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Rapid degradation kinetics of amyloid fibrils under mild conditions by an archaeal chaperonin

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ABSTRACT

Amyloid depositions containing exceptionally stable β-sheet rich protein aggregates, called fibrils are associated with prevalent and incurable neurodegenerative diseases. Chaperones are proteins that facilitate protein folding in both eukaryotes and prokaryotes. We found that a cold-adapted mutant ATP-dependant chaperonins (Hsp60) from a hyperthermophilic archaeon binds to and fragments insulin fibrils very rapidly with local targeted entry points. Individual fragments swell and the fibrillar β-sheet is quickly transformed into a mix of α-helical and unordered protein structures. After further incubation, the fragments coalesced, forming large amorphous aggregates with poly-disperse topologies. This finding represents a new approach to the disassembly of refractory protein aggregates under physiological conditions.

1. Introduction

Amyloid depositions, containing exceptionally stable β-sheet rich protein aggregates called fibrils, are associated with several debilitating neurodegenerative diseases. The role of amyloid fibrils in these maladies remains elusive. A large body of evidence suggests that amyloid fibrils are toxic species, which cause misfolding and aggregation of native functional proteins. According to a current hypothesis, amyloid fibrils are warehouses of misfolded proteins, that entrain toxic species that, left unchecked, would cause neurodegenerative diseases [1]. Consequently, therapeutic and pharmaceutical strategies target the appearance and possibly deconstruction of amyloid fibrils. The latter strategy is difficult to implement *in vivo* since the extreme stability of most amyloid structures dictates the use of harsh conditions that are not clinically applicable. Several small molecules have been reported to prevent monomeric protein aggregation during the initial stages of fibrillation [2,3] . Some of these molecules are purported to arrest a protein aggregation by competitive binding to the fibril oligomers. At the same time, mature amyloid fibrils resist small molecule attack. Harsh dispersion conditions, such as a strongly alkaline medium, [4] low temperature, and high pressure, [5] are required to disintegrate mature fibrils formed from full-length proteins. Recent findings supporting the suggestion that pre-amyloid species are more toxic than the amyloid deposits themselves. For example, amyloid β (Aβ) dimers and oligomers have higher toxicity than fibrils, during extracellular delivery to cell lines or *in vivo* when injected into rat brain [6,7] . Moreover, fibril precursors, such as proto-filaments and proto-fibrils of insulin have higher toxicity than mature fibrils [8]. If this is true, intra and extra cellular conversion of the misfolded proteins and their oligomers into highly ordered amyloid forms may in fact diminish the level of these toxic species and therefore be protective.

In this study, we address the factors affecting the dispersion of amyloid fibrils formed from human insulin. A common clinical condition, known as injection amyloidosis, is observed when a high local concentration of insulin causes its under-skin aggregation in the form of fibrils [9,10] . Insulin is a very well-studied peptide hormone that has two polypeptide chains linked by two inter-chain and one intra-chain disulfide bonds. In native form, insulin has mainly α-helical secondary structure. Upon aggregation into fibrils, native insulin undergoes structural change into β-sheet rich conformations. Often, insulin *in vitro* fibrillization results in the formation of morphologically heterogeneous fibrils, a phenomenon known as fibril polymorphism [11].

The interactions of heat shock proteins with amyloid fibrils have drawn significant attention in recently years. However, most of these studies focus on one group of heat shock proteins with small molecular mass, so called small heat shock proteins [12,13]. Many small heat shock proteins have been reported to disassemble fibrils or prevent the fibrillation process *in vitro* [14]. For example, small

Abbreviations: Pf, *Pyrococcus furiosus*; Hsp60, heat shock protein; Cpn, chaperonin; CD, circular dichroism.

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heat shock protein 104 from yeast disassembled mature prion fibrils *in vitro* [15,16]. Hsp104 changes the secondary structure of PrP fibrils from β-sheet to a random coil [17]. MTT tests confirmed that Hsp104 treated PrP maintained the same level of toxicity as untreated protein fibrils [18]. Human small heat shock proteins have been found to inhibit the assembly and elongation of alpha-synuclein amyloid fibrils [17]. Several other heat shock proteins have been reported to show a similar impact on fibrils [19]. For instance, heat shock protein 70 and Hsp90 modulate the assembly of alpha-synuclein amyloid fibrils [20–23]. Human heat shock protein 60 (also known as Chaperonin) cooperates with heat shock protein 70 preventing huntingtin protein from fibrillation [19].

Chaperonins are divided into Group I, represented by GroEL found in all bacteria, mitochondria and chloroplasts, and Group II complexes, occurring in eukaryotes and archaea. Group I chaperonins require a co-chaperone, GroES, to facilitate protein folding, however group II chaperonins can function without a co-chaperone. Functional double-ring complexes of group II chaperonins usually consist of two or more different subunits [24,25]. The chaperonin complex from *Pyrococcus furiosus* (Pf), in common with most hyperthermophiles [26], is composed of identical subunits and therefore is minimally complex [24,27]. Pf chaperonin is exceptionally stable, but has very limited activity below 50 °C. We have recently demonstrated that a hyperstable chaperonin with mutations that increase flexibility of the carboxyl terminus is highly active at relatively low temperatures [28]. Here we report a comprehensive investigation of the kinetic mechanism of insulin fibril deconstruction by this mutated Cpn.

2. Materials and methods

2.1. Insulin fibril preparation

Bovine insulin (60 mg/ml), purchased from Sigma–Aldrich, St. Louis, MO, was dissolved in water with a final pH adjusted by concentrated HCl to pH 2.5. The protein solution was incubated at 70 °C for 2 h. The insulin fibrillation process was terminated by reducing the temperature to ~25 °C. Non-aggregated protein was removed by a sample centrifugation at 14.000*g* for 20 min. After a supernatant was removed, a gelatinous phase, dominated by mature fibrils, was re-dispersed in HCl solution with pH 2.5. The centrifugation–redispersion procedure was repeated twice.

2.2. Chaperonin preparation

Pf Cpn was cloned and expressed as previously reported [24,28] . The supernatant extracts were heated at 70 °C for 30 min, and then they were purified to homogeneity by two steps of anion exchange: HiTrap™ Q HP cartridge from Biorad (Hercules, CA) and Bio-Scale™ macro-prep high Q cartridge from GE healthcare (Uppsala, Sweden).

2.3. Chaperonin-fibril reaction

Insulin fibrils were mixed with Cpn (0.05 mg/ml) in 20 mM sodium acetate buffer, pH 6.0 with 2 or 200 mM ATP. Mg ions, essential for Cpn activity, were added to the buffer together with sodium chloride to the final concentrations 1 and 50 mM, respectively.

2.4. Atomic force microscopy (AFM)

An aliquot of the analyzed solution was re-suspended in sodium acetate buffer, pH 6.0 with a 1:400 dilution factor (V/V). A drop of this solution was placed onto freshly cleaved mica and incubated for 2 min followed by removing of the solution excess. Finally, mica surface was dried under a nitrogen flow. AFM scanning was performed immediately in AC tapping mode using MFP-3D™ Bio Asylum Research microscope (Asylum Research, CA, USA) with Olympus AC160 tips.

2.5. Scanning electron microscopy (SEM)

For each sample 20 μL of analyzed solution were diluted in 1:400 ratio by distilled water and deposited on a 200-mesh copper grid. Staining with 1% uranyl acetate was performed in 10 min after the deposition. Samples were imaged on Zeiss Gemini Ultra 55 SEM (Oberkochen, Germany) in InLense mode with 5 kV EHT.

2.6. Far-UV CD spectroscopy

Far-UV CD (190–250 nm) spectra were measured using a JASCO J-810 (Jasco, Japan) spectropolarimeter at room temperature. For protein concentrations of ~1 mg/ml, a 0.02 cm path length cell was used. The CD spectra were acquired with 100 nm/min scan speed at 1 nm step and 2.0 nm bandwidth. Five spectra were accumulated and averaged for each sample. Protein secondary structure composition was evaluated using CCAplus software package. Four principal protein secondary structure components from CDPro package were taken to de-convolute the analyzed CD spectrum.

2.7. Thioflavin T (ThT) fluorescence assay

ThT fluorescence assays were performed on Fluorolog spectrofluorometer (HORIBA Jobin Yvone, Edison, NJ) at room temperature. The sample aliquot of 20 μL was mixed with 3 ml of Thioflavin T (25 μM, Sigma–Aldrich, St. Louis, MO) in 10-mM Phosphate buffer (pH 7.4). ThT emission spectra were recorded between 465 and 550 nm at 450 nm excitation using a 1 × 1 cm rectangular cell. The solution was stirred using a magnetic bar for several minutes before fluorescence measurements. The total of three spectra were measured for each sample and then averaged.

3. Results and discussion

3.1. Chaperonins modify and fragment insulin fibrils

Insulin fibrils prepared according to the literature procedure are shown in Fig. 1A and E. They conformed to the shape and size of 25 nm in width, around 8 nm in height (thickness) and with a variable length from 100 nm up to a several microns [11]. Fibrils were centrifuged and re-dispersed in pH 6.0, 20 mM sodium acetate buffer in the presence of Cpn (0.05 mg/ml) and ATP (2 mM) at 37 °C. The kinetics of fibril transformation caused by the Cpn activity were probed by AFM and SEM. Solution aliquots were taken 5 min, 15 min and 30 min after the reaction started. As evident from AFM images in Fig. 1B and C the insulin fibrils fragmented in a periodic pattern within 5 min of exposure to Cpn (Fig. 1B) and were swollen and foreshortened with significantly lower height (~6 nm) and width up to 200–400 nm (Fig. 1F and G, indicated by red arrows). The periodicity of initial processing by Cpn is reminiscent of the non-continuous formation of insulin fibrils reported by Knowles et al. [29]. Interestingly, white image features on the edges of the clamps mimic the outline of the original fibrils (Fig. 1B). Most likely they are fibrillar regions that were not melted by Cpn because the reaction was quickly terminated. These observations confirm that Cpn changes the fibril architecture, melting the fibril core and finally forming an amorphous protein mass from regular β-sheet structure. Microscopic observations of this phenomenon confirm the fibril swelling before fragmentation. A longer exposure of insulin fibrils to Cpn results in their