

The protein-folding problem has been a keenly investigated subject of research owing to the ubiquity of these molecules in life processes as well as in industrial applications. Ever since the seminal studies of Anfinsen (1973) showing that the amino acid sequence of the protein dictates the three-dimensional structure, several efforts have been put forth towards understanding the protein folding reaction.

Although initial studies indicated that long-range interactions were needed to stabilize the secondary structural elements of proteins (Taniuchi and Anfinsen, 1969), the detection of structure in peptides led to a shift in the perception with regard to the interactions that stabilized structures in protein molecules. Studies of protein fragments has therefore been sought to understand the possible initiation sites as well as the events that occur prior to the formation of the fully folded native-like structure. Although, the literature is replete with examples of fragment complementing systems, most of them employ only two interacting fragments (Richards and Vithyathil, 1959; Williams and Shoelson, 1993).

Given the diversity of folds and sequences in protein molecules, the folding mechanism is expected to vary, therefore generating the interest in studying additional systems towards elucidating their mechanisms. We performed studies with Glutathione-S-transferase of *Schistosoma japonicum*, derived from pGEX-KG vector in an attempt to elucidate its folding strategies, using fragment complementarity as the tool. The biotechnological uses as well as its importance in cellular detoxification led to it being an important candidate for folding studies. Since literature suggested that only peptides with  $\alpha$ -helical tendencies achieved structural conformation in isolation (Dyson et al., 1992), GST with its relatively higher helical content was a suitable candidate for protein fragment complementarity studies. Limited proteolysis of GST was performed using SC in order to generate fragments for purification and further studies. Results indicated that the molecule exhibits several nicking sites as a consequence of proteolysis. Moreover, the presence of more than two fragments in the proteolyzed molecule generated further enthusiasm in using it as a fragment complementing system. Identification of the sites of proteolysis by amino terminal sequencing suggested that one of the cut-site was present at a region between the end of a helix and a loop region. Interestingly, the second site of proteolysis appeared at a region mapped to the middle of a  $\alpha$ -helix. This observation is contrary to the hitherto understood concept of proteolysis occurring at sites showing a higher degree of flexibility than the secondary structural elements. Since proteolyzed

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molecules depict the importance of tertiary interactions in the folded conformation of a molecule, the nicked GST molecule was taken up as such for further investigations pertaining to the consequences of proteolysis on GST. Size exclusion chromatography using Superdex 200 column suggested that the nicked form had a quaternary status similar to that of the native conformation. However, the slightly lower elution volume of the nicked molecule than that of the native form suggested that proteolysis had led to a minor increase in the hydrodynamic radius of the molecule, thereby suggesting that the interactions responsible for maintenance of the dimeric status of GST remained unperturbed as a consequence of proteolysis. Furthermore, the tertiary structure of the molecule was investigated using fluorescence spectroscopy. Excitation of the nicked form of GST using a wavelength of 295nm resulted in an emission maxima close to that obtained for the native counterpart. Studies performed to probe the changes in the microenvironment of the nicked molecule employing acrylamide quenching indicated that the tryptophan population in both species of GST exhibited similar accessibility to the quencher. Additionally, ANS binding studies revealed that the nicked molecule displayed surface hydrophobicity similar to that of the native GST. It can therefore be concluded that minimal perturbation of the tertiary structure is displayed as a consequence of proteolysis. Enzymatic activity of molecules has been often employed as a sensitive probe for analyzing structural changes. Data indicated that the nicked molecule displayed nearly 62% of the activity seen for native GST. Investigations performed to ascertain the change in secondary structural content as a consequence of proteolysis showed that the nicked molecule had a lowered  $\alpha$ -helical content. This could be attributed to the perturbation of structure in the helix bearing the cut-site. Conformational stability of the nicked molecule was assessed using CD as well as fluorescence spectroscopy in the presence of urea as well as GdmCl. Data indicated that the nicked molecule exhibited a compromised stability as a consequence of depletion in the peptide bond content. Interestingly, the transition curves obtained for the nicked molecule in the presence of GdmCl using CD and fluorescence were fairly coincident, in contrast to that obtained from urea melting studies. Thereby suggesting that the nicked species followed a two-state transition in the presence of GdmCl, as against the multi-phasic pathway followed with increasing urea concentrations. It can therefore be concluded that the nicked molecule apparently unfolds by different pathways in urea and GdmCl.

Furthermore, attempts made to purify the fragments of GST using reverse-phase chromatography resulted in the generation of a single peak corresponding to the native GST as evidenced from the SDS-PAGE. Similar observations of the religated product had been found by Ray *et al.* (1999) and Vogel and Chmielewski (1994). Furthermore, attempts to purify the fragments using ion-exchange chromatography resulted in the formation of religated GST. Since the fragments failed to dissociate under the purification conditions used, fragment complementation was attempted by denaturing the non-covalent complex using GdmCl and refolding it back by serial dilution with the refolding buffer. However, refolding could not be achieved. It can be reasoned that the presence of denaturants would have led to an enhanced accessibility of the proteases for cleavage, therefore leading to formation of smaller fragments that may not be able to form larger structures. Furthermore, the presence of cleavage site in the helix may be another reason for the non-covalent complex not refolding back. Attempts to crystallize the nicked molecule did not yield results.

Since reformation of the peptide bond in the presence of salts has not been demonstrated thus far, our results showing religation under ion-exchange conditions were novel and worth pursuing. Salts have been known to play an important role in biological systems. Importantly, ions have been known to affect the structure of proteins owing to their direct interactions with the molecules or as a consequence of altering the water structure around the molecule. Studies were carried out with proteolyzed GST to understand the efficacy of salts in the formation of peptide bonds. A variety of salts were tested for their relative abilities in facilitating peptide bond formation. Results demonstrated that the order of ease of peptide bond formation with respect to the cations was:  $Mg^{2+} > Li > Na = K$ . Since the charge densities of the cations increases in a similar fashion, it was concluded that the ease of religation was facilitated by an increase in the surface charge density of the cations. This has strong connotations with the Hofmeister series. Furthermore, we investigated the role of protease in mediating peptide bond formation in the presence of salts. Experiments carried out to demonstrate the change in proteolytic activity of SC revealed that there was a minimal perturbation of the activity as a consequence of addition of salts. This is contrary to the organic co-solvent milieu wherein religation was earlier observed, thus hinting that the protease had little role to play in religation. Furthermore, protease linked to agarose beads was used for experiments wherein salts were added to the protease-deficient supernatant. Since

religation occurred under those conditions, it is concluded that protease may not be playing a role in religation of severed peptide bonds.

Studies were further performed to assess changes in the three-dimensional structure of lysozyme as a consequence of proteolysis, using X-ray crystallography. Initial crystallization trials resulted in the formation of crystals that were shown to be composed of religated lysozyme molecules. Since the conditions under which these crystals were obtained, contained PEG as well as NaCl, religation was attributed to them. It was therefore sought to grow crystals in the absence of precipitating agents. Variable concentrations of sulphuric acid were added to the reservoir and the coverslips containing drops of proteolyzed lysozyme were inverted on them. Crystals of proteolyzed lysozyme were found after a few days. However, the low quality diffraction obtained from these crystals was of little use in structure solution. In order to obtain better quality crystals, variable concentrations of PEG were employed. Crystals containing nicked lysozyme obtained after a few days was used for diffraction and further structure solution. 2Fo-Fc map computed using the structure factors showed that the residue Gly71 exhibited no density. Furthermore, secondary structure rendering followed by visualization of the final model showed that the site of proteolysis mapped to the loop region. Since there was no global; change detected as a consequence of proteolysis, the robustness of tertiary interactions in holding together the structure is further highlighted.

Since proteases were extensively used in the work, a chance observation was made pertaining to the usage of protease inhibitor PMSF in facilitating quenching of the proteolysis reaction. Interestingly, addition of 2-3mM concentrations of PMSF led to religation of the nicked species. Given the universal use of this reagent in quenching proteolysis reactions, the results were of immense significance. The ubiquity of the reagent in facilitating religation was studied using hydantoinase, GST, lysozyme, RNase A and the fusion protein GST-Eps8SH3. Results showed complete religation of proteolyzed lysozyme, GST and hydantoinase. However, RNase A failed to religate. Since proteolysis of RNase A lead to fragmentation at the flexible region between Ala 20 and Ser 21 residues. It was therefore hypothesized that the inherent flexibility of the loop region caused an increase in the entropic barrier, hence preventing formation of the peptide bond. To further validate this argument, GST-Eps8 SH3 was used as a substrate for the proteolysis-religation reaction. Results showed that addition of PMSF led to the formation of GST but no fusion protein was detected, therefore indicating that religation

was indeed a function of entropy. Furthermore, lysozyme as well as GST was treated with PMSF in a protease-deficient milieu. Results showed that the nicked molecule is efficiently religated, therefore indicating that protease may not be needed for religation. Furthermore, validation of this observation was provided by the religation seen of the nicked lysozyme in crystals consequent upon addition of PMSF. Since the usage of AEBSF, a water-soluble analog of PMSF also resulted in religation, the phenomenon was extended to sulfonylating agents. Investigations were therefore performed to gain insights into the mechanism of religation mediated by the protease inhibitors studied. Reports that addition of sulfonylating inhibitors to proteins resulted in non-specific reactions with the lysine, tyrosine residues as well as the free amino terminus was instructive in supporting the perspective that religation is facilitated by lowering of the enthalpic barrier due to the binding of the sulfonylating agent to the free amino terminus at the site of proteolysis. Additionally, literature indicated that the sulfonylating agents act as efficient leaving groups.

In light of the literature available as well as the results obtained, religation in the presence of sulfonyl inhibitors appear to be a consequence of lowering of the enthalpic barrier towards bond formation due to the activation of the amino terminus and the lowering of the entropic barrier due to the proximity of the reacting termini.

Overall, the endeavour presented in this dissertation highlights the importance of tertiary interactions in stabilizing structures of proteolyzed intermediates. Additionally, a novel perspective is presented towards the understanding of the *in vitro* synthesis of peptide bonds.