

ABri peptide associated with familial British dementia forms annular and ring-like protofibrillar structures

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ABBREVIATIONS: AD = Alzheimer's disease; CD = circular dichroism; HFIP = 1,1,1,3,3,3-Hexafluoro-2-propanol; HPLC = high performance liquid chromatography; MS = mass spectrometry; TFA = trifluoroacetic acid; AFM = atomic force microscopy

Abstract

Amyloid plaque deposition involves the aggregation of normally soluble proteins into insoluble amyloid fibrils (fibrillization) and proceeds through intermediates with distinct morphologies, including spherical aggregates, protofibrils, and mature fibrils. Recently, a novel annular protofibril-like intermediate with unique pore-like properties was produced by α -synuclein, A β -Arctic and amylin, which are proteins associated with Parkinson's disease, Alzheimer's disease, and type-II diabetes. The observation of annular structures coupled with size selective channel-like activity by these proteins suggests that these structures may be responsible for vesicle permeability by ion-channel formation. Using atomic force spectroscopy, we report here that the ABri peptide associated with familial British dementia produces similar annular and ring-like protofibril structures during the following sequence of events: spherical aggregates (0.4–1.5 nm height) \rightarrow chain-like protofibrils (1.5–2.3 nm height) \rightarrow ring-like protofibrils and annular protofibrils (1.5–2.3 nm height). This suggests that ABri fibrillization occurs in a similar fashion to other amyloidogenic proteins and that the annular protofibrillar structures may represent a common amyloid intermediate.

Introduction

Amyloid formation is a key pathological event of many diseases and the mechanisms by which proteins aggregate into amyloid plaques remains unknown. Identification of the cytotoxic and pathogenic intermediates that are produced during amyloid formation is the subject of intense research efforts. Although several quaternary structures, such as spherical aggregates, protofibrils, and mature fibrils have been identified, the role of these intermediates in the disease pathology is uncertain. The observation of a sub-population of protofibrils with annular morphology coupled with size-dependent channel-like activity by α -synuclein, A β -Arctic, and amylin have suggested that the annular protofibrils disrupt neuronal membranes by a pore-like mechanism involving formation of ion-permeable channels^{1–4}. Previous studies with α -synuclein and the A β peptides showed that the annular structures have heights similar to spherical aggregates, which are approximately 11 nm for α -synuclein and 1 nm height protruding above the embedding lipid bilayer surface for the A β peptide and are comprised of 3–6 spherical subunits^{3,5}. The exact role of such protofibrillar structures in the amyloid assembly process is still under debate.

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We have undertaken comprehensive biophysical studies of the ABri peptide associated with familial British dementia (FBD) utilizing an array of spectroscopic techniques that includes atomic force microscopy (AFM) for the characterization of fibril morphology⁶. The ABri peptide is the first example of an amyloid molecule created *de novo* by the abolishment of the stop codon in its precursor that features 266 amino acids (BRI-266) and is codified by a single gene located on the long arm of chromosome 13⁷. The FBD is primarily a cerebrovascular disease, where the amyloid deposition is associated with extensive neurofibrillar degeneration and dementia in the fifth decade of life. The ABri amyloid peptide is formed by the 34 C-terminal amino acids of a mutant precursor protein BRI-277 as a result of a furin-like processing⁸. Previous studies established that the intramolecular disulfide bond and the C-terminal extension are required for the formation of amyloid-like β -sheet structures and that the ABri peptide induces apoptotic cell death while the wild type is non-toxic to cells⁹. We report here that the ABri peptide produces classical linear protofibrils and annular protofibrils as seen with other amyloidogenic proteins which supports the notion that these annular protofibrils may be a common intermediate in protein amyloidosis.

Methods

Peptide synthesis

The ABri peptide (34 amino acids) has the following primary sequence: ¹<Glu-²Ala-³Ser-⁴Asn-⁵Cys-⁶Phe-⁷Ala-⁸Ile-⁹Arg-¹⁰His-¹¹Phe-¹²Glu-¹³Asn-¹⁴Lys-¹⁵Phe-¹⁶Ala-¹⁷Val-¹⁸Glu-¹⁹Thr-²⁰Leu-²¹Ile-²²Cys-²³Ser-²⁴Arg-²⁵Thr-²⁶Val-²⁷Lys-²⁸Lys-²⁹Asn-³⁰Ile-³¹Ile-³²Glu-³³Glu-³⁴Asn, with a pyroglutamate (<Glu) at the N-terminus and a disulfide bond between Cys5 and Cys22. The ABri peptide was synthesized using t-BOC chemistry (Dr. James I. Elliott, W. M. Keck Protein Chemistry Facility, Yale University), or prepared in our own laboratory using standard Fmoc protocols. The peptides were purified by HPLC and characterized with both mass spectrometry and amino acid analysis. Disulfide bond formation was achieved by on-resin oxidation of the peptide with iodine in methanol solution¹⁰ and the MS data for the oxidized ABri peptide was 2 mass units less than the reduced peptide, thus establishing that oxidation did indeed take place (MALDI-MS for the ABri peptide m/z 3935.3; molecular weight excluding the CF₃COO⁻ salts 3935.5).

Peptide solutions

A stock solution of the ABri peptide (2.0 mg, 0.43 μ mole) was prepared in 1.0 ml of distilled 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (2:1, mg/ml),

with dissolution promoted by sonication (Model SC-101TH, Sonicor) at 25°C for about 15 min. The ABri peptide is soluble in the HFIP solvent and remains monomeric for periods up to one month at 5°C. The aggregation state was rigorously established using analytical ultracentrifugation, sedimentation velocity measurements, where fresh peptide solutions in acetate buffer yielded a single species fit that corresponded to monomeric ABri. An aliquot (0.13 ml) taken from the HFIP stock was evaporated to dryness under a fine stream of dry N₂ gas and redissolved in sodium acetate buffer solution (1.0 ml, 10 mM) at pH 4.9. The ABri peptide solution (56 μ M) was aged at 22°C and periodically monitored by AFM.

Atomic force microscopy

Imaging was performed on a Nanoscope IIIa multi-mode scanning probe microscope (Digital Instruments). Aliquots (10 μ l) were withdrawn from the aggregating ABri peptide solutions in acetate buffer and applied to the surface of freshly cleaved, muscovite mica and allowed to stand at room temperature for 60 s. The mica substrate was tilted, in a manner such that the lower edge was in contact with an absorbent material. The mica surface was rinsed with doubly-distilled water (2 \times 50 μ l) to remove loosely bound peptide (to prevent multi-layer of sample coverage), then blown dry with a stream of dry N₂ gas and imaged immediately. The AFM measurements were carried out in the TappingModeTM under ambient conditions using etched, single-beam silicon cantilevers (length 125 μ m) and a nominal spring constant (20–100 N/m). The following scanning and feedback control parameters were utilized: RMS drive amplitude, 30–300 mV, set point amplitude 0.5–1.2 V, drive frequency 270–320 kHz, and a scan rate 1–2 Hz. All experimental results were reproduced three times.

Results and discussion

Morphological characterization of ABri aggregates

At neutral pH 7.1–7.3, the ABri peptide has limited solubility and produces spherical and amorphous aggregates, while at slightly acidic pH 4.9, spherical aggregates, intermediate-sized protofibrils, and larger-sized mature amyloid fibrils are detected by AFM⁶. Although it is possible to encourage *in vitro* fibrillization at neutral pH by the addition of ABri peptide seeds⁶, the present studies were done at pH 4.9, which were more straightforward.

Due to our HFIP sample pretreatment protocol, the ABri peptide aggregation in acetate buffer (pH 4.9) always started from a well-defined monomeric state as analyzed by sedimentation velocity analytical ultracentrifugation.

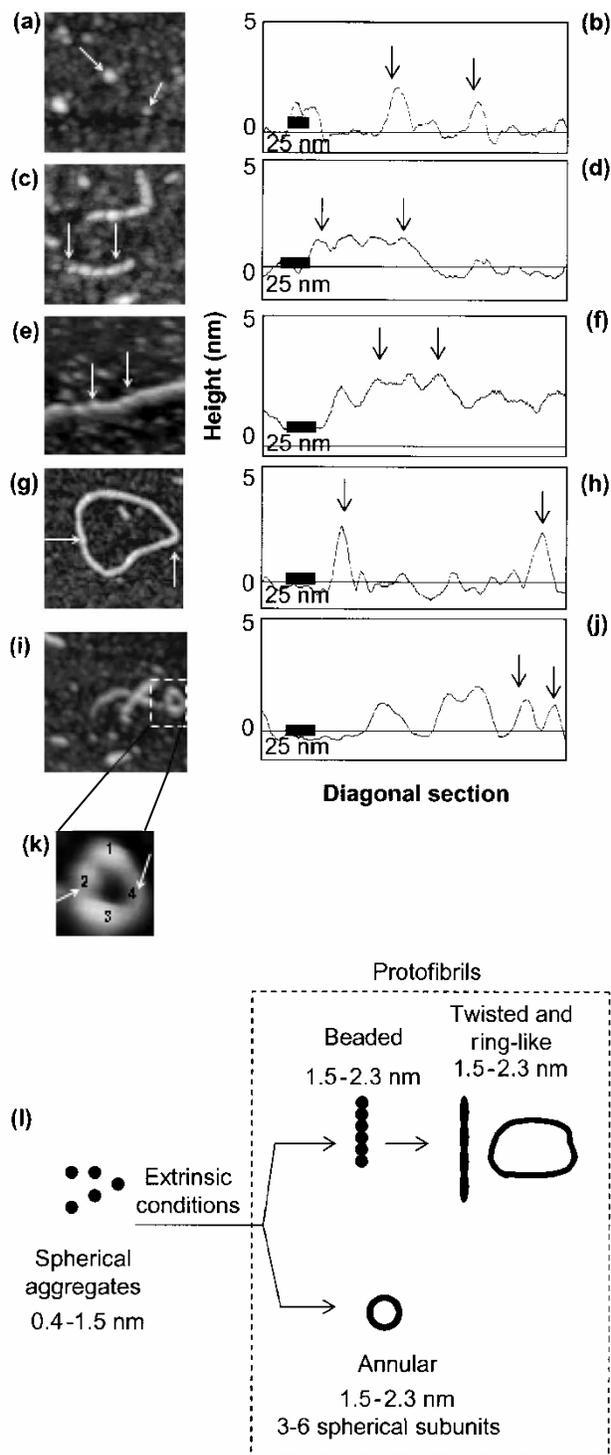


FIGURE 1: AFM-images featuring protofibril morphologies from aggregating ABri peptide solutions (Z-range 10 nm for all images, scan area $(250 \text{ nm})^2$, scale bar 25 nm for all images). The arrows on the images correspond to the arrows on the sections. (a) Spherical aggregates; (b) axial section showing the height (1.4 and 0.9 nm) of the spherical aggregates; (c) chain-like protofibrils consisting of a linear

trifugation (data not shown), which alleviated potential problems related to seed-induced aggregation. The AFM analysis of the freshly prepared solutions showed the absence of any detectable aggregates. With incubation at room temperature, the solutions aggregated with the production of four morphologically distinct species: (1) spheres, (2) chain-like protofibrils, (3) annular structures and (4) ring-like structures. During the first 24-hr of incubation, the aggregates were predominantly spherical with heights in the range of 0.4–1.5 nm (Figure 1a and b). The spherical aggregates assembled into beaded, chain-like protofibrils with average lengths of 250 nm (Figure 1c and d). After 6 day incubation, protofibrils became elongated with average lengths of 500 nm and heights of 1.5–2.3 nm (Figure 1e and f). High-resolution AFM demonstrated that the protofibrils were twisted and exhibited 25–32 nm periodicity along their long axis, as obtained from analysis of axial sections taken from 50–70 protofibrils.

Ring-like and annular protofibril formation

Besides the protofibrils, after 6 day incubation, two additional structures were detected, which were the annular and ring-like protofibrils. The ring-like protofibrils had heights and periodicities similar to the elongated protofibrils and inner diameters of 100–150 nm (Figure 1g and h). The annular structures had smaller diameters (outer 30–32 nm and inner 8–13 nm), a centralized pore-like depression, and heights identical with chain-like protofibrils (Figure 1i and j). Closer examination of the individual annular structures revealed that they were composed of at least three and up to six subunits (Figure 1k).

The classical protofibrils were present in larger quantities than the annular structures, and multiple images ($5 \mu\text{m}^2$) taken at each time point consistently showed 5–6 annular and ring-like structures per image. For the multiple images, a minimum of three different areas were imaged and repeated twice at each time point. Mature fibrils appeared after longer, 2-week incubations without noteworthy depletion of the protofibrils.

array of spherical aggregates; (d) axial section across the protofibril showing the periodicity (25 nm) and height (1.5 nm); (e) elongated twisted protofibril; (f) axial section showing the height (2.3 nm) and periodicity (30 nm); (g) ring-like protofibril; (h) diagonal section showing the inner diameter (180 nm) and height (2.3 nm); (i) annular protofibril; (j) diagonal section across the annuli showing the height (1.4 nm) and the pore-like depression; (k) off-line zoom of annuli showing four sub-units; (l) possible sequence of steps involved in ABri aggregation and fibrillization.

Mechanistic implications

The identification of discrete intermediates in the ABri aggregation pathway is essential for elucidating the mechanism of amyloid formation and toxicity. A conceivable mechanism for ABri aggregation based on the AFM data could initially involve the formation of spherical aggregates. In the initial step, the spherical aggregates act as building blocks and combine into beaded chain-like protofibrils and/or annular structures (Figure 1). Once produced, the chain-like protofibrils could undergo further assembly to produce mature fibrils and ring-like structures. The selection of a particular pathway is highly dependent on the environmental conditions, including the ABri peptide concentrations that when sufficiently high favor mature fibril formation. The membrane permeabilization of α -synuclein, A β -Arctic and amylin has been attributed to formation of ion-channels by pore-like protofibrillar intermediates^{1,4}. Membrane associated, pore-like structures of A β have also been observed by AFM. Soluble oligomeric species of ABri have been reported to be more cytotoxic than the fibrils and it is interesting to speculate that the annular structure of ABri presented here may be responsible for such properties, although it remains to be established if the ABri annular structures exhibit pore-like properties.

Another important issue concerns the pH, which for the present studies was conducted at pH 4.9 instead of more physiologically relevant pH 7.2–7.4. Our reason for using pH 4.9 relates to the enhanced ABri aggregation and fibril formation, which at neutral pH would require seeding⁶. Thus, production of the ABri annular structures *in vivo* presumably would require either some preaggregated seed peptide material or possibly passage of the ABri peptide through intracellular organelles such as late endosomes or lysosomes, where the pH can be quite acidic. Because the main clinical hallmark of FBD is cerebral hemorrhage, not dementia, which is accompanied by amyloid ABri deposits in arterial walls and basement membranes, the interaction of the ABri with biological surfaces and/or acidic micro-environments may be involved in the β -aggregation process and formation of annular structures. Additional studies aimed at unraveling the environmental conditions that influence ABri annuli formation, the possible interconversions among them, and the relevance of such structures *in vivo* are currently underway in our laboratory.

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