Neeraj Dhaunta (2011). *Kinetically-stable macromolecules studies of extremophile proteins and a protein aggregate.* Ph.D. Thesis. CSIR-IMTECH, Chandigarh/ Jawaharlal Nehru University, New Delhi: India.
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SUMMARY OF THE THESIS

Studies on homologous proteins from organisms living under different environmental conditions provide critical information about the mechanism by which a protein modulates its stability in concert with the environmental conditions that are best suited for the growth of the source organism. In general, proteins from hyperthermophiles are very stable, many factors have been argued to contribute to the stability of hyperthermophile proteins. Most of these proteins defy determination of the thermodynamic parameters of stability, and are kinetically stabilized to retain their structure and function at higher ranges of temperatures through a slowing down of unfolding. For a protein that show a high degree of kinetic stability, measurement of thermodynamic parameters becomes quite difficult as they fail to unfold/refold under *in vitro* conditions, and only a small fraction of proteins from thermophiles undergo fully reversible changes so as to make it possible to measure free energy changes (ΔG) and other thermodynamic parameters during the process of unfolding/refolding.

On the basis of sequence and structure comparisons with the homologous proteins from mesophile and psychrophile, some features have been identified that may contribute to the extreme stability of thermophile proteins. In our lab, we have been exploring the role of some of these features in controlling the overall stability a protein. In a previous work from our lab we were able to demonstrate the role of salt bridges and autonomy of substructures in stabilization of a hyperthermophile protein. In continuation of the previous work on the topic of protein stability, this thesis is an attempt to further unfurl some of nature's best kept secrets which govern protein folding and stability, so as to help us expand our understanding about the factors responsible for differential stability of homologous proteins.

The main objectives of the work presented in this thesis thesis are: a) to study the effect of engineering salt bridges on the stability of a protein of psychrotroph origin (which normally doesn't have a scope for any such interactions). b) Simultaneously, we wished to explore two proteins from *P.furiosus* (a hyperthermophile), one in terms of its stability and resemblance to δ-crystallin (argininosuccinate lyase), and another in terms its similarity with a well studied kinetically stable protein (alpha lytic-protease) from *L.enzymegenes*. c) We also

wanted to test a hypothesis of possible role of an antibody in recovering proteins from an aggregate.

The work undertaken in this thesis is divided into six chapters, with each chapter having its separate reference section. The first chapter is an introduction to various concepts in the field of protein folding, protein stability and protein aggregation that have relevance to the work presented in various chapters of this thesis. The second chapter is a record of all the chemical and general techniques that have been used in the lab for conducting the work undertaken in this thesis. Details about the materials and methods specific to each chapter are mention in appropriate sections of that chapter.

Chapter 3 entitled: **"Stabilizing *Methanococcoides burtonii* Triosephosphate isomerase (MbuTIM) through protein engineering"** describes a novel finding of kinetic thermal stability in Triosephosphate isomerase (TIM) from a psychrotroph organism *Methanococcoides burtonii* expressed in *E.coli*, and further enhancement of its kinetic stability by introducing three ion-pairs (two-ion pair networks) in the helix of its 4^{th} $\beta\backslash\alpha$ unit. Not many proteins from psychrophiles/ psychrotrophs are known to sustain high temperatures. To our surprise, MbuTIM was an exception. DSC and CD thermal melt data suggested that MbuTIM has an apparent T_m (temperature of melting) of 73°C which is very high for a protein that is designed by nature to function in an organism with an optimum growth temperature of 23°C. Following kinetics of unfolding of MbuTIM as a function of temperature, we were even more surprised to find that it showed kinetic stabily. Provided an increased period of incubation at temperatures below its apparent Tm, MbuTIM unfolds at a very slow rate, suggesting that high thermal stability of MbuTIM owes to its high kinetic stability.

In continuation of a previous work from our laboratory, where we destabilized an otherwise highly thermostable *P.furiosus* TIM by disrupting two ion-pair networks between its 3^{rd} and 4^{th} $\beta\backslash\alpha$ unit, and to further validate the role of salt-bridges in stabilization of thermophile proteins, we wanted to explore the effect of introducing PfuTIM-like ionic interactions in the psychrotroph TIM (MbuTIM), which in its native sequence does not have scope for any such interactions. Using splicing by overlap extension (SOE) PCR, we engineered the scope for the

formation of 3 PfuTIM-like ion-pairs in MbuTIM (note that PfuTIM has 4 ion-pairs). With an apparent T_m of 78°C, the mutant MbuTIM (MutMbuTIM) had more thermal stability than the wild type MbuTIM. Kinetics of temperature-dependent unfolding of MutMbuTIM showed that it had more kinetic stability as compared to MbuTIM. DSC thermal melt showed that both MbuTIM and MutMbuTIM have 2 independently unfolding transitions indicating the presence of two independently-unfolding subdomains; further, in MutMbuTIM there was a shift in the positions of both the unfolding transition peaks towards higher temperatures. Gel filtration profile of both MbuTIM and MutMbuTIM show that both of them are hexamers, which along with autonomy of its sub-structures could be one of the reasons for high thermal/kinetic stability of MbuTIM. In this chapter, we successfully demonstrated the role of ion-pair interactions in modulating (increasing) the stability of MbuTIM.

Chapter 4 entitled: **"A study of *Pyrococcus furiosus* argininosuccinate lyase (PfuASL)"** describes expression and characterization of PfuASL and studies on its resemblance with δ-crystallin, which is a major eye lens protein found in birds and reptiles. We expressed the gene in *E.coli*. Far UV-CD and FTIR spectra indicated that it was fully folded with characteristic high α- helical structure. Though, PfuASL is a protein from a hyperthermophile it shows moderate thermal (in comparison to a hyperthermophile protein) and no kinetic stability. Activity profiling with sodium salt of argininosuccinic acid show that it had T_{opt} for activity at 80°C. Above 85°C PfuASL tends to form visible aggregates.

Gel filtration studies on PfuASL indicate that it is hexameric in nature, classically; members of the ASL superfamily are tetramers. In this regard, PfuASL is probably the first hexameric member characterized in the classically conserved tertameric ASL superfamily. Tryptophan fluorescence studies indicate that PfuASL had an emission doublet like that of delta crystallin from chick lens. At lower protein concentrations (≤ 0.1mg/ml) the peak at longer wavelength dominates and at higher concentrations the order gets reversed. Two tryptophan excitation maxima indicate that PfuASL is an asymmetric hexamer; this is because each of its monomers contains a single tryptophan residue, and, for a symmetric hexamer all tryptophans will have similar solvent accessibility that will show as a single fluorescence excitation peak. In an independent experiment (not part of this thesis) where we wanted to see whether it is possible to take PfuASL to high concentrations (like that

of δ-crystallins) we managed to concentrate it to a concentration of 150mg/ml without any visible aggregation. The crux of the chapter is that PfuASL is a novel hexamer of a classically conserved tetrameric ASL superfamily, with high thermal stability (in general). It has δ-crystallin like tryptophan fluorescence spectra (emission doublet), suggesting an asymmetric assembly of monomers in the native hexamer form.

Chapter 5 entitled: "**A study of an Alpha-lytic protease (ALP) homolog from *Pyrococcus furiosus***" describes studies on a thermophile homolog of *L.enzymogenes* alytic-protease. After much trouble, we finally were able to express PfuALP from a gene optimized for expression in *E.coli*. By using alignment softwares we predicted catalytically important residues and assigned putative boundaries between the pre, pro and the protease regions. PfuALP gene was cloned without its putative pre-region (signal sequence).

When purified under denaturing conditions, we observed three bands in the elution profile of PfuALP: one of correct size (48 kDa unprocessed), one of 38kDa, and last one of 25kDa (processed protease). With N-terminal sequencing of 25kDa band, we were able to assign the exact junction between the pro (N-terminal) and the protease (C-terminal) regions. N-terminal sequencing of 38 kDa band revealed that it starts with first amino acid from the N-terminal of the ALP; therefore, it lacked a significant portion from the C-terminal end of ALP (mature protease), but still it was able to show autocatalysis (when purified from the wild type clone and not form the active site serine mutant clone). As expected of a hyperthermophile enzyme, PfuALP showed high rate of autocatalysis at 80°C as compared to 37°C. With mutational studies we were able to predict that S366 and not S368 is the part of PfuALP's "His-Asp-Ser" catalytic triad, since the S366A mutation resulted in complete loss of its autocatalytic activity whereas protein from S368A mutant of PfuALP was catalytically active. The 38kDa band was also present in protein purified from S366A mutant (catalytically inactive) of PfuALP, which shows that 38kDa band is a result of some *E.coli* proteases mediated cut in PfuALP. We also showed that one ALP molecule cannot proteolyse another.

When expressed individually, pro was found to be well folded and quite thermostable. But, we were not able to express the mature protease independently. It

shows that at least under mesophile conditions, pro-peptide of PfuASL in indispensable.

Chapter 6 entitled: **"Attempts to recover beta-2-microglobulin from kinetically stable aggregate by scFv antibodies"** describes an attempt to explore a hypothesis of recovering protein from a protein aggregate with the help of antibody that binds to some specific epitope on the native protein which is not present on the aggregate, but, might be presents on some conformationally flexible molecules which are part of the conformational equilibrium of molecules on the surfaces of protein aggregates. To test this hypothesis we choose an aggregation-prone protein, beta-2-microglobulin (β2m). Phage display library was used to generate scFv antibodies against native β2m (in 20mM Tris buffer of pH 8). After 4 rounds of biopanning we screened phages from 48 clones for their ability to bind to native β2m. Out of the 48 clones screened, phages from the 14 best binders were allowed to infect HB2151 cells for soluble expression of scFv. Although we could see expression of scFvs on the SDS PAGE, but, these antibodies never gave positive results when used for ELISA against native β2m.