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Amino acid sequence determinants in self-assembly of insulin chiral amyloid superstructures: Role of C-terminus of B-chain in association of fibrils

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ABSTRACT

Formation of chiral amyloid superstructures is a newly recognised phenomenon observed upon agitation-assisted fibrillation of bovine insulin. Here, by surveying several amyloidogenic precursors we examine whether formation of such entities is unique to bovine insulin. Our results indicate that only bovine, human, and porcine insulins are capable of chiral superstructural self-assembly. A tiny covalent perturbation consisting in reversal of Pro^{B28}-Lys^{B29} residues in a human insulin analog is sufficient to prevent this process. Our study suggests that insulin's dimer-forming interface – specifically the B-chain's C-terminal fragment – may acquire the new role of a molecular velcro upon lateral alignment of individual fibrils into superstructures.

Structured summary of protein interactions:
BI and **BI** bind by infrared spectroscopy (View interaction)
HI and **HI** bind by atomic force microscopy (View interaction)
HEWL and **HEWL** bind by circular dichroism (View interaction)
BI and **BI** bind by circular dichroism (View interaction)
α-LAC and **α-LAC** bind by circular dichroism (View interaction)
BI and **BI** bind by atomic force microscopy (View interaction)
α-LAC and **α-LAC** bind by atomic force microscopy (View interaction)
HI and **HI** bind by circular dichroism (View interaction)
PI and **PI** bind by circular dichroism (View interaction)
PI and **PI** bind by atomic force microscopy (View interaction)
HEWL and **HEWL** bind by atomic force microscopy (View interaction)

1. Introduction

Aggregation of misfolded protein molecules may lead to lower-free-energy assemblies termed amyloid fibrils [1,2], which are composed of β-sheets arranged perpendicularly to the fibril axis. In living organisms, formation of amyloid fibrils is often associated with so-called *conformational diseases*, such as Creutzfeldt–Jakob disease or Alzheimer's disease [3]. However, in vivo amyloidogenesis is not always linked to medical disorders. Several cases of functional naturally occurring amyloid-like aggregates have been reported in recent years. Among them, there are yeast prions which activate dormant genes helping to withstand environmental stress [4], and fibrillar forms of transmembrane protein Pmel17 required for maturation of melanosomes [5]. Meanwhile, specific properties of in vitro-grown amyloid fibrils make them promising candidates for new nanomaterials and this has led to considerable research efforts in this direction. Examples include fibril-based conductive nanowires [6] and enzymatically-degradable templates for fabrication of silver and platinum nanocables [7,8].

Structural transitions accompanying protein aggregation do not end on the conformational level, but continue on the levels of tertiary and quaternary structures leading to superstructural organisation of amyloid fibrils. Some well-known examples include spherulites – birefringent spherical structures first described by Krebs et al. [9], nematic liquid crystal phases assembled from lysozyme fibrils [10], and chiral superstructures of insulin fibrils first

Abbreviations: AFM, atomic force microscopy; BI, bovine insulin; CD, circular dichroism; FT-IR, Fourier transform infrared; HEWL, hen egg white lysozyme; HI, human insulin; ICD, induced circular dichroism; Ins-Core, Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val insulin amyloid core peptide; α-LAC, bovine α-lactalbumin; Lispro, Lys^{B28}-Pro^{B29} human insulin analog; PI, porcine insulin; PLGA, poly-L-glutamic acid; ThT, thioflavin T

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characterized by our group [11,12]. These meso- and microscopic entities are not only fascinating objects for fundamental research, but they are also inspiring a spectrum of possible applications such as scaffold materials for surface enhanced Raman scattering spectroscopy [13].

The problem of relationship between protein primary structure and propensity to form fibrils is of paramount importance. Studies conducted on sequenceless amyloidogenic polypeptides by Fändrich and Dobson suggested that formation of amyloid fibrils is a common trait of proteins as polymers, and is primarily driven by interactions between polypeptide main chains [14]. From this perspective, the amino acid sequence merely increases or decreases rate of aggregation, and affects structural details of grown fibrils [15,16]. However, even if formation of amyloid fibrils is a generic property of proteins, it remains unclear whether the higher-level superstructures are also accessible to fibrils from any amyloidogenic protein precursor. This study is aimed at addressing this question for the case of chiral superstructures of insulin amyloid fibrils that have been reported in our earlier works [11,12].

Chiral superstructures of bovine insulin amyloid are formed upon vortexing of the native protein at low pD, and in the presence of charge-compensating chloride ions. These fibrillar assemblies exhibit strong chiroptical properties. For detection of such species, we employed a common achiral amyloid-binding dye, thioflavin T (ThT) [17]. An extrinsic Cotton effect at around 450 nm with a '+' or '−' sign is enforced upon binding of ThT to aggregates [18] called henceforth +ICD and −ICD, respectively. These two types of amyloid assemblies differ not only in the sign of the ICD signal but also in morphology and abnormally intensive positive or negative far-UV CD spectra. The strong chiroptical properties could possibly originate from long range coupling of oriented transition moments [19]. Our previous studies have shown that the chiral assemblies are formed through a rapid lateral association of individual fibrils [20].

Recently, the problem of chirality of individual amyloid fibrils has started to attract much attention – especially in the context of relationship between the L-chirality of amino acid residues and fibril's twist [21], but also in studies on influence of environmental factors (e.g. pH) on fibril handedness [22,23]. In a number of investigations, extrinsic Cotton effects induced in bound ThT have been examined [24–26], yet in none of them the ICD intensity levels were comparable to those detected for the complex of ThT with insulin amyloid chiral superstructures.

Insulin is the key peptide hormone in the glucose metabolism. As a monomer, it is composed of two chains, 21-residue-long A-chain and 30-residue-long B-chain linked together by two disulfide bonds [27] (Fig. 1). Insulin is produced and stored in pancreas as a hexamer stabilized by Zn^{2+} ions while its active form is monomeric. Each hexamer is composed of three dimers. Insulin dimer is stabilized by antiparallel β-sheet between hydrophobic B-chain's C-terminal fragments of two monomers. Residues B8–B9, B12–B13, B16, and B23–B28 have been demonstrated to play important roles in the stabilization of native dimers [28,29]. Successive dissociations of hexamers and dimers are the key steps in biological activation of the hormone, and – during early stages of fibrillation. Insulin not only readily forms fibrils in vitro, but its aggregates also tend to accumulate subcutaneously in diabetic patients [30]. Several ideas on insulin amyloid structure and self-assembly pathways of insulin protofilaments and protofibrils have been put forward so far. These models are based on crystal structure of monomeric despentapeptide form of protein [31], cryo-electron microscopy images of fibrils [32], X-ray scattering on helical oligomers [33], and crystal structure of an insulin-derived amyloidogenic core peptide [34]. However, structure of a single insulin protofilament is still being debated. The goal of this study is to assess which parts of insulin's primary structure are critical for the assembly of chiral amyloid superstructures. Our findings are discussed in the context of the existing fibril models.

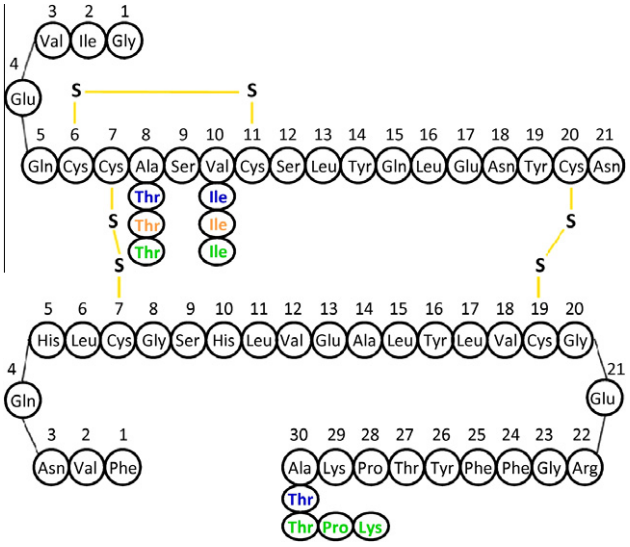

Fig. 1. Amino acid sequences of different types of insulin: BI (black), HI (blue), PI (orange), and lispro (green). Disulfide bridges are marked in yellow.

2. Materials and methods

2.1. Preparation of samples

Bovine insulin, hen egg white lysozyme, bovine α-lactalbumin (type I) and poly-L-glutamic acid sodium salt (M.W. 20.5 kDa) were purchased from Sigma. Human insulin was a gift from Dr. Piotr Borowicz (Institute of Biotechnology and Antibiotics in Warsaw). Porcine insulin and lispro analogue were ordered as reference standards from U.S. Pharmacopeial Convention and LGC Standards, respectively. Ins-Core peptide was custom-synthesized by Bachem S.A. at >98% purity. Fibrils described in Fig. 2 were prepared through 48-h-vortexing at 1400 rpm, or 72-h-long incubation at 60 °C of 0.2 wt.% bovine insulin in D_2O (H_2O has been substituted with D_2O due to following FT-IR measurements) with or without 0.1 M NaCl, pD 1.9 ('pD' was pH-meter readout uncorrected for isotopic effects). Samples analysed in the following figures were prepared through 48-h-long vortexing at 1400 rpm/60 °C of 0.2% solution of protein (0.25% for Ins-Core peptide) in the presence of 0.1 M NaCl, pD 1.9. The only exception was poly-L-glutamic acid for which pD was set to 4.3 and no NaCl was added. All samples prepared for agitation had identical 0.6-ml volumes and were placed in 2 ml Eppendorf probes. Vortexing was performed in Eppendorf Thermomixer Comfort accessory.

2.1.1. ICD, ThT fluorescence and FT-IR measurements

Protein concentrations for ICD measurements were 0.075 wt.%, while ThT molar concentration was kept at 70 μM. Measurements were carried out on Jasco J-815 spectrophotometer in a 1-cm quartz cuvette. Concentrations of proteins and ThT for fluorescence measurements were set at 0.03% and 40 μM, respectively. Fluorescence spectra were collected on AMINCO Bowman Series 2 luminescence spectrometer equipped with a 4-mm quartz cuvette. FT-IR measurements were carried out at the initial protein concentration using CaF_2 transmission cell and 25 μm Teflon spacers. Measurements were performed on a Nicolet NEXUS FT-IR spectrometer equipped with a MCT detector cooled by liquid nitrogen; 256 interferograms of 2 cm^{-1} resolution were co-added for a single spectrum. All spectroscopic experiments were carried out at room temperature.