ABSTRACT

Aggregation is a poorly understood phenomenon- only recently begun to be studied from a structural biochemical point of view. In the present study we addressed several questions relating to aggregation of globular proteins towards furthering of the understanding of aggregation and manipulating its occurrence. The term 'aggregation' is used here to indicate formation of large intermolecular associates of proteins brought about by changes in conformation, in turn brought about by changes in physical or chemical conditions, which are irreversible. Aggregates studied here are therefore the result of conformational alteration of protein molecules brought about by thermal denaturation, chemical perturbation, exposure to extremes of pH, or exposure to damaging radiations (UV).

In the second chapter, we have tried to look at the structural requirements of a small heat shock protein, α–crystallin that govern its ability to function as a chaperone. In dilute solution, α–crystallin is a large heteromeric aggregate of approximately forty subunits of two sequence-related polypeptides α–A and α–B crystallins. The chaperoning activity of α–crystallin was known to be temperature dependent, where the aggregation prevention activity of the protein is only observed at a temperature of above 30°C. α–B crystallin is found to possess sequence homology to the small heat shock proteins towards its C-terminal end. We reckoned that α–crystallin undergoes a temperature dependent (switch) structural change to expose this smHSP homologous domain at elevated temperature to gain a chaperone-like activity, through the dissociation of its N-terminal segment from the C-terminal domain. We therefore excised away the N-terminal segment and purified the

C-terminal domain to examine whether the domain, in isolation, can function as a molecular chaperone. The excised heat shock domain was found to fold into a beta-sheet, small molecular weight multimer displaying extensive hydrophobic surfaces, however without retention of its aggregation-preventing activity. The domain, instead of preventing aggregation facilitated it by joining in the aggregate formation. Further, we have found that native alpha crystallin is not a chaperone in the lens, and that its ability to prevent the aggregation of beta and gamma crystallin depends on concentration and not on stoichiometry; suggesting that its structure and organization in the lens may be different from that in dilute solutions.

In the third chapter, we addressed the question of where polar residues of the molecules in the interiors of aggregate locate, and whether interiors of aggregate are solid-like native proteins or porous. The answer to both these questions emerged from studies in which bovine carbonic anhydrase (BCA) was thermally aggregated, and a hydrophobic dye (ANS) was used as a probe of accessibility, to demonstrate that interiors of heat induced aggregates of BCA are porous and not solid-like, and likely to be awash with solvent. It appears from these studies that only a small proportion of the hydrophobic stretches that get exposed initially during thermal unfolding are used in the process of intermolecular association in aggregates. It also appears from these studies that upon cooling, there is a folding-like consolidation of structure within aggregates, as observed by increase in the ANS fluorescence yield. This increase in the ANS fluorescence yield was observed to be about the same extent regardless of whether ANS was added prior to or after aggregation had taken place.

In an attempt to find whether a particular kind of non-covalent interaction is predominant in aggregates, we found that in thermal aggregates of BCA, aggregation happens mainly due to hydrophobic interactions between the partially structured hydrophobic stretches that gets exposed during incubation at high temperature. In a follow-up of this observation, in the fourth chapter we demonstrate that by physically occluding the hydrophobic patches on partially-structured BCA (at nearly 500 fold molar excess of hydrophobic dye (ANS) over protein), aggregation is completely inhibited. ANS at such high concentrations keeps the destabilized protein in a folding-competent state as removal of ANS from heated samples by extensive dialysis results in refolding to native structure. Qualitatively similar results were obtained for another protein alpha-lactalbumin, but for yet another protein lysozyme, the results were the opposite. In the case of lysozyme instead of any inhibition of aggregation there was an enhancement in the aggregation upon inclusion of ANS. These results have been analysed and rationalized in terms of the stabilization of aggregation-prone intermediates by bound dye *vis-a-vis* the binding of the surfaces involved, or not involved, in aggregation. The exposed hydrophobic surfaces on lysozyme would appear not to be involved in aggregation.

In the fifth chapter we screened peptides from a phage displayed peptide library (both 7-mer and 12-mer) having affinity (complementarity) towards exposed surfaces on aggregation-prone, heated BCA, with the aim of obtaining some information about the stretches of the native protein that are responsible for intermolecular contacts in the aggregates, and also with the hope of using the same for blocking aggregation (as these peptides could bind to surfaces responsible for aggregation). After successive biopanning and amplification we identified two peptides [that were represented

maximally (about 60% of the total selection)], and had these synthesised. We then used these peptides to examine their effect on aggregation. Reagents binding aggregates could potentially recognize partially-folded intermediates and either inhibit, or abet, aggregation, depending on the site of binding. Instead of observing any inhibitory effect with both peptides there was an increase in the aggregation of BCA when heated along with the selected peptides.

In the sixth chapter we screened a phage-displayed library of antibodies to select antibodies that could potentially bind to thermally destabilized aggregation-prone BCA molecules. We took this approach taking into consideration the results obtained from the previous chapter. In the previous chapter the observation of abetting aggregation of thermally denatured BCA by small peptides (selected for binding to partially-structured BCA, from phage displayed peptide library) was qualified by reasoning that small peptides do not restrict to any particular structure, and often attain a structure through induced fit to potential binding surfaces. Antibodies on the other hand are large molecules that can assume their own structures and therefore could constitute 'moulds' capable of binding structural epitopes on the aggregation-prone BCA molecules, thereby preventing association of such molecules, when present in molar excess of concentrations. With this view antibodies against thermally-denatured aggregation-prone BCA were selected from a phage-displayed antibody library. Attempts were made to produce antibodies from the selected phage clones in large amounts to be used in subsequent *in vitro* aggregation studies, but all attempts failed with mutation in the coding region of the antibody gene.

In the seventh chapter, in order to select peptides that mimic surfaces responsible for thermal- and UV-induced aggregation

of gamma II crystallin, we obtained custom made overlapping 14-mer peptides (with 2 amino acid overlaps) scanning the entire length of the gamma II crystallin polypeptide. Use of these peptides at 50, 100, 200 and 400 fold molar excess over protein showed various levels of aggregation (enhancement/inhibition). At 400 fold molar excess, there was as much as 60% inhibition effected by some of the peptides in the thermal aggregation of gamma II crystallin, whereas when some of these peptides were included in protein solutions and exposed to UV induced aggregation, they showed different results as compared to that seen for thermal aggregation.

We also studied the folding behaviour of a 377-residue long, cysteine-less, single domain TIM barrel fold endoxylanase obtained from a mesophilic *Bacillus* sp. The protein was found to be alkalistable and thermophilic (optimum activity at high temperature). It unfolds through a partially structured intermediate, displaying enzymatic activity. It also produces a refolding intermediate, which is obligatorily formed, partially-structured non-molten globular, thermally stable, enzymatically inactive, and extremely long-lived, refolding back to native active enzyme over several tens of hours. Interestingly, even though a demonstrable intermediate occurs on the folding pathway of this protein, there was no detectable aggregate in both unfolding and refolding reactions, indicating that while aggregation may require the presence of intermediates, the occurrence of intermediates (even long-lived ones) does not guarantee the occurrence of aggregation. However, this work is largely presented in the thesis of a collaborating student and not included for presentation here.