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**SUMMARY OF THE THESIS**

Stability studies of proteins generally focus on assessments of the behavior of proteins during thermal or chemical denaturation, with a view to estimating the stabilities of their three-dimensional structures in thermodynamic terms. Hyperthermophilic proteins, however, do not allow the making of rigorous thermodynamic assessments of stability. Regardless of whether they are purified from their source organisms, or produced through heterologous expression and purified from mesophilic hosts, they turn out to be so extremely structurally stable that they are either completely resistant to denaturant-mediated or heat-mediated unfolding, or turn out to unfold inordinately slowly; so slowly, in fact, that the time taken to reach equilibrium (to allow any estimations of melting temperature, or free energies associated with unfolding) far exceeds all feasible experimental timescales (e.g., hours or days) by many orders of magnitude (e.g., years, or centuries). Therefore, with these proteins, it is difficult if not altogether impossible to assess typical protein stability parameters. Furthermore, because the unfolding of these proteins (when achieved) is found to be mostly irreversible, doubts remain about whether the unfolding process is microscopically reversible at all, casting doubts on any standard interpretations of unfolding data in the absence of any evidence of refolding. Notwithstanding this, the high stabilities of these proteins are clearly of both fundamental interest, as well as potentially applicability (in the chemical and pharmaceutical industries) from the operational and practical viewpoints.

Where parameters estimated under conditions of conformational equilibrium are unavailable, one alternative manner of assessing heat stability is through assessments of 'kinetic stability', i.e., through measuring of the slowness of the unfolding reaction. Ultimately, for any protein in which different conformational states are kinetically 'locked-in', and where inter-conversions amongst states take extremely long lengths of time, thermodynamic considerations - while still fully applicable - lose relevance, and kinetic considerations assume greater importance, although, of course, it may be readily admitted that the true definition of 'stability' must always remain a thermodynamic one. This shift of attention from 'true stability' to operational stability may be further explained as follows: if a protein population retains its native state for several days even after it has been effectively thermodynamically destabilized (i.e., where conditions have already been put in place to ensure that, given a few months, the equilibrium will shift towards the unfolded state) kinetics becomes more relevant than thermodynamics, if the activity required to be performed by the protein takes only a few minutes or hours. Thus, from a purely operational viewpoint, it does not matter if a protein is not thermodynamically stable under a given set of conditions (e.g., a very high temperature, or the presence of a denaturant), if its unfolding is as slow under these conditions as to become totally irrelevant to the specific task being performed.

Thus, therefore, in the very near future, understanding and applying kinetic unfolding to be of great importance, and this is the emphasis of this entire work: (a) exploring the mechanisms by which high kinetic stability is achieved in hyperthermophilic proteins, (b) exploring the role played by various ionic interactions, (c) exploring the structural autonomy of sub-domain elements of structure, i.e.,
substructures, such as e.g., super secondary structural elements, and (c) examining whether the unlocking of kinetic stability can be achieved by thermo-chemical means, as well as whether such unlocking allows hyperthermophile proteins to display conformational behavior that is more controlled by pure thermodynamic considerations such as e.g., cold denaturation at room temperature. In addition, (d) in this thesis, we have also explored ways and means of avoiding obstacles in the expression of hyperthermophile proteins in mesophile hosts; by devising new strategies and showing that they succeed. The bulk of the work has been carried out with Triose phosphate isomerase of *P. furiosus* (PfuTIM) as an experimental system, but we have also worked with *P. furiosus* Rubredoxin (PfRd), Lysophospholipase, a putative Alpha lytic protease analog, and other proteins like Amylase, Trehalose synthase, or Arginosuccinate Lyase.

**Attempts to overexpress *P. furiosus* proteins in *Escherichia coli***: It is now evident that there are significant problems associated with the overexpression of hyperthermophile-derived proteins in mesophile hosts. Since such overexpression is important for structural and biochemical characterization of hyperthermophile proteins, for their use in industry, it is necessary to address these problems through exploration of the extent of the problem, and the application of new ideas and approaches. Thus, we carried out a detailed set of trials of overexpression in *E.coli* of six different ORFs of *P. furiosus*, a hyperthermophilic organism in **chapter 3**. To screen for, and optimize, the overexpression, we chose to work with following strategies: (a) use of multiple vector-host combinations; (b) optimization of conditions for overexpression in different hosts of three different genes cloned into pET-22a expression vector; (C) expression of two genes through a novel pET-23a based vector incorporating a PfuTIM-insertion, as fusions with PfuTIM; and (D) finally, de novo designing of an optimized synthetic gene encoding a putative protease (PF0305) that was never previously successfully overexpressed, despite many attempts in the laboratory. Remarkably, we obtained successful overexpression of lysophospholipase, trehalose synthetase and the putative protease, amongst the chosen six ORFs with different strategies from amongst the abovementioned strategies. Our studies reveal that m-RNA stability is more important than codon biasness.

**Purification, refolding and characterization of a putative lysophospholipase from *P. furiosus***: We got successful overexpression and purification of a putative lysophospholipase (PF0480) encoded by the *P. furiosus* genome has previously been cloned and expressed in *Escherichia coli*. **In chapter 4**, we have presented the biochemical and biochemical characterization of this protein. Studies involving structure determination established the enzyme to be an esterase; however, owing presumably due to failure to deposit into extraction-unfriendly inclusion bodies, purification and refolding have thus far on been done. In this chapter, we describe the successful recovery and refolding of the enzyme from inclusion bodies. Light scattering suggests that the enzyme is a dimer, or trimer, in circular dichroism and fluorescence spectroscopy show, the enzyme has mixed beta/alpha structure and well-buried tryptophan residues. Changes in temperature over the temperature range of 30-80 °C, as well as changes in the range of 0-50 % (v/v) of water mixtures with organic
solvents such as methanol, ethanol and acetonitrile. The enzyme is confirmed to be an esterase (hydrolyzing p-NP-acetate and p-NP-butyrate) and also shown to be a lipase (hydrolyzing p-NP-palmitate), with lipolytic activity being overall about 18-20 fold lower than esterase activity. Against p-NP-palmitate the enzyme displays optimally activity at pH 7.0 and 70 °C; notably over fifty percent activity is retained at 70 °C in the presence of 25% acetonitrile. The high organic solvent stability and thermal stability suggest that this enzyme may have useful biodiesel-related applications or applications in the pharmaceutical industry, once yields are optimized.

Partial destabilization of native structure facilitates cold denaturation in *P. furiosus* Triosephosphate isomerase (PfuTIM): We have chosen Triosephosphate isomerase (TIM) of *P. furiosus* as an experimental system in which to explore the existence of the kinetic stability. Cold denaturation is a phenomenon seen in many different proteins; however, it is not seen in all proteins and there have been no reports so far of its occurrence in hyperthermophile proteins. We propose that, due to kinetic stability, it fail to do so, therefore once the determinants of the kinetic stability, would be removed sufficiently it will appear to show the cold denaturation. In chapter 5, using PfuTIM, a recombinant trios phosphate isomerase (TIM) from the hyperthermophile archaeon, *P. furiosus* (Pfu), we show that heating of this protein through the low temperature side of its thermal denaturation transition in the presence of a denaturant (GdmCl) gives rise to partially-disordered conformational ensembles retaining a high degree of native-like secondary and tertiary structure. These partially-disordered conformational ensembles undergo cold denaturation upon cooling to room temperature, and gain of structure as they are heated back to temperatures approaching the boiling point of water. The ability of GdmCl to destabilize a hyperthermostable protein sufficiently to cause it to display cold denaturation behavior provides support to existing ideas concerning the role played by electrostatic interactions in stabilizing hyperthermophile proteins kinetically.

"Trishanku" states of *P. furiosus* Rubredoxin (PfRd): generated through forced cold-denaturation and refolding: Thermo-chemical perturbation of the highly kinetically-stable native structures of *P. furiosus* Triosephosphate isomerase (PfuTIM) homooctamers display partial cold-denaturation and heat-renaturation in proportion to the extent of initial structural perturbation. In chapter 6, we describe the application of thermo-chemical perturbation to a much smaller, and monomeric, hyperthermophile protein, the 53 residues-long *P. furiosus* Rubredoxin (PfRd), one of the most thermostable proteins known to man. Like PfuTIM, PfRd displays cold-denaturation after initial thermo-chemical perturbation. However, PfRd requires considerably higher temperatures as well as higher concentrations of guanidinium hydrochloride (GdmCl) to do so, and its cold-denaturation behavior is incompletely like that of PfuTIM. This allows PfRd to access multiple partially-structured states that are themselves highly kinetically-stable. Since PfRd trapped in this conformation is not able to access the fully-unfolded or fully-folded states, we refer to "Trishanku" unfolded states (or TUIs) after a mythical ruler (Trishanku) who got trapped between physical and non-physical states of consciousness, and if refolding of TUIs through removal of GdmCl results in the formation of partially-refolded, monomeric, non-native states that we refer to as "Trishanku" intermediates (or TRIs) which, like TUIs are kinetically-
trapped. TRIs differ from each other, and from native PfRd as well as from the TUIs, in respect of their structural content and characteristics. TRIs also differ from native PfRd in their susceptibility to proteolysis.

*Attenuation of ionic interactions profoundly lowers the thermal stability of P. furiosus Triosephosphate isomerase (PfuTIM): In chapter 7, we find that PfuTIM shows comparable structural contents at pH 3.0, 7.0 and 10.0, but profound differences in structural stability, evident in its heat- and cold-denaturation behaviour; increase in ionic strength through addition of salt compensates partially for these changes. A mutated form of PfuTIM lacking 4 key charged residues involved in ionic interactions networks, similarly displays a structural content identical to PfuTIM but profound differences in structural stability (amounting to the conversion of a hyperthermophile to a thermophile protein, with significant loss of kinetic stability), evident in heat- and chemical-denaturation experiments. Ionic interactions thus appear to contribute significantly to PfuTIM's kinetic stability. Thus, the effect of salt bridges in the thermodynamic stabilization of local sub-domain structures could translate into significant increases in the autonomy of sub-domains, and consequently in significant decreases in the cooperativity of the unfolding process, to effect significant increases in kinetic stability.*

*Autonomy of beta-alpha substructural modules in P. furiosus Triosephosphate Isomerase (PfuTIM): explaining the mechanism for kinetic stability: In chapter 8, we show that different quarter barrel sized fragments of PfuTIM, have significant secondary structure, when they are genetically excised and produced as independent polypeptides. Interestingly, all fragments show a considerable amount of thermal resistance to unfolding, with some consolidation of structure upon heating, which is reversible upon cooling for some fragments, but not for all fragments (showing hysteresis). This data highlights the importance of substructural autonomy in thermal-kinetic stability. We propose that the inordinately high stability of hyperthermophilic protein is achieved by augmenting the stability of substructures to reduce their interdependence and cooperativity, during unfolding.*