Abstract

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Title of the thesis:

Interrupting the formation of harmful amyloid fibrils of human insulin with different co-solvents and small molecules

Conformational changes of the therapeutic proteins like insulin leads to aggregation followed by deposition are detrimental to human system. Various external influences like pH, temperature, solvent or long-term storage may induce the transformation of native folds of insulin into non native cross-β-sheet rich fibrous structures. This process of protein aggregation is called amyloid fibrillation or amyloidosis. Including insulin, other protein viz., Transthyretin, β2-microglobulin, Apolipoprotein A etc. may undergo this type of fatal transformations. Amyloidosis collapses the functional properties of proteins and causes various neurodegenerative disorders like Alzheimer's disease, Parkinson's disease, Huntington's diseases, Spongiform encephalopathy etc. Diabetic patients taking regular insulin injections are commonly affected with the pathogenic deposition at the injection site followed by poor absorbance, reduced insulin functionality, cytotoxicity and immunogenicity. Additionally, pharmaceutical and clinical formulations, storage and delivery of insulin requires critical care against amyloid generation.

It is necessary to understand the mechanism of insulin aggregation and the ways that can potentially prevent amyloid formation. The insulin amyloid inhibitors should be specific enough to avoid interfering with the normal processes under physiological conditions. Naturally occurring compounds like micronutrients, vitamins, amino acids expected to show little/no side effects were chosen to proceed with the investigations of the present work. The stepwise approaches to attain the goal under the scope of the thesis were as follows. Firstly, the physical parameters (pH, temperature, duration of thermal exposure) were optimized to get the most aggregated/ amyloid form of insulin in vitro. The effects of micronutrients in the form of metal ions (Cu^{2+} and Fe^{3+}), water soluble B complex vitamins (vitamin B_1, B_6 and B_{12}) and sulphur containing amino acids (cysteine and methionine) on insulin amyloidosis were investigated with different in-vitro and in-silico approaches. In this regard, the influence of fluorinated co-solvent on insulin fibrillation was also examined.

Insulin composed of A and B polypeptide chains which are greatly stabilized by inter and intra-chain disulfide bonds. The fluorescence property of it is majorly rendered by the structural Tyrosine residues. The hexameric form of insulin found in its market available therapeutic formulations should be converted to monomeric form at acidic pH to proceed with the said objectives as the stability consideration of the monomeric form is vital in the functioning of insulin in vivo and also this form is susceptible to form amyloid structure.

Chapter1 deals with the kinetics of insulin fibrillation along with the effects of metal ions Fe^{3+} and Cu^{2+}on it. Fe^{3+} is more effective in inhibiting the heat induced amyloid generation than Cu^{2+}. Fe^{3+} maintains the alpha helical conformation of insulin by binding it with greater affinity. $\Delta G°$ for its binding is -7.02 kcal/mol where that for Cu^{2+} is -6.31 kcal/mol. Cu^{2+} has shortened the lag phase by promoting self-association of insulin but decreases the propensity of its aggregation. Fe^{3+} treated insulin, under aggregated condition produces small spherule and 'worm-like' globular morphology of insulin as detected under FESEM imaging but Cu^{2+} causes 'needle shaped' fibrillar morphology. Being transition metal ions, both of them

possess d-electrons and are assumed to form either octa or hexa-conjugated complex with water in aqueous media of interaction. They preferably access with protein side chains through surface H-bonds. Besides this, being very good heat and electrical conductors, these metal ions somehow have neutralized the thermal incubation effect on protein structure. The metal coordination sites are aspartate/asparagine, glutamate, histidine and cysteine residues of insulin as found in molecular docking simulations.

Chapter 2 explores the effect of vitamin B_1, B_6 and B_{12} on insulin aggregation. Vitamin B_1 potentially retains the monomeric alpha helix rich conformation of insulin in aggregating conditions whereas vit.B_6 mediated inhibition is feeble. In contrast, vit.B_{12} aggravates the aggregation process. The thiazolium ring of Vit.B_1 is assumed to favourably interact with insulin with H-bond and electrostatic interactions. The sulphur group present in vit.B_1 may form inter molecular disulphide bond within two or more vit.B_1. This assembly in turn interacts with insulin side chains and serves as a shield to prevent more insulin molecules to come closer during aggregation. The resulting polar environment created in presence of vitamin B_1 disfavours the hydrophobic association and burying event of non-polar residues of insulin. Vit.B_6 makes similar interactions but with lesser stability. Unlike the rest, vit.B_{12} induced the said aggregation of insulin. The bulky structure of Vit.B_{12} cannot fit itself to interact with insulin and induces the protein misfolding by hampering the stability and thus aggregation is favoured.

Chapter 3 addresses the influence of fluorinated co-solvents, 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) and 2, 2, 2-trifluroethanol (TFE) during the course of insulin aggregation. Maintenance of hydrophobicity of protein is critical in the inhibition of its amyloidogenic behaviour. Unlike HFIP, TFE greatly lengthened the Lag phase up to 150 minutes and thus assumed to delay the onset of fibrillation. Overall, HFIP at 10% v/v interaction ratio provided better protection to insulin monomeric form against the fibrillation. HFIP being a volatile solvent disrupts assembly of unfolded monomers to generate amyloid fibrils. Additionally, in aqueous solution the acidity of HFIP was enhanced which in turn maintained the monomer predominating form of insulin due to the protonation of its His residues. The possible formation of HFIP micro droplets at the amphoteric protein-alcohol interfaces in the aqueous medium can explain the results of the present study. However, above 10% v/v of fluorinated alcohol, the aggregation of insulin may take place. Then the HFIP mediated insulin fibrils gives needle shaped crystal-like morphologies whereas TFE mediated are of branched higher aggregates of insulin as revealed in Transmission Electron Microscopic imaging.

Chapter 4 gives a brief account of insulin aggregation in presence of sulphur containing amino acids. Cysteine and methionine when employed to interact with insulin freely but cannot protect it against heat induced fibrillation. In the process of mature fibril formation, insulin first unfolds itself followed by refolded in an erroneous manner to generate the seed of nucleation. Here the data suggests, the unfolding of insulin is followed by a random coiling under the influence of Cys and Met individually. Increase in amount of beta-sheet and beta-turn rich species is prevalent in the aggregates. At higher interacting ratio with insulin, Cys produces larger particles than Met. However, at lower interacting ratio of Cys, the particle size gets smaller than that of in presence of Met.

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