Abstract

INDEX NO. 240/16/Chem./25

Title of the thesis:

Investigation of the interactions and aggregation mechanism of human insulin and bovine β-lactoglobulin in the presence of different chemical and physical modulators

Proteins are a crucial component of life, and their unique three-dimensional structure is largely determined by their amino acid sequence and thermodynamic factors. Unfortunately, when the balance of cellular homeostasis is disrupted, proteins can undergo a structural change known as misfolding. Misfolded proteins often take on non-native forms that can aggregate into harmful clumps. This phenomenon is linked to a range of disorders in the human body, with neurodegenerative diseases being of particular concern. The challenge of protein instability is a significant barrier to developing biopharmaceutical formulations. In order to address this challenge, it is important to have a thorough understanding of the physical mechanisms involved and the strategies available to protect proteins from degradation. When proteins misfold, they can transform from their natural structure to non-natural structures that can aggregate into organized fibrils. This process is implicated in over 35 human diseases, including Alzheimer's disease, Parkinson's disease, Huntington's disease, and sickle cell anemia, which makes it a significant challenge for the medical technology and pharmaceutical industries. Protein aggregates refer to the fibrils that contain misfolded proteins, which tend to form cross-β-sheet structures called amyloids. During thermal aggregation, pH-dependent conformational changes take place in several proteins, such as β-lactoglobulin (β-lg), α-lactoglobulin, immunoglobulin, bovine serum albumin, lysozyme, and insulin, leading to the development of amyloid fibrillar structures.

My research work is focused on the interaction and aggregation processes of two proteins, human insulin and β-lactoglobulin, with various physical and chemical modulators. Human insulin is a widely studied protein and serves as an essential model to investigate the underlying mechanisms of amyloid formation. Its intricate structure comprises two polypeptide chains, the A chain of 21 amino acids and the B of 30 amino acids, connected by two disulfide bonds. Furthermore, I have selected β-lactoglobulin as a model carrier protein owing to its pH-dependent opening, encapsulating property, unique acidic pH sensitivity, and bioavailability. The protein's core structure pattern is composed of an α-helix and eight antiparallel β-sheets. Additionally, the protein features a highly reactive -SH group located at Cys-121, embedded in the protein's hydrophobic core. Moreover, it contains two tryptophan residues, Trp19 and Trp61, which are responsible for its fluorescence properties. **Chapter 1** provides a general overview of proteins, covering topics such as protein misfolding, aggregation, and the consequences of protein aggregation. It also discusses factors that accelerate the fibrillation process. Additionally, this chapter includes a brief overview of two selected proteins, human insulin, and β-lactoglobulin, along with their literature review and biological applications. **Chapter 2** explores the interaction between human insulin and hydrophobic and hydrophilic molecules. Two Schiff's base compounds have been synthesized, one with a hydrophobic side chain and the other with a hydrophilic side chain. Our research findings reveal a slight but noteworthy preference for insulin towards hydrophilic molecules over hydrophobic ones. These results suggest that insulin can bind with hydrophilic molecules in a manner similar to glucose. At a temperature of 283 K, we found the binding constant to be 6.64×10^4 M^{-1} for hydrophilic molecules and 4.99×10^4 M^{-1} for hydrophobic molecules. The hydrophobic molecule binds to the hydrophobic pocket of the insulin surface, while hydrophilic molecules interact with the polar surface of insulin. An interesting observation is that the binding of the hydrophobic molecule significantly alters the secondary structure of insulin compared to

hydrophilic molecules, leading to a decrease in insulin stability in the presence of hydrophobic molecules. **Chapter 3** demonstrates the effect of three biologically important salt anions on insulin aggregation. Specifically, we investigated the self-assembly formation of insulin induced by salts differing in their anions, namely NaI, NaOAc, and $NaNO_3$, at low physiological concentrations (in the micromolar range). The results reveal that the efficacy of this process follows the order $I^- > CH_3COO^- > NO_3^-$. Notably, the anion-driven aggregation of insulin does not conform to either the Hofmeister series or electroselectivity series at very low salt concentrations. Instead, we propose that the binding of anions at low pH to the positively charged residues of insulin is determined by a mechanism where the salt anions promote the fibrillation of insulin and modify the morphology of the monomeric precursor molecule. The nucleation and fibril elongation are both controlled by electrostatic forces and hydrophobic interactions. **Chapter 4** addresses the impact of Ultraviolet (UV) light exposure on human insulin. In this experimental investigation, monomeric insulin samples were subjected to UV light at an excitation of 276 nm for varying durations - 1 minute, 3 minutes, 7 minutes, and 15 minutes. By employing the multi-spectroscopic method, it was observed that UV exposure leads to the aggregation of Human Insulin. Furthermore, the aggregation becomes more pronounced with an increase in exposure duration. The exposure induces structural modifications in the protein by altering its secondary structure and exposing hydrophobic sites on the surface of the protein, resulting in protein aggregation. The effect is most notable for native Human Insulin under UV light exposure for 15 minutes, followed by 7 minutes, 3 minutes, 1 minute, and human insulin monomer. **Chapter 5** demonstrates the impact of the polarity of coumarin molecules on their binding with beta-lactoglobulin. This investigation involves the production of two derivatives of coumarin-3-carbamide that contain chloride and electron-donating groups within a single molecule (C3A2). According to previous literature reports, the chloride groups in the coumarin moiety demonstrate a strong binding affinity to β-lg. In this research work, I have synthesized two molecules: one with -OMe and -Cl groups (C3A2) and the other solely with the -Cl group. Both molecules had -Cl groups attached to the aromatic ring at amide nitrogen. I examined the protein interactions with β-lg and found that C3A2 had a superior binding capacity compared to C3A1. Both compounds bind to β-lg through an endothermic process driven by entropy. During binding, C3A1 has a preference for the hydrophobic surface of the protein, while C3A2 interacts with the calyx of the protein. This binding results in conformational changes in the protein. As a result, the presence of -Cl and -OMe groups in coumarin-3-carbamide creates a polar nature in the molecule that enhances the β-lg binding process.

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