Biochimica et Biophysica Acta 1844 (2014) 1881–1888

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Elongation of amyloid fibrils through lateral binding of monomers revealed by total internal reflection fluorescence microscopy

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ARTICLE INFO

Article history:
Received 19 March 2014
Received in revised form 13 June 2014
Accepted 17 June 2014
Available online 12 August 2014

Keywords:
Amyloid fibril
Islet amyloid polypeptide
Surface diffusion
Total internal reflection fluorescence microscopy
Type II diabetes

ABSTRACT

Amyloid fibrils are fibrillar aggregates of denatured proteins associated with a large number of amyloidoses. The formation of amyloid fibrils has been considered to occur by nucleation and elongation. Real-time imaging of the elongation as well as linear morphology of amyloid fibrils suggests that all elongation events occur at the growing ends of fibrils. On the other hand, we suggested that monomers also bind to the lateral sides of preformed fibrils during the seed-dependent elongation, diffuse to the growing ends, and finally make further conformation changes to the mature amyloid fibrils. To examine lateral binding during the elongation of fibrils, we used islet amyloid polypeptide (IAPP), which has been associated with type II diabetes, and prepared IAPP modified with the fluorescence dye, Alexa532. By monitoring the elongation process with amyloid specific thioflavin T and Alexa532 fluorescence, we obtained overlapping images of the two fluorescence probes, which indicated lateral binding. These results are similar to the surface diffusion-dependent growth of crystals, further supporting the similarities between amyloid fibrillation and the crystallization of substances.

1. Introduction

Amyloid fibrils are fibrillar aggregates of denatured proteins with a diameter of approximately 10 nm and length of several μm, and are predominantly composed of cross β-structures in which the direction of polypeptide chains are perpendicular to the fibril axis [1,2]. Amyloid fibrils are associated with a large number of amyloidoses and more than 30 amyloidogenic peptides or proteins have been found including amyloid β peptide (Aβ) associated with Alzheimer's disease, islet amyloid polypeptide (IAPP) with type II diabetes, and β_2-microglobulin (β2m) with dialysis-related amyloidosis. On the other hand, peptides or proteins not directly related to diseases have been shown to form amyloid fibrils in vitro under certain conditions [1]. Moreover, various amyloid-like structures formed under physiological conditions, including curli fibrils [3], chorion proteins [4], some peptide hormones [5], and type I antifreeze protein [6], are proposed to be functionally important. Therefore, clarifying the mechanism of amyloid fibrillation is of critical importance not only for developing strategies against those diseases, but also for advancing our understanding of proteins.

The formation of amyloid fibrils is generally considered to occur by nucleation and elongation [1,7–9]. The nucleation step, in which several monomers associate, is thermodynamically unfavorable. Once the nucleus is formed, elongation proceeds rapidly. Consequently, the kinetics of the spontaneous formation of amyloid fibrils is represented by a sigmoidal curve with a lag phase. The addition of pre-formed fibrils as seeds into the reaction mixtures shortened the lag phase. These phenomena are similar to the crystallization of substances, which indicates that the formation of amyloid fibrils and crystals share common mechanisms even though their morphologies are distinct [7,10].

The linear morphology of amyloid fibrils suggests that all elongation events occur at the growing ends of fibrils, although the distinct roles of the two ends are still unclear [11]. In a template-dependent dock–lock mechanism, the first phase, "dock", addition of monomers to the growing ends of amyloid template is followed by the second phase, "lock", stabilization of the deposited peptides [12,13]. Single fibril observations of elongation have also suggested that the growing ends are the only place where monomers or oligomers bind to fibrils [14–16]. On the other hand, the branching of fibrils has been suggested to occur by the formation of a new growing end at the inside of preformed fibrils. Branching is one type of secondary nucleation, in addition to that produced by breaking preformed fibrils [17–20].

On the basis of the kinetic analysis of elongation monitored by tryptophan fluorescence [21] and NMR [22,23], we suggested that monomers also bind to the lateral sides of preformed fibrils. These

Abbreviations: AFM, atomic force microscopy; Aβ, amyloid β; β2m, β_2-microglobulin; DMSO, dimethylsulfoxide; FRET, fluorescence resonance energy transfer; HFIP, 1,1,1,3,3,3-hexafluoroisopropanol; HPLC, high performance liquid chromatography; IAPP, islet amyloid polypeptide; ThT, thioflavin T; TIRFM, total internal reflection fluorescence microscopy

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http://dx.doi.org/10.1016/j.bbapap.2014.06.014
1570-9639/

molecules that bound to the non-growing ends subsequently diffuse on the lateral surface, reach the growing ends, and finally induce conformation changes to form mature fibrils. Since amyloid formation is the result of the exclusion of peptides or proteins from solvent above their solubility limits [24], such a two-step elongation through lateral binding and diffusion on the surface is likely to occur. "Surface diffusion" of the bound monomers on a terrace (corresponding to the lateral surface) before growth at the step or kink (corresponding to the growing ends) is an important elementary step in the crystallization of substances [25]. Similar non-specific binding and surface diffusion have been proposed as important for the interaction between DNA binding proteins and DNA [26–28].

We used IAPP to examine the possibility of lateral binding during the elongation of fibrils (Fig. 1). IAPP [29], also called amylin [30], is a 37-residue peptide with a molecular weight of 3.9 kDa. The COOH-terminal residue is amidated and one intramolecular disulfide bond exists between Cys2 and Cys7. IAPP assumes a dominantly random coil conformation in solution [17]. However, CD and NMR studies have shown that IAPP forms a transient amphipathic helix in the NH_2-terminal region upon binding to micelles or membranes [31–33]. This helical intermediate has been proposed to be important for the formation of amyloid fibrils [34]. On the other hand, the COOH-terminal region is unstructured. The amyloid fibrils of IAPP were shown to be deposited in the extracellular spaces of the pancreas in patients with type II diabetes [35]. Since amyloid deposits have been observed in approximately 95% of diabetic patients, but are rarely found in non-diabetic individuals, it has been hypothesized that IAPP fibrillation is involved in the pathogenic development of the disease [36,37].

To observe the amyloid fibrils of IAPP, we used total internal reflection fluorescence microscopy (TIRFM). We previously developed a technique for the real time observation of amyloid fibrils at a single fibril level by combining TIRFM and amyloid-specific thioflavin T (ThT) fluorescence [14–16,38]. The direct observation of amyloid fibrils provided a range of important information such as the growth rate, morphology, and elongation direction of amyloid fibrils in real time at the single fibril level. Regarding glucagon, the frequent branching of fibrils during elongation has been observed [20]. If another fluorescence dye could be used in addition to ThT fluorescence, it may be possible to distinguish the molecules in preformed fibrils and intermediate molecules in the process of elongation.

Here, we prepared IAPP modified with a fluorescence dye, Alexa532. By monitoring the process of elongation with ThT and Alexa532 fluorescence separately, we succeeded in obtaining overlapping images of the two fluorescence probes. The images obtained indicated the lateral binding of monomers before the formation of mature fibrils, suggesting that surface diffusion common to crystallization is also valid for the formation of amyloid fibrils.

2. Materials and methods

2.1. Materials

IAPP peptides were purchased from the Peptide Institute, Inc. (Japan). ThT was obtained from Wako (Japan). Buffer, salts, and solvents were obtained from Nacalai Tesque Co. Ltd. (Japan). Alexa Flour 532 carboxylic acid succinimidyl ester was purchased from Life Technologies (U.S.A.).

2.2. Amyloid fibrils of IAPP

Lyophilized synthetic IAPP was dissolved in 10 mM HCl containing 80% (v/v) 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) for complete dissolution. This approach efficiently removed any preformed IAPP aggregates. After lyophilization to remove the HFIP, the peptide was dissolved in 10 mM HCl (pH 2.0) at 100 μM as a stock solution. The

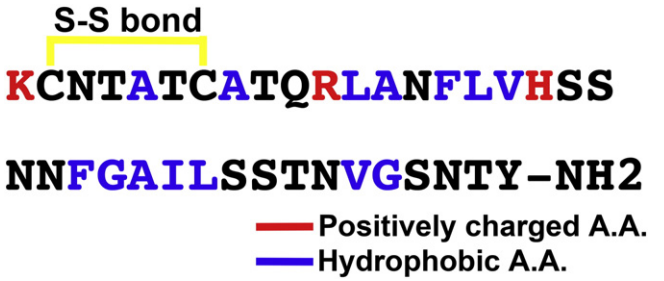

Fig. 1. Primary sequences of human IAPP. IAPP contains disulfide bonds between 2 and 7. All peptides are C-terminally amidated.

formation of fibrils was initiated by diluting the stock solution into the reaction buffer. The final conditions were 25 μM IAPP in 10 mM HCl containing 10% (v/v) HFIP. The peptide solutions were incubated at 25 °C.

2.3. Peptide labeling

Succinimidyl esters react with non-protonated aliphatic amine groups. IAPP has two amine groups; the N-terminal α-amino group and ε-amino group of the lysine residue. To specifically label the α-amino group, the reaction was carried out at pH 8.0 at which the α-amino group deprotonated while the ε-amino group did not.

IAPP was dissolved in 90% (v/v) dimethylsulfoxide (DMSO) to a concentration of 250 μM and was diluted to 100 μM with 20 mM HEPES buffer (pH 8.0) containing 380 μM Alexa Fluor 532 carboxylic acid succinimidyl ester and 0.1 mM EDTA. The solution was incubated for 17 h at room temperature in the dark under gentle stirring. The solution was then lyophilized, dissolved in 80 μl of 80% (v/v) HFIP, and then purified by HPLC. The molecular mass of IAPP-Alexa532 was confirmed by MALDI-TOF-MS (Bruker Daltonics, Germany).

2.4. Reversed phase HPLC

The purification of IAPP-Alexa532 was performed on a high performance liquid chromatograph (HPLC) (Gilson Inc., U.S.A.) equipped with COSMOSIL PACKED COLUMN Cholester (4.6 × 250 mm, Nacalai Tesque, Japan). The linear gradient was achieved by a water–acetonitrile system containing 0.05% trifluoroacetic acid with the acetonitrile increment from 20 to 60% for 45 min at 0.5 ml/min. The injection volume of the sample was 10 μl.

2.5. Atomic force microscopy (AFM)

Atomic force microscopy (AFM) images were acquired with a Digital Instruments Nanoscope IIIa scanning microscope at room temperature (Bruker AXS, Japan). A 5 μl sample solution was put on freshly cleaved mica. After air drying, measurements were performed in an air-tapping mode. The scan rate was 0.5 Hz, and images were obtained in a 5.0 × 5.0 μm area with 512 × 512 points.

2.6. TIRFM

The TIRFM system used to observe amyloid fibrils was developed based on an inverted microscope (IX70, Olympus, Japan) as previously described [39]. The ThT molecule was excited at 442 nm by a helium–cadmium (He–Cd) laser (IK5522R-F, Kimmon, Japan). IAPP-Alexa532 was excited by an Argon laser (Spectra-Physics, Japan). The laser power was 20–60 milliwatts (mW) (Argon laser: 20 mW, He–Cd laser: 40–60 mW), and observation period was 0.2–3 s. The fluorescence image was filtered with a bandpass filter (Argon laser: U-MWIGA3, Olympus, Japan, He–Cd laser: D490/30, Omega Optical, U.S.A.) and visualized using a digital steel camera (DP70, Olympus, Japan).