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SUMMARY OF THE THESIS

"The choice of a structural strategy for thermal stabilization of hyperthermostable proteins depends on the evolutionary history of the organism."-Berezovsky *et. al.*, 2005.

Archaea is the family of very old living members of this earth. These organisms developed in the conditions that are very harsh in comparison with today's environment; in which no other organism, except themselves, can survive. At that time, presumably the environment on the earth was "reducing" due to abundance of hydrogen and deficiency of oxygen. The sea, which is the birth place of these first organisms, was presumably boiling and protecting them from dryness and hazardous radiations. The simple reason which "clicks" in the mind regarding their survival in harsh conditions is the unbreakable coordination between the hyperthermostable components of these organisms. The hyperthermostable components include high GC rich DNA and very thermally stable proteins, called as hyperthermophilic (phile = loving) proteins. These "hyperthermophile" organisms are still living on small places on this earth which mimic the harsh conditions of the primitive earth. *Pyrococcus furiosus* is one of these organisms and was originally isolated from geothermally-heated marine sediments with temperatures between 90 °C and 100 °C collected at the beach of Porto Levante, Vulcano Island, Italy.

Pyrococcus furiosus rubredoxin (PfRd) is a hyperthermostable protein which is famous for its stability. This is a very small protein having only 53 amino acids and possesses an iron sulfur cluster, a three stranded beta sheet and a tight hydrophobic core made up of mainly aromatic rings. Archea are the first to possess the iron sulfur cluster since the iron-sulfur clusters themselves are the most ancient type of prosthetic group. PfRd has the simplest type of iron sulfur cluster in which sulfurs of four cysteines join the iron with four coordinate bonds in a tetrahedral geometry.

Thermodynamically, any stable system is a "fine" energetic equilibrium state. The states become more stable when they have as low an energy as possible, since the shift in equilibrium will need more energy input. Addition of sufficient energy, in the form of thermal or chemical free energy, to cause shift in equilibrium, takes the system towards instability. A possible way to avoid the shift in equilibrium is to slow down the rate of shift in equilibrium as much as possible. This slowing down the kinetics of attainment thermodynamic equilibrium shift defines the kinetic stability of the system. A system with high kinetic stability is far more operationally stable than when it was stable

thermodynamically. It is the kinetic stability which compares the stabilities of two systems when they are thermodynamically marginally stable. It is clear from the discussion that the "time" factor is the distinguishing factor between thermodynamically and kinetically stable proteins; which is of the order of ~5 minutes for proteins. Proteins are generally marginally thermodynamically stable, and therefore, a hyperthermophile protein cannot be stable owing only to thermodynamic stability, since they are found in the places of high thermal energy. These proteins have to retain their structure in order to function to maintain the life of the possessing organism, in that instability-causing environment. They must possess some way of getting more stable which is other than pure increase of thermodynamic stability. PfRd has just 53 amino acids but has almost all the factors which contribute to its stability. These factors are also supposed to make PfRd so stable that it cannot be destabilized till 200 °C.

Chapter 1 (Review of literature) summarises all the possible factors which contribute to the high structural stability of PfRd. This chapter starts with a brief introduction about *P.furiosus* and rubredoxin with further description of PfRd's structure and the function. The function of PfRd is mainly defined by iron sulfur cluster's redox status. Functions of various rubredoxins are described briefly and the role of PfRd is supposed to be one or more of them. There are many factors which are reported to contribute in hyperthermostability and may be contributing in the kinetic stability of PfRd. Apart from these, the role of solvation and the lack of cooperativity also seems to play important roles in its kinetic stability. Iron sulfur cluster's role in the folding and the stability is also described. Some similarities were found by the multiple sequence alignment of rubredoxins from thirty five mesophilic and thermophilic bacterial sources; which suggest that combinations of the residues at particular positions stabilize the protein.

Chapter 2 tries to describe the role of iron in the stability of PfRd. Apo PfRd made by the TCA precipitation method had less stability than native PfRd. We attribute the difference in the stability to the method of making the apo form. TCA method is a harsh method in which protein was precipitated by unfolding. After that the protein is refolded back in a suitable buffer in order to refold it. This method has an advantage of guaranteed removal of the iron but on the other hand, breaking of maximum or all stabilizing interactions occurs, which may not necessarily be reversed when the protein is refolded. To examine whether the method of making of apo PfRd determines the stability, apo PfRd was made by four other different methods, numbered in descending order of the harshness used. From the study it becomes clear that the iron is not entirely necessary in the maintenance of the structure, even

it helps in the formation of the structure. PfRd can survive at that higher temperature even without the iron as evident by the studies on apo-IV.

Chapter 3 establishes the role of various commonly used chemicals on the redox status of iron and removal of iron from the iron sulfur cluster of PfRd. EDTA, 2-mercaptoethanol, ferrozine and Gdm.HCl are very commonly used chemicals. From literature it is clear that EDTA oxidizes ferrous iron while 2-mercaptoethanol reduces ferric iron. Besides these properties, these are chelators of iron and perform their redox acts mainly by binding to iron; however, EDTA oxidizes ferric iron without binding to it. Ferrozine is a chelator of reduced iron and forms a magenta coloured complex after binding to ferrous iron in the 3:1 stoichiometric ratio (ferrozine : iron). This complex is large and it could be isolated by gel filtration chromatography. Gdm.HCl has no role in deciding the redox status of iron. The reduction of ferric iron by 2-mercaptoethanol was again established by ferrozine assay. The above components, in combination not only affect the redox status of iron in the iron sulfur cluster of PfRd but also they tend to remove it from the cluster, even without the use of heat and chemical denaturant. EDTA is a stronger chelator than ferrozine and can titrate the iron from ferrozine. Iron sulfur cluster is buried and shielded and use of Gdm.HCl is necessary for the iron to be removed and chelated. On the other hand EDTA, in combination with 2-mercaptoethanol, removed iron without the use of Gdm.HCl. This formed the basis of the two mild methods of formation of apo forms which are described in chapter 2. Our conclusion was that the gently made apo-PfRd was as stable as native PfRd.

Chapter 4 tries to unveil the reasons behind non-occurrence of cold denaturation in PfRd in presence of 2-mercaptoethanol. Cold denaturation causes unfolding of a protein which occurs due to lowering the temperature below the temperatures at which that protein is formed. Hyperthermophile proteins do not show cold denaturation. The protein has to be partially destabilized in order to show the cold denaturation. This was earlier evidenced by the study on PfuTIM which showed cold denaturation on heating and then cooling in the presence of Gdm.HCl. Similarly PfRd also showed the cold denaturation on heating and then cooling in the presence of Gdm.HCl. While PfuTIM showed cold denaturation during only one heating-cooling cycle, PfRd showed it in subsequent cycles also, which gave rise to kinetically-locked partially-unfolded intermediates. Addition of 2-mercaptoethanol at any stage of the heating cooling cycles inhibited the occurrence of cold denaturation. The access of 2-mercaptoethanol to the cluster happened only above ~60 °C. Although, no evidence of formation of disulfides was seen in the process of preparing cold-denatured forms but it could

one of the reasons of non-occurrence of cold denaturation upon addition of 2-mercaptoethanol besides reduction of iron. The study signifies the role of reducing environment in the maintenance of the structure of iron sulfur cluster and PfRd.

There have been some mutational studies done on PfRd. All of the above studies were done to reveal the basic components which decide the factors behind the extreme structural stability of PfRd. These studies revealed that unzipping of β-sheet results in the unfolding of PfRd. It is an obvious statement which can be made regarding the structural stability of PfRd because the β-sheet "encloses or brackets" all of the structure of PfRd. In another study on exchanging sections of PfRd and rubredoxin from *C.pasteurianum* with each other, it was shown that the role of the N-terminal is the most promising in determining the stability of PfRd. In PfRd most of the interactions including salt bridges, hydrogen bonding and hydrophobic interactions are mainly found in the N-terminal region. Hydrophobic interactions are shown not to play the only role in determining the kinetic stability. These studies showed that there must be the interactions between β-sheet region and the hydrophobic core which decide the kinetic stability of the PfRd. The role of iron sulfur cluster was also explored by mutating the cysteines holding the iron. All of these studies try to explore some of the regions which contribute to the stability of PfRd; and give explanations according to the "obvious" forces which contribute to the stability of the PfRd. Although roles of some amino acids in N-terminal region and hydrophobic region are explored but no study has earlier explored the roles of individual amino acids covering all the parts of PfRd, in determining the role in the structural integrity or the stability of PfRd. Chapter 5 and 6 describe the role of some individual amino acids in determining the structure and the stability of PfRd besides the role of solvation.

Solvation is the interaction between the peptide group of a polypeptide chain and environmental water. It plays major a role in unfolding of a protein. At high temperatures, the broken water structure and higher flexibility can cause the solvation of the peptide backbone. The solvation can be prevented by the "neighbouring residue effect" which arises due to the bulkiness of the side chains of the seven amino acids namely, Trp, His, Tyr, Phe, Ile, Thr and Val. These residues are able to reduce the solvation of peptide chain up to 1-6 amino acids neighbouring to them. The effect is more prevalent in the regions of the protein which contain high propensity of formation of PP-II structure.

The seven amino acids mentioned above are spread at 13 places in all over the small sequence of PfRd which contains only 53 amino acids. The possibility of the reduced solvation of peptide backbone due to these seven amino acids is worth exploring. To make the study convenient, the whole study is subdivided in to two parts. Chapter 5 deals with role of three aromatic side chain residues namely Trp, Tyr and Phe in avoiding the solvation. Chapter 6 deals with the role of the three aliphatic side chain residues namely Thr, Ile and Val for the same reason. It was found that the aromatic residues, in comparison to aliphatic residues, have a much larger role in maintaining the structure and the stability of PfRd (probably due to their packing using ring to ring interactions). Two major structural determinant amino acids have been found out which are F41 and W 48.

Chapter 7 is a General Materials and Method chapter which describes different materials and method used in the studies during the whole period. The material and methods, which are specific to the chapters, are described in the subsequent chapters.