ELUCIDATING THE ROLE OF OLIGOMERS ON INSULIN AGGREGATION USING BIOPHYSICAL METHODS

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Abstract

Protein misfolding and abnormal fibrillation underlie many neurodegenerative conditions, such as Alzheimer’s and Parkinson’s disease. Insulin, which is composed of two covalently bonded peptide chains, exists in vivo mostly in a native hexameric state but becomes amyloidogenic under certain conditions: at high temperature with neutral pH (7.4) and agitation or with low pH (1.6) and quiescence. To investigate the mechanisms that drive insulin aggregation, we monitor its self-assembly into fibrils by kinetic fluorescence spectroscopy, which uses Thioflavin T (ThT), a fluorescent dye that binds to the -β structure of amyloid fibrils. At low pH, insulin behaves similarly to other amyloid proteins, kinetic rate of fibrillogenesis increases with concentration. At neutral pH, we observe an increase of the kinetic rate of fibrillation with low insulin concentration (2.5 – 25 µM), whereas at higher concentrations (25 – 100 µM) the opposite trend is observed. To explain this observation, we utilize photo induced cross-linking of unmodified proteins (PICUP) and Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE) to determine the oligomeric population of pre-fibrillar stages of insulin self-assembly. Preliminary results show a shift toward larger oligomers at insulin concentrations in the vicinity of 25 µM. As self-assembly advances and fibrils start to form (as observed by ThT fluorescence), PICUP/SDS-PAGE shows progressively decreased oligomer abundances. Insulin aggregation is also monitored via atomic force microscopy (AFM) to investigate differences in morphology between the two methods used to induce aggregation and the corresponding time evolution of oligomeric species. Our results are consistent with oligomer formation that is on the pathway to fibril formation, thereby elucidating a key interplay between oligomers and fibrils in insulin aggregation.

Experimental Design

Sample Preparation: by dissolving human insulin in 10 mM Sodium Phosphate buffer (pH 1.6, 7.4) at a stock concentration of 100 µM. Dilutions to concentrations of 2.5 – 100 µM (ε = 6710 M⁻¹cm⁻¹). Diluted samples are incubated.
- Acidic (pH 1.6) – Aggregation driven by T = 60°C
- Neutral (pH 7.4) – Aggregation driven by T = 60°C and agitation 155 rpm

Kinetic Thioflavin T (ThT) Fluorescence is used to determine the aggregation rate as well as lag phase of fibril formation.
- Molar ratio of Insulin:ThT is 1:1

Photo-Induced Cross-Linking of Unmodified Proteins (PICUP) is a process used prevent dissociation of oligomers by denaturants in gel electrophoresis. Tyrosine residues are covalently bonded by excitation and subsequent relaxation of photosensitizers and oxidizers, respectively.
- Oxidizer – 200 mM Ammonium Persulfate (APS)
- Photosensitizer – 10 mM Tri2,2′-bipyridine dichlororuthenium(II) hexahydrate
- Quencher – 0.5 M Ethylenediaminetetraacetic acid (EDTA)

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) allows for the determination of oligomeric species during the aggregation process.
- 10-20% Tricine Gel
- SilverQuest Silver Staining Kit

Atomic Force Microscopy (AFM) enables visualization of morphology of aggregates.
- 10 µL aliquots applied to freshly cleaved mica for 2 mins
- Rinsed with 50 µL Milli-Q dH₂O
- Dried with compressed air and vacuum desiccation
- Tapping mode.

Conclusions
- Insulin aggregation is monitored at pH 7.4 during lag phase and fibril formation.
- PICUP/SDS-PAGE shows that a shift toward larger oligomers at insulin concentrations in the vicinity of 25 µM. As self-assembly advances and fibrils start to form (as observed by ThT fluorescence), PICUP/SDS-PAGE shows progressively decreased oligomer abundances. Insulin aggregation is also monitored via atomic force microscopy (AFM) to investigate differences in morphology between the two methods used to induce aggregation and the corresponding time evolution of oligomeric species. Our results are consistent with oligomer formation that is on the pathway to fibril formation, thereby elucidating a key interplay between oligomers and fibrils in insulin aggregation.

Introduction

Subdermal insulin aggregates are known to form in Diabetes patients in injection-localized amyloidosis [Dobson (2006) Annu. Rev. Biochem.]. It has been shown that stabilization with Zn²⁺ ions significantly slows the formation of these aggregates [Noormägi (2010) Biochem J]. This is a now a standard practice in pharmaceutical production and distribution. Contrary to most amyloid forming proteins, such as amyloid-β or α-synuclein, decreased insulin concentrations may accelerate fibrilization [Ahmad (2003) Biochemistry]. The assembly pathway is not fully understood; investigating pre-fibrillar insulin may provide insight into fibril formation.

Insulin is a 52 residue protein consisting of two helical polypeptide chains. Three disulfide bonds are present: A6 – A11, A7 – B7, and A20 – B19 (Fig. 1). It is important to note that there are four tyrosine (Y) residues present in insulin, allowing for photo induced cross-linking. Insulin adopts a hexameric native state, composed of three dimer, able to be stabilized via Zn²⁺ ions or high peptide concentrations (~10⁻² M). Insulin is able induced into a fibrillar state with specific conditions, e.g. acidic pH, agitation, heating.

Figure 1: (above) Insulin secondary structure with the disulfide bonds and bromines – PDB ID 1UZ9 (left) Insulin A and B chain primary structure.

A chain = GIVEQCCCTKSLSYOLENVCN
B chain = FYNGHLC8GSHLVEALY1VCGGERGF7-PKPT

Figure 2. ThT Intensity vs. Time for (a) 2.5 – 20 µM and (b) 25 – 100 µM pH 7.4 at T = 50°C and 155 ppm, and (c) 10 – 100 µM pH 1.6 at T = 60°C

Figure 3. Oligomer size distribution of insulin (10 – 75 µM) per incubation period at pH 7.4, T = 60°C, and 155 ppm.

Figure 4. AFM images of 100 µM Insulin (10 mM KCl buffer at pH 1.6) incubated at T = 60°C for (a) 1 hr (b) t = 4 hr and (c) t = 6 hr.