensively used as a tool to probe the conformational features of protein molecules (Hubbard, 1998). Although, results generated from studies of several systems may provide insights into the details of protein folding, caution is prescribed in extrapolating them to other systems that have not been studied so far, therefore rendering it necessary to study systems themselves in order to understand the generation of their native conformation from the primary sequence.

Glutathione-S-transferase (EC2.5.1.18) from Schistosoma japonicum (Sj26GST) was first identified as a major antigen capable of producing host-immunity in mice (Smith et al., 1986). It has been noted by Armstrong (1991) to be a constituent of an important superfamily of enzymes involved in Phase II metabolism of electrophilic compounds. GST has been
found to catalyze the S-conjugation reaction between the thiol group of glutathione and an electrophilic moiety of xenobiotic and endobiotic toxic compounds (Armstrong, 1991). Owing to the high solubility of cytosolic GSTs, and the fact that it could be easily purified using glutathione affinity chromatography, pGEX gene fusion expression systems came in vogue (Smith and Johnson, 1988). Crystal structures of Sj26GST suggest that each subunit in homodimeric GST contains two structural domains: an N-terminus domain with a \( \beta_3\beta_2\alpha \) folding topology (domain I; residues 1-84) and an all-helical C-terminal domain (domain II; residues 85-217) (McTigue et al., 1995; Lim et al., 1994). Considering the immense physiological role played by SjGST in detoxification and the wide spread role in biotechnology, conformational stability studies were first performed by Kaplan et al. (1997) in order to probe its conformational dynamics and subunit folding and assembly.

Here we sought to understand the folding characteristics of native GST by fragment complementarity using proteolysis as a tool to generate fragments of the molecule. Structure of the nicked molecule of GST was probed using various spectroscopic methods and the results obtained were compared with those of the native counterpart, thereby providing insights into the stability determining factors of the nicked species. Refolding characteristics of the nicked GST (in chemical denaturant) were studied to gain insights into the role of tertiary interactions in determining the native conformation.

3.2 Results

3.2.1 Preparation of intact GST and generation of fragments

Plasmid vector, pGEX-KG, a derivative of pGEX-2T (Figure 3.1A), was used to generate the GST molecule using the protocol as described in the materials and methods section. A 15% SDS-PAGE was run to visualize the various steps towards purification of GST (Figure 3.1B). *Schistosoma japonicum* GST (SjGST) derived using this vector has an additional sequence of amino acids (PGISGGGGGILDSMGRLELKLNS) towards the C-terminal end of the polypeptide. Therefore, the net molecular weight of the dimeric protein synthesized from this vector was 57024.20 Da (as calculated using the tools available at:
Fig. 3.1: **Expression and purification profile of GST.** (A) Parent vector of pGEX-2T showing the gene encoding GST from *Schistosoma japonicum* placed immediately after the tac promoter site (adopted from http://www.amershambiosciences.com). (B) Samples collected at various stages of purification were subjected to 15% SDS-PAGE followed by staining with Coomassie Brilliant Blue. Lane 1: uninduced, lane 2: induced, lane 3: pellet, lane 4: supernatant, lane 5: flow through, lane 6: wash, lane 7: elution, lane 8: purified GST, lane 9: marker.
Proteolysis was performed using various w/w ratios of substrate (GST) : protease (Subtilisin Carlsberg (SC)) at variable temperatures in order to optimize conditions for the proteolytic reaction. It was observed that an optimum reaction could be achieved within 5 min. from the initiation of the reaction, in the presence of 50mM Tris pH 8.0 at 37 °C. Gel profile shows complete proteolysis to have occurred with the generation of two discernible fragments at mobilities close to 18.4 kDa and 14.4 kDa (Figure 3.1C). Additionally, a set of unresolved fragments is seen towards the end of the gel front. Furthermore, an aliquot of the reaction obtained at 5 min. was subjected to analysis using the tricine SDS-PAGE system employed for the resolution of low molecular weight proteins. Gel profile shows further resolution of the smear into three distinct bands apart from the other two bands that were obtained on the glycine SDS-PAGE (Figure 3.1D).

3.2.2 Sequence determination of GST fragments

PVDF-blotted membranes of GST fragments obtained from Tricine SDS-PAGE were subjected to N-terminal sequencing by Edman’s degradation method. The fragment G2 corresponding to a molecular weight of 14.4 kDa shows the sequence MLGGCPKE at its amino terminus (Figure 3.2). Considering the average molecular weight of an amino acid to be 110 Da, the putative C-terminal can be computed to have GGFTAQWGQ residues towards the end. Sequencing results of fragment G5, indicate the presence of residues, DVVLMDP towards the N-terminus of this fragment. Interestingly, this sequence appears to be a part of the fragment G2, considering the approximate molecular weight of ~4.0 kDa exhibited by this fragment, thereby suggesting that this fragment may be the result of secondary cleavages. Since the amino-terminal sequences of two fragments, G1 corresponding to 18.4 kDa and fragment G3 could not be obtained, analyses of these fragments could not be proceeded with. Data shows that the fragment, G4 corresponds to the N-terminal of intact GST bearing an amino acid sequence of MSPILGYW. Since this fragment has a higher mobility than the G2 fragment, it could be the derivative of a larger fragment. To gain further insights into the structural implications of fragment generation, the crystal structure of GST derived from Schistosoma japonicum (PDB code: 1Y6E) was
Fig. 3.1: Proteolysis profile of GST-Subtilisin Carlsberg (SC) reaction mixture. (C) GST-SC reaction was performed at a ratio of 100:1 w/w and the reaction was quenched by addition of SDS-Lamelli buffer and subjected to 15% SDS-PAGE. Lane 1: marker, lane 2: control GST, lane 3: reaction after 1 min., lane 4: reaction after 5 min. (D) GST-SC reaction mixture was collected at 5 min. from the start of the reaction was quenched using Tricine gel sample buffer and subjected to 10% Tricine SDS-PAGE. Lane 1: marker, lane 2: control GST, lane 3: proteolysis reaction mixture at 5 min.
Fig. 3.2: **Sequence-structure representation of GST fragments.** Depiction of the amino-terminus residues of the fragments resolved on Tricine gel in addition to the graphic representation of GST molecule showing the sites of proteolysis (Gln 80-Met 81) and (Leu 159 – Asp 160).
visualized using the molecule visualization software, ICM (www.molsoft.com). Visualization of the monomeric GST showed that the fragment G2 generated by proteolytic cleavage of the peptide bond between residues Gln 80 and Met 81 was mapped to a region between the end of a solvent exposed helix and a flexible loop connecting another helix (Figure 3.2). Further, the fragment G5 was found to have been generated as a consequence of proteolysis between Leu 159 and Asp 160 which interestingly happens to be present in the middle of an α-helix.

3.2.3 Gel filtration studies of the non-covalent complex

Size exclusion chromatography was performed in order to probe the quaternary structure of the nicked GST molecule. 0.2 mg/ml of the nicked species was applied onto a Superdex 200 column of 28 ml bed volume, pre-equilibrated with 50mM Tris pH 8.0. Experiments were performed at a flow rate of 1 ml/min. Gel filtration chromatogram shows that the proteolyzed molecule appears at an elution volume of ~14.0 ml as evidenced from a single peak generated at that volume (Figure 3.3). In addition, a small, broader contour is observed at an elution volume of ~22.6 ml. Calculations were performed to estimate the relative mobility of the nicked species (Ve/Vo) in order to gain information pertaining to the molecular size of proteolyzed GST. Comparison of the relative mobility of the nicked species (Ve/Vo = 1.448) with that of the standard molecular weight markers showed that the elution volume of the nicked species corresponds to a molecular size equivalent to approximately 60 kDa. Furthermore, gel filtration experiments performed under similar conditions with native GST indicated that the molecule elutes at ~14.2 ml elution volume, therefore suggesting that the nicked species possesses a hydrodynamic volume greater than that of the native counterpart (Figure 3.3). Estimation of the relative mobility of native GST (Ve/Vo = 1.468) further displays the changed mobility of the molecule as a consequence of proteolysis. Additionally, the interactions responsible for maintainance of dimeric state of the molecule remain largely unperturbed, thereby leading to a quaternary structure similar to that of the native molecule.
Fig. 3.3: **Size exclusion chromatography studies.** Samples prepared for size exclusion analysis were applied onto Superdex 200 column and the eluant was observed at 280nm. Gel filtration chromatogram shows the elution profile of (A) native GST, and (B) nicked GST. Inset shows the calibration curve for Superdex 200.
Chapter 3

3.2.4 Intrinsic fluorescence studies of nicked GST

Fluorescence spectroscopy has been widely used as a tool to gain information related to changes in the conformation of protein molecules. About 1.75 μM concentration of proteolyzed GST was subjected to an excitation wavelength of 295 nm and emission spectra were recorded from 300 nm to 400 nm at a scan speed of 120 nm/min. Results showed that the proteolyzed molecule had an emission maxima of 341 nm in comparison to 340 nm obtained for the native molecule under similar experimental conditions (Figure 3.4(A)), thereby indicating that both, nicked molecule and the native GST have their tryptophans (Trp8, Trp41, Trp201, Trp205) in a fairly similar environment. Since, there is little change in the emission maxima of the proteolyzed species from that of the native molecule, it can be concluded that the tertiary structure of the proteolyzed GST molecule remains unperturbed. However, an increased quantum yield of nicked GST in comparison to that of the native form is representative of the tryptophan population residing in a more non-polar environment after proteolysis. Additionally, it can also be inferred that non-specific proteolysis of GST does not cause large scale perturbation of the global structure of the native molecule.

3.2.5 ANS binding studies of nicked GST

8-anilino-1-napthalenesulfonic acid (ANS), an amphipathic dye with hydrophobic naphthalene and phenyl groups has been widely used as a probe to ascertain the existence of equilibrium and kinetic intermediates in protein folding. ANS binding to intact and proteolyzed GST was facilitated by mixing of the protein samples (1.75 μM) with a 200 fold molar excess of the hydrophobic dye. ANS dye concentration was estimated spectrophotometrically using an extinction coefficient of 5000 M⁻¹.cm⁻¹. Following incubation with the dye for approximately 1 min., emission spectra were collected from 400 nm to 600 nm using an excitation wavelength of 395 nm. Spectra obtained for the two molecules were corrected for inner filter effect from the excess dye by subtracting with the values obtained for a reference blank containing only buffer and ANS. Results show that there is an increase in the ANS intensity upon binding to the protein samples in addition to a blue shift.
Fig 3.4: **Fluorescence spectroscopic studies.** (A) Spectra showing the emission profile of samples collected over a wavelength of 300nm to 400nm, employing an excitation wavelength of 295nm. Plot shows an increase in the intensity of the nicked GST accompanied by a minimal shift of the emission maxima when compared with that of native GST. (B) Surface hydrophobicities of samples were probed using a 200 fold molar excess of ANS over the protein. Similar emission spectra were observed for nicked as well as native GST.
in the spectra of both native as well as nicked GST, when compared with the ANS-buffer sample (Figure 3.4(B)). Comparative emission spectra of the native and nicked GST suggests that the two species of GST have nearly similar surface hydrophobicities.

### 3.2.6 Acrylamide quenching studies of nicked GST

Acrylamide quenching studies have been performed not only to assess the topological features of macromolecules, but also to determine the accessibility of tryptophan residues consequent upon conformational changes in the protein molecule. Proteolyzed GST was subjected to acrylamide quenching studies, to gain information pertaining to the changes in the environment of the reporter tryptophan residues. The ratio of final fluorescence intensity (I) to the initial intensity (I₀) was plotted against the quencher concentration to obtain the Stern-Volmer plot. Data was linear-fitted (with a y-intercept of 1) in order to obtain the value of the Stern-Volmer constant, Kᵥ (Figure 3.5(A)). In case of proteolyzed GST, Kᵥ assumes a value of 7.2 mole⁻¹ which is greater than the Stern-Volmer quenching constant obtained for native GST (Kᵥ = 5.8 mole⁻¹). Further, Kᵥ⁻¹ values calculated to estimate the concentration at which 50% quenching occurs, indicate that for proteolyzed GST, Kᵥ⁻¹ (0.14 M⁻¹) is slightly lower than that obtained for native GST (Kᵥ⁻¹ = 0.17 M⁻¹), therefore suggesting that the fluorophores in the nicked molecule may be slightly more accessible to the quencher than in the native GST molecule. Additionally, the plot of nicked GST shows a proclivity towards the x-axis, indicating the presence of fluorophores in different environments. Surprisingly, this is not seen in case of the native GST molecule. To further check the fraction of initial fluorescence (I₀) accessible to the quencher, a modified Stern-Volmer plot was done, wherein the ratio of (I₀/I₀-I) was plotted against the reciprocal of acrylamide concentration (Figure 3.5(B)). The value of the fraction (1/I₀) obtained for nicked GST was 0.9976, whereas that for native GST was 1.01. Therefore, the fraction of fluorophores accessible for quenching with acrylamide was similar in both cases (I₀ = 1). Overall, data obtained from fluorimetric studies suggested that large scale changes did not occur in the molecule as a consequence of proteolysis.
Fig. 3.5: Acrylamide quenching studies. Plots represent data obtained from quenching studies performed with native and nicked species of GST. (A) Stern-Volmer plot generated for determination of the Stern-Volmer constant ($K_D$) for nicked (●) and native GST (○). (B) Modified Stern-Volmer plot to determine the fractional accessibility of fluorophores. (All linear plots had $R^2$ in the range of 0.90 to 0.97).
3.2.7 Activity determination of nicked GST

In addition to determination of the integrity of the three-dimensional structure, changes in the thiol-conjugation ability of GST molecule as a consequence of non-specific proteolysis was investigated by calculating the specific activity of the proteolyzed species and comparing it with the value obtained for its native counterpart. Native GST (1.65 μg) and the nicked molecule (3.0 μg) were subjected to CDNB assay (as discussed in materials and methods) and the absorbances obtained at 340nm were averaged and plotted against various time points for which data were collected (Figure 3.6). We defined 1 unit of GST activity as the amount of GST that will produce an increase in absorbance at 340 nm (ΔA₃₄₀) of 0.1 absorbance units per minute (AU/min) under the assay conditions as described in the experimental procedures. Further, specific activity calculations showed that the nicked GST had a specific activity of 1025.0 U/mg in comparison to 1651.52 U/mg obtained for the native molecule. In other words, the nicked molecule is found to retain 62 % activity of the native molecule. Perturbation of the active-site GST is therefore indicated from the data.

3.2.8 Secondary structure determination of nicked GST

Investigations were carried out to ascertain changes in the secondary structural content of the nicked GST. Far-UV circular dichroic spectrum of nicked GST indicates the presence of two negative bands corresponding to 208nm and 222nm wavelengths of the circularly-polarized light used for the scan, thereby suggesting the presence of substantial α-helical content when compared to the spectrum obtained for the native GST molecule (Figure 3.7). Detailed analysis of the spectra suggests that the mean residue ellipticity of the nicked molecule at 208nm (-7.54 x 10³ deg.cm².dmol⁻¹) and 222nm (-5.92 x 10³ deg.cm².dmol⁻¹) were lower than those observed at 208nm (-9.81 x 10³ deg.cm².dmol⁻¹) and 222nm (-7.47 x 10³ deg.cm².dmol⁻¹) for the native molecule. Calculations carried out to estimate the fraction of α-helical content in the two forms of GST, suggests that post-proteolysis, the GST molecule shows a lowering of the α-helical content by nearly 31.5%. Further calculations were performed to obtain the ratio of [θ]₂₂₂/[θ]₂₀₈, an index of the interaction amongst helices.
Fig. 3.6: GST activity assay profile. Plots showing the activity profiles of native and nicked GST. (A) Changes in the absorbance at 340nm of native (1.65 μg) and nicked GST (3.0 μg) plotted as a function of time. (B) Plot showing the relative specific activities of native (natGST) and nicked GST (nkGST).
Fig. 3.7: **Circular dichroic studies.** Far-UV CD spectra were obtained to gain insights into the secondary structural content of nicked and native GST. Plot shows the mean residue ellipticities of nicked and native GST as a function of the wavelength of circularly polarized light.
in a molecule. The nicked molecule bears a ratio of 0.79 in comparison to 0.76, the value obtained for the native GST. This clearly suggests that the interactions amongst helices in the nicked molecule is similar to that found in the native GST.

3.2.9 Urea induced denaturation studies of nicked GST

Conformational stability studies have been widely used to ascertain the stability of molecules as a result of modifications of the protein molecules themselves or in response to changes in the milieu of the molecules under study. Proteolyzed GST was subjected to chemical denaturation by application of varying concentrations of urea to the reaction mixture and observations were made using fluorimetric techniques to follow changes in the tertiary structure. The observed physical parameter (emission maxima) was averaged and plotted against urea concentrations to generate the transition curve. Figure 3.8 (A,C) summarizes the effect of increasing concentrations of urea on the fluorescence emission maxima of native and nicked GST molecules. No significant alteration of the tryptophan emission was observed up to ~3.5M urea in the case of native GST. However, between 3.5M and 5.5M urea, transition is seen to occur with the tryptophans nearly exposed at ~5.5M urea concentration. Increasing the concentration of denaturant beyond 5.5M does not result in significant changes in the emission maxima. Furthermore, changes in the fluorescence emission maxima of the native molecule shows a sigmoidal dependence on denaturant concentration. Similar studies performed with nicked GST showed that the unfolding as measured by tryptophan emission maxima begins at ~ 1.0M and complete unfolding is seen to occur at ~ 4.0M. Additionally, plots suggested that the nicked molecule bears a lower C_m value than that of native GST. Since the nicked and native GST molecules showed a significant minima at 222nm in circular dichroic studies, observations at that wavelength were carried out to study changes in the secondary structural content of molecules subsequent upon addition of urea to samples. For native GST, initiation of unfolding occurs at ~2.0M with complete denaturation facilitated at a urea concentration of ~5.0M (Figure 3.8 (B)). Similar studies performed with nicked GST indicated that denaturation begins at low concentrations of urea and complete loss of secondary structure can be observed at ~3.5M.
Fig 3.8: **Urea-induced unfolding transition curves.** Plots show the change in tryptophan emission maxima and $[^\theta]_{222\text{nm}}$ of samples with a corresponding change in the urea concentration. (A) Changes in emission maxima of native GST upon incubation with increasing concentrations of urea. (B) Changes in observed ellipticity at 222nm of native GST with increasing concentrations of urea. (C) Changes of tryptophan emission maxima of nicked GST with increasing urea concentrations. (D) Changes in observed ellipticity of nicked GST with increasing concentrations of urea.
(Figure 3.8 (D)). Moreover, the nicked GST showed a broader transition curve in comparison to that of the native form. Furthermore, the transition curves obtained from CD and fluorimetric data were non-superimposable, therefore implying that unfolding of nicked GST in urea follows a multiphasic pathway.

**3.2.10 GdmCl-induced denaturation studies of nicked GST**

Further studies were performed using guanidinium chloride (GdmCl) to investigate the conformational stability of the nicked form in a relatively strong denaturant. Investigations performed to observe changes in emission maxima using fluorimetric techniques indicated that the nicked molecule shows signs of a relatively open conformation at GdmCl concentrations as low as 0.4M. Furthermore, complete unfolding of nicked GST is seen to occur at ~1.0M denaturant concentration (Figure 3.9(D)). Similar studies performed with native GST showed that the transition, as observed fluorimetrically, begins at ~1.0M and is completed at ~3.0M concentration of denaturant (Figure 3.9(B)). Comparison of the transition curves obtained by plotting the emission maxima against GdmCl concentrations highlight the compromised conformational stability of the nicked form in comparison to that of the native molecule. Furthermore, studies were performed to estimate the changes in secondary structural content consequent upon increasing concentrations of GdmCl. Loss of secondary structure was observed to initiate at ~1.0M and complete unfolding was found to occur at ~3.5M for the native form, whereas the nicked GST begins transition at ~0.15M and complete loss of secondary structure was seen to occur at ~1.0M (Figure 3.9 (A,C)). Additionally, the nicked molecule exhibits a sharper transition curve than the native counterpart, further underlining the susceptibility of the nicked conformation towards denaturants. Calculations were performed using the linear extrapolation method to obtain the unfolded fraction of nicked GST against GdmCl concentrations. Data shows that the transition curves obtained through circular dichroic studies and fluorimetric investigations are fairly coincident, therefore suggesting that unfolding of nicked GST using GdmCl is a two-step process (Figure 3.10).
Fig. 3.9: *GdmCl induced unfolding transition curves*. Plots show changes in observed physical parameters against GdmCl concentrations for nicked and native GST molecules. (A) Transition curve showing changes in observed ellipticity at 222nm of native GST against increasing concentrations of GdmCl. (B) Changes in tryptophan emission maxima of native GST against increasing concentrations of GdmCl. (C) Unfolding transition curve of nicked GST showing changes in ellipticity at 222nm upon increasing concentrations of GdmCl. (D) Changes in tryptophan emission maxima of nicked GST upon increasing concentration of GdmCl.
Fig. 3.10: GdmCl induced changes in fraction of denatured nicked GST. Graphical representation of the denatured fraction of nicked GST against variable GdmCl concentrations. Plot shows coincidence of the GdmCl induced transition curves obtained for proteolyzed GST using circular dichroic and fluorimetric methods.
3.2.11 *Thermal denaturation studies of nicked GST*

Thermal denaturation studies were performed using CD spectroscopy to estimate the stability of the nicked GST towards temperature. Plots of the observed parameter, [θ]_{222} against temperature show a transition curve having a T_m of ~60.5 °C for the nicked molecule (Figure 3.11). However, in comparison the native GST bears a T_m of approximately 62 °C. Detailed investigation of the transition curves revealed that in case of nicked molecule, thermal melting is seen to begin well below the T_m, at approximately 53.5 °C. However, denaturation of native GST was seen to initiate near the T_m of the molecule, at approximately 58.0 °C, therefore suggesting that unfolding of nicked GST by thermal denaturation involves the denaturation of the various secondary structural elements independent of each other, unlike in case of native GST where loss of structure is seen to occur in a cooperative manner. Overall, data pertaining to thermal denaturation studies of the nicked molecule suggests that peptidic cleavages in the molecule lead to a concomitant decrease in the stability of the molecule.

3.2.12 *Attempts towards purification of fragments of GST*

Fragment complementarity studies of GST involved purification of the fragments generated post-proteolysis. Reverse-phase HPLC analysis of the proteolysis mixture was carried out on a Sephasil C18 column fitted onto a Shimadzu HPLC system. Separation of fragments was affected at a flow rate of 1.0 ml/min. employing a linear gradient of acetonitrile in water supplemented with 0.05% TFA as the counterion and the effluent was monitored by recording the absorbance at 218nm. Results suggested that separation of fragments did not occur under the reverse-phase purification conditions, instead a single peak was found to occur in the chromatogram. SDS-PAGE profile of the eluant showed that the peak corresponds to a band with mobility similar to that of native GST, therefore rendering this method of fragment purification useless (Figure 3.12). Similar observations have earlier been made with proteolyzed fragments of *Plasmodium falciparum* TIM and rabbit muscle TIM (Ray *et al.*, 1999; Vogel and Chmielewski, 1994). To facilitate purification of
Figure 3.11: Thermal denaturation studies. Native and nicked forms of GST were subjected to increase in temperature and concomitant changes in raw ellipticity were observed.
Figure 3.12: **Reverse-phase HPLC purification of fragments of proteolyzed GST.** Purification of fragments of proteolyzed GST was attempted using reverse-phase chromatography (A) Chromatogram depicts a single peak for the reaction mixture, (B) 15% SDS-PAGE indicates the proteolytic status of the eluant (Lane 1: control GST, lane 2: eluted fraction).
fragments, ion-exchange chromatography was utilized as an alternative method. Since the nature and composition of the fragments generated by proteolysis were not known, experiments using ion-exchange chromatography to purify fragments was not a feasible approach. However, to check whether this technique could be employed to generate purified fragments, proteolyzed GST was applied onto both cation as well as anion exchange columns in separate experiments and the elution was performed with a linear gradient of NaCl, surprisingly, the fragments failed to separate under these conditions (data not shown). Since, the sizes of the fragments generated post-proteolysis were close to each other, gel filtration chromatography as a means of separating the fragments was ruled out.

3.2.13 Refolding studies of nicked GST from Guanidinium chloride

Since attempts to purify fragments of the nicked species of GST did not yield results, denaturation of the reaction mixture in GdmCl, followed by renaturation by serial dilution was used as a method to study the characteristics of the fragment complexation reaction in GST. Considering that denaturation occurs to greater extent in GdmCl than in urea, proteolyzed GST was subjected to concentrations of GdmCl as high as 1M followed by incubation of the denatured sample at 25 °C for approximately 1 hr. CD and fluorescence measurements were performed to ensure that the fragments were completely denatured prior to refolding. Renaturation was initiated by performing serial dilutions of the proteolyzed mixture in GdmCl with the refolding buffer (50mM Tris, pH 8.0 and 1mM DTT). The spectra were corrected by multiplying the dilution factor with the intensities obtained for the spectrum. Plot of the emission maxima against the final concentration of denaturant in the diluted samples showed that renaturation had not occurred even when the final GdmCl concentration was brought down to 0.1M (Figure 3.13(A)). This suggests that no detectable tertiary structure forms as a consequence of lowering the GdmCl concentration. Investigations were performed to gain insights into the formation of secondary structure consequent upon dilution of denatured GST fragments in the refolding buffer. Data again shows that there is no detectable gain in the secondary structure as a consequence of dilution (Figure 3.13(B)). Additionally, experiments were performed to assess the ability of nicked
Fig. 3.13: Refolding profiles of nicked GST from GdmCl. Proteolyzed GST in 1M GdmCl was subjected to refolding and structural changes were monitored using fluorescence and CD. (A,B) Refolding of nicked GST from 1M GdmCl was carried out by serial dilutions of the denatured sample in the refolding buffer. (C,D) Nicked GST was refolded from different GdmCl concentrations to 0.1M in separate experiments and the extent of refolding was observed by fluorimetry and CD.
GST to refold to its native conformation from varying concentrations of GdmCl in separate experiments. Concentrations of GdmCl used for denaturation ranged from 0.9M to 0.2M in steps of 0.1M. Furthermore, refolding was affected by dilution with appropriate volumes of the refolding buffer such that the final concentration of denaturant in all cases was 0.1M and changes in structure was observed using CD and fluorescence. Plots suggested that the nicked molecule shows considerable ability to refold back from GdmCl concentrations up to 0.6M, however the nicked molecule exhibited no refolding from much higher concentrations of GdmCl. (Figure 3.13 (C,D)).

3.2.14 Crystallization trials of proteolyzed GST

To corroborate the biophysical and biochemical data obtained for nicked GST, experiments were designed to gain insights into the changes in the three-dimensional structure of the native molecule as a consequence of proteolysis. Preliminary crystallization trials of proteolyzed GST were performed using the 96 random conditions of Magic 96 matrix, with 4 °C and 25 °C (room temperature) as the ambient temperatures for trials. 2µl of the nicked GST (10 mg/ml) was mixed with an equal volume of the reservoir solution of each well on a siliconized cover slip that was subsequently inverted over the well. Trials did not yield any crystals of the nicked species. In another set of experiments, a grid was set up around the conditions wherein crystals of native SjGST derived from pGEX-3X vector were obtained (McTigue et al., 1995). Proteolyzed GST at concentrations of 10 mg/ml and 20 mg/ml was mixed with reservoir solution containing 100mM Sodium Acetate buffer pH 5.6 and 20-75% saturated ammonium sulfate solution and the drops were equilibrated by hanging drop vapour diffusion method with the same reservoir buffer at the temperatures employed earlier. Unfortunately, no crystals could be obtained of proteolyzed GST. Attempts to crystallize the non-covalent complex were accomplished using variable concentrations of sulphuric acid and PEG 400, 1000, 3350 and 8000 as the reservoir solution. However, no crystal formation could be seen.
3.3 Discussion

The importance of tertiary interactions in holding together the structure of protein molecules is best illustrated by nicked molecules. In these variants of the native molecule, the loss of peptide bond(s) as a consequence of proteolysis has been found to lead to structures which exist as non-covalent complexes that have been shown to bear native-like structures, therefore highlighting the importance of long-range interactions in determining the conformational stability of proteins. Since most fragment complementarity systems studied involve only two interacting fragments, it would be of interest to study fragment complementary systems with more than two interacting components. Moreover, considering the importance of GST in the cellular milieu, an understanding of the folding characteristics of this molecule gains importance. Studies performed with these objectives showed that non-specific proteolysis of the native GST molecule leads to the generation of a non-covalent complex wherein several peptide bonds have been cleaved. SDS-PAGE profiles showed the generation of two clearly discernible fragments accompanied by a set of unresolved peptides, therefore suggesting the occurrence of multiple cleavage sites for proteolysis. Further resolution of the smear showed the presence of three fragments in addition to the ones that were observed on the SDS-PAGE. Subsequent characterization of the fragments by N-terminal sequencing and structural analysis of the cut-sites revealed that one of the fragments was generated as the result of proteolysis at a region between the end of the helix and a flexible loop (G5 fragment) whereas, the second cut-site was mapped to the middle of a helix. This observation is contrary to the generally accepted perspective of conformational flexibility being a major factor in determining proteolysis. Since the fragment, G5 is suggested to be the result of secondary cleavage of the G2 fragment, proteolysis affected at the loop region (Gln 79 and Met 80) might be considered to bring about a lowering of the conformational rigidity of the helix bearing the residues Leu 158 and Asp 159, thereby increasing the proteolytic susceptibility. Decrease in the \( \alpha \)-helical content, as suggested by CD lends considerable support to this perspective. Furthermore, intrinsic fluorescence studies suggest a minor perturbation of the tryptophan environment, with the nicked species having a slightly more open conformation than the native one. In addition to intrinsic fluorescence
studies, quenching experiments suggest a minimal perturbation of the microenvironment of the tryptophan population, further accentuating the robustness of long-range interactions in holding the non-covalent complex together, post-proteolysis. Investigations carried out with ANS showed that the surface hydrophobicities of the nicked GST were similar to that of the native conformer. Analysis of the crystal structure of GST reveals that proteolysis of the native molecule would not lead to changes in the microenvironment of the fluorophores as these moieties are distantly placed from the sites of proteolytic cleavages, therefore corroborating the spectrofluorimetric data suggesting that proteolysis of native GST does not lead to large scale conformational changes. Additionally, activity studies with the nicked and native forms show that non-specific proteolysis leads to lowering of the specific activity of nicked GST, suggesting subtle changes in the active site profile.

Gel filtration data suggests that proteolysis of GST leads to an increase in the hydrodynamic radius of the nicked form, therefore indicating that the proteolyzed GST had a slightly open conformation when compared with that of the native GST. Additionally, proteolysis does not seem to change the interactions responsible for maintenance of the dimeric status of the nicked form. Overall, characterization of nicked GST using biophysical and biochemical techniques showed that subtilitic cleavage of GST leads to the formation of a multiply nicked dimeric, non-covalent complex with subtle changes in the tertiary structure and relatively more profound changes in the secondary structure.

Stability of the multiply nicked structure assessed using chemical denaturants show that the nicked molecule exhibits a lower stability towards denaturants when compared with the native molecule. Data also reiterates the higher strength of GdmCl in bringing about denaturation when compared with urea as the chemical perturbant. Additionally, non-coincidence of the transition curves of the nicked GST in urea suggests that unfolding of the nicked form follows a multi-phasic pathway with indiscernible transition points unlike native GST that follows a two-state transition. Interestingly, data obtained from GdmCl induced transitions of nicked GST show a reasonable coincidence of the transition curves from the two spectroscopic techniques employed, therefore suggesting that the nicked molecule follows a two-state transition in the presence of increasing concentrations of GdmCl with concomitant unfolding of the tertiary and secondary structure of the protein during the
process. This is in contrast to the unfolding transition curves obtained for urea-induced unfolding studies of nicked GST, thereby suggesting that the two denaturants facilitate unfolding of the nicked species by different pathways.

In addition to studies performed with chemical denaturants, increase in temperature was used to facilitate unfolding of the native and nicked GST. The relatively lower $T_m$ of nicked GST suggests a lowering of the stability of GST post-proteolysis. Moreover, the broader transition of the melting curve for nicked GST indicates that the unfolding of nicked GST is non-cooperative in contrast to the sharper transition curve observed for native GST. Further, attempts made to purify fragments of GST surprisingly yielded religated GST. Protease-assisted religation of nicked protein substrates, in the presence of organic cosolvents has been extensively investigated (Vogel and Chmielewski, 1994; Vogel et al., 1996). Interestingly, proteolyzed rabbit muscle TIM when subjected to HPLC separation in the presence of acetonitrile, resulted in the formation of religated species with a mobility similar to that of the native molecule. Similar results were obtained by Ray et al. (1999) with Plasmodium falciparum TIM. Our results pertaining to reverse-phase separation of GST fragments suggested that reverse proteoytic condensation of nicked GST occurred under HPLC conditions, thereby rendering the method useless in purifying fragments of nicked GST.

Furthermore, methods employing dilution from higher denaturant concentrations were used to attempt refolding of the denatured nicked fragments. Results showed that dilution experiments did not yield the native non-covalent complex, therefore indicating that once the non-covalent complex had fallen apart, long-range interactions that facilitated formation of the non-covalent species could not be regained upon dilution to lower denaturant concentrations. In addition, no secondary structural elements are observed to form suggesting that local interactions that result in the formation of secondary structures were not stabilized post-dilution. Taken together these results suggested that dilution of the denatured non-covalent complex do not result in stabilization of the interactions important for structure formation. Additionally, the possibility of the denatured complex exhibiting more sites for proteolysis at higher denaturant concentrations, therefore resulting in the generation of smaller fragments cannot be neglected.
However, diluting denatured samples from different denaturant concentrations to lower concentrations of the perturbant showed that the nicked molecule when subjected to denaturant concentrations that are low, exhibits the ability to get back to its non-covalent complex-like state. Similar tendencies are however lost at higher perturbant concentrations, therefore showing that the non-covalent interactions can be regained to a large extent when the perturbant concentrations are low.