A number of studies have been attempted to understand the mechanism by which a globular protein transforms into a building block of amyloid (Booth, Sunde *et al.* 1997, Chiti, Webster *et al.* 1999). Lysozyme is a well characterized protein, rich in α-helical and β-sheet secondary structure which upon heating forms amyloids (Artymiuk and Blake 1981).Existing literature suggests that first the native structure of protein is lost, which makes it competent to stack in amyloid state (Arnaudov and de Vries 2005, Buell, Dhulesia *et al.* 2011).Also, it has been shown that besides protein concentration, NaCl concentration in the buffer and temperature affect the rate of amyloid formation (Fujiwara, Matsumoto *et al.* 2003, Hill, Robinson *et al.* 2009, Hill, Miti *et al.* 2011). Despite extensive studies, it still remains unclear whether loss of native structure precedes association, association precedes loss of native folding or both the processes occur simultaneously leading to amyloid association. Our Dynamic Light Scattering experiments brought forth that temperature-driven transition of lysozyme from globular monomeric state to associated form occurs *via* an intermediate state in the temperature range of 40-55°C. SEC-MALLS experiments revealed that low order associated species are formed during intermediate state followed by high order associations. Mapping of Amide I stretching frequencies confirmed that total secondary structure goes down with loss of α-helical content when the low order associations are formed. Also, the onset of large order association is accompanied by substantial gain in β-sheet architecture. Further heating led to higher order associations of disordered particles towards fibrillar architecture. Congo red staining confirmed the fibrils present in samples of these experiments are of amyloidic nature.

For getting insights into the process of amyloid formation we tried to crystallize lysozyme at temperatures where intermediates are formed. Crystallization set-up to obtain

diffractable crystals of lysozyme at 45°C, brought forth that lysozyme existed in three-to-four forms: tetragonal and orthorhombic crystals, and spherulites with needle like crystals and thin fibrous spikes coming out of central white core, in a single drop. Similar spherulitic structures were previously observed in case of bovine insulin and HEWL (Krebs, Macphee *et al.* 2004, Heijna, Theelen *et al.* 2007). Amyloidic nature of fibrous spherulitic outgrowths has also been reported (Krebs, Macphee *et al.* 2004). Diffraction of the needle shaped crystals allowed us to visualize the heat induced dimeric and trimeric state of lysozyme. Though there are some examples of lysozyme dimers in crystalline state, none showed the heat-induced anti-parallel arrangement seen through our structure (PDB ID: 4R0F). Moreover, through this study we report the first structure of lysozyme trimer (PDB ID: 4DC4). Analysis of trimer structure revealed that the active site of lysozyme gets occupied, which is reflected in the compromised bactericidal activity under the conditions where intermediates were formed.

ThT staining of spherulites and TEM images of thin spikes confirms that both spherulite core and thin spikes are composed of amyloid fibrils. Moreover, presence of weakly associated lysozyme molecules in needle shaped crystals and amyloidic nature of thin spikes show that decrease in dimensions of spherulitic outgrowths lead to stacking of more protein molecules together which ultimately form amyloidic fibrils (present as spikes emerging out of spherulites). Thus, they may represent different stages of amyloid formation pathway. Also this observation supports pre-existing hypothesis that the outgrowths from core of spherulites are amyloidic in nature (Krebs, Macphee *et al.* 2004). Structural information from thinner needle shaped crystals (which we were not able to

diffract) can provide interesting insights into other intermediate stages formed during lysozyme amyloid formation.

Presence of species matching the dimensions of weakly associated dimer and trimer structure through solution scattering experiments further supported crystallization results. This study also shows that equilibrium exists between the formation and dissolution of associated species in solution. This equilibrium state in turn depends on salt concentration in buffer and temperature. Importance of associated state found in our studies was further proved by using needle-shape crystals (composed of associated protein molecules) as seeds for amyloidic associations. This experiment supported our view that the associated molecules are "on pathway" ensembles of amyloid formation pathway.

DLS and crystallization experiments with known aggregation inhibitors like arginine, benzyl alcohol and sucrose provided insights into how these small molecules inhibit the formation of intermediate step or spherulite upon heating. Crystallographic details brought forth that aggregation inhibitory molecules bind to lysozyme at the sites/interfaces which are involved in forming heat induced associations and thus correlate with absence of intermediate states or spherulites during crystallization attempts. Overall, our experiments support that heat-induced amyloid formation of lysozyme is initiated by low-order association of protein retaining its globular shape and native-like secondary structural content. After association, lysozyme loses its secondary structure while being part of associated species. This can be seen in the rapid gain of β-sheet content in protein molecules as the dimensions of the scattering particles increased in solutions. The observations made with this study are summarized in the form of a mechanism (**Figure**

.1), which concludes that low order association precedes formation of non-native tructures which in turn gain sheet architecture to form amyloid fibrils.

Gaining visual insight into the pro-, pre- and amyloid organization, particularly hose formed by full-length proteins have remained a challenge. Incremental insights, nainly by indirect methods, have helped us conjure a picture of what probably happens as a erfectly globular protein like lysozyme transforms into a building block of β-amyloid. Precedence till now, teaches us that non-native shapes of globular protein including lysozyme associate to gain cross β-sheet structures to eventually form β-amyloid fibrils. It remains unclear how the partitioning of charged residues on surface and hydrophobic core gets disturbed or redistributed to achieve non-native structures, particularly when a good portion of lysozyme also forms α-helical structures. Non-specific changes in this partitioning might lead to non-native structures which might just aggregate. Formation of specific higher order associations which represent fibrils and get stained by Congo red and Thioflavin T, are indicators that their precedence or origin is *via* specific precursors. In other words, random events cannot lead to formation of specific resultant shapes/organizations. Our experiments conclude that prior to loss of native structural content, low order associations occur when lysozyme is exposed to higher temperatures. Probably, the associated states provide that solvent-shielded environment which allows restructuring to occur in a specific manner without leading to rapid misfolding or initiation of random aggregation. Physiologically, or in cellular environment, local concentration of amyloid forming proteins is actually higher than what are usually considered during representative biophysical experiments. This could be one limiting factor in correlating observed biophysical results with biology. In our experiments, involvement of higher

Figure 5.1

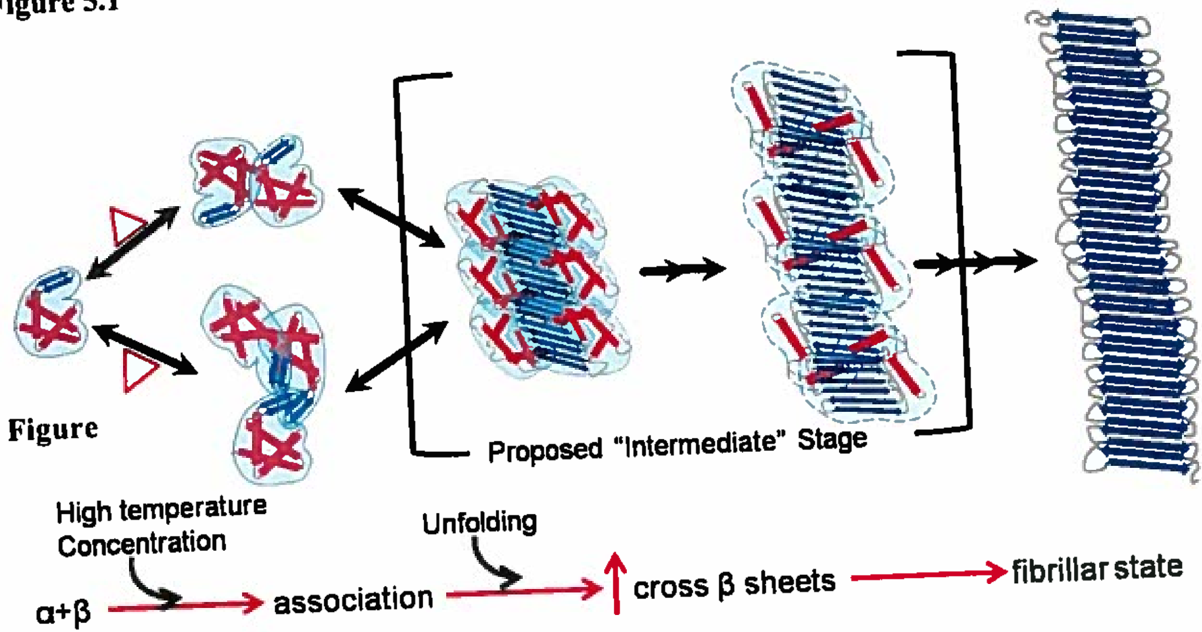

Figure 5.1 Schematic representation of the mechanism proposed by us that association of native globular proteins precede loss of native structure, which then associate to form large order species with gain in β-sheet architecture. (Blue arrows and red cylinders in this schematic diagram represent β-sheets and α-helical order).