Abstract

Proteins fold into their native three dimensional structures to accomplish a diverse range of biological functions with great specificity. Many factors and stages of quality control ensure the folding of the protein to its correct native state. However, under some circumstances, owing to the misfolding or incomplete folding, proteins fail to acquire the native state and undergo aggregation. Aggregation results when a polypeptide chain trying to fold establishes non-local contacts and comes into contact with amino acids of other polypeptide chains and interacts with them. Protein aggregation and folding processes are in kinetic competition with each other and both the processes are driven by similar stereospecific interactions, occurring intramolecularly during folding, but intermolecularly during aggregation. The result of this competition is sensitive to many physical and chemical factors and sometimes even slight changes shift the equilibrium away from folding towards the aggregation causing "loss-of-function" of normal biological proteins and "gain-of toxic activity" in aggregates.

The process of aggregation involves protein-protein interactions but many additional events accompanying such protein-protein interactions are necessary to qualify the product of such interactions to be called an aggregate. Different kinds of aggregates show variation in their structural contents ranging from completely non-native like to almost native like protein conformations, and this variation is expected to differentially impact the success of efforts taken in order to recover natively folded proteins from aggregate. Many methods have been employed to block protein aggregation, and a method that works efficiently with one system need not be successful with another system. Some proteins require the application of more than one of these methods to block aggregation, even to a small extent.

A mechanistic understanding and knowledge of various types of useful protein-protein interactions necessary for protein folding on the one hand, and of various factors and forces abeting/stabilizing aggregation and concomitant structural changes, on the other hand, can greatly help in the understanding of protein folding and aggregation. Protein aggregation obstructs *in vitro* protein research and causes serious pathological conditions as well as technical problems in biotechnological

applications. Analysis of the factors causing, and processes accompanying, protein aggregation is expected to be of great aid in attempting, to counter the problems arising because of it.

In the present study, we have tried to understand some aspects related to protein aggregation by examining and/or manipulating the aggregative behavior shown by polypeptide chains either during, or after structure formation, or due to the destruction of formed structures by exposing proteins to destabilizing influences. The broad objective of this thesis is to use rational protein engineering methods and an assortment of analytical techniques to manipulate and monitor the protein aggregation process, or lack of it, in some physiologically or pharmaceutically important proteins relevant to disease processes. We have employed different systems to address different aspects of aggregation; one being a cytokine, Interleukin 2 (IL2), and another being a group of proteins called the lens crystallins that are mainly present in eye lens fibre cells. Besides these two systems, we have done an analysis of the thermal stability and temperature-induced aggregation of a therapeutic antibody adalimumab.

This thesis is divided into six chapters with lists of references given at the end of each chapter. **Chapter 1** gives an introduction to protein aggregation including the different types of protein-protein interactions, conformational changes accompanying them, nuances of different types of aggregations, structural formats in aggregates, forces responsible for stabilization of aggregation and strategies for blocking aggregation. **Chapter 2** details the materials and methods commonly applicable to all the aspects of the study. Each of the remaining chapters has a separate brief "introduction" section and "materials and methods specific to this study" section. It must be appreciated that since, aggregates are largely intractable to structural-biochemical (and especially conformational) spectroscopic studies, many

In **Chapter 3** entitled "**Conformational studies of soluble and solid aggregates of native IL-2 and some designer variants engineered to reduce aggregation**", we have tried to engineer a recombinant IL2 molecule (known to be active but extremely prone to aggregation), to display a lower tendency to aggregate, by employing a combination of structural analyses, intuition and molecular modelling followed by protein engineering. However, precipitation of all the "designer" variants of IL2 upon refolding, in absence of any denaturants and additives, indicates the inexorable aggregation of all of them. Along with attempts to design a less aggregation-prone recombinant IL2 molecule, our objective was to study IL2 aggregates and see the structural contents in the aggregates of native as well as different mutant forms.

FTIR based characterization of the conformational states of the aggregated forms of native IL2 and its variant forms shows a variation in the alpha helical and beta sheet contents among different forms. Even the variant forms obtained by single point mutations are considerably different in secondary structure content, suggesting some positions are significant for the helix-forming propensities of the molecule. We concluded that formation of helical structures must have preceded aggregation, which further argues that helix-formation must have been based on intrinsic structural propensities, dictated by local, sterically-driven, backbone-backbone interactions.

In **Chapter 4** entitled "**Conformational studies of soluble protein matrices in the form of highly concentrated solutions of alpha-B, beta-B2 and gamma-B crystallins**", we have examined crystallins, the structural proteins of the eye lens, at physiologically relevant concentrations. Lens crystallins form a highly transparent and refractive medium in the lens, by tight packing at very high concentrations in the lens fiber, which is necessary for the proper focusing of light and to minimize

protein concentration, which appear to be similar in nature in all three of them. We suggest that, the unusual associative properties of crystallins owe to concentration dependent conformational reorganization occurring in such a way that the new conformation forms an extended structure with inter-molecular interactions.

In **Chapter 5** entitled **"Conformational studies of solid aggregates of gamma-B crystallin produced through different physico-chemical treatments"**, we have examined γB crystallin aggregates formed under four different conditions and addressed the question of whether any conformational reorganization has taken place during aggregation by comparing the conformational contents of native γB and its four differentially aggregated forms. FTIR study shows that there are structural differences between the various precipitated forms of γB-crystallin, as well as significant commonalities, both amongst the different precipitated forms and between these and the spectra obtained for native γB-crystallin in aqueous solution. All four aggregates have distinctively different morphological characteristics, evidenced by EM studies,which appears to be in keeping with their distinctively different secondary structural contents.

In **chapter 6** entitled **"A study of temperature induced conformational changes in a therapeutic antibody Adalimumab"**, we have examined the thermal stability and occurrence of thermal aggregation in a therapeutic, anti-TNF alpha monoclonal antibody, adalimumab. DSC, CD, FTIR and DLS were used to monitor temperature-induced changes in conformation and the conclusion was drawn that antibody unfolding occurs concomitantly with aggregation, in a sequential manner, due to the presence of many domains. Also, we obtained indications that domain dissociation largely precedes domain unfolding, upon comparing data from DSC with data from FTIR spectroscopy, CD and DLS. The major purpose of this study was to develop and test multi-technique approaches that can be used to probe the stability of