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Ultrasonication-dependent formation and degradation of α-synuclein amyloid fibrils

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ABSTRACT

Ultrasonication can be used to break the supersaturation of α-synuclein, a protein associated with Parkinson's disease, at pH 7.4 above the critical concentration of fibrillation, thereby inducing the formation of amyloid fibrils. We speculated that ultrasonication could also be used to depolymerize preformed fibrils below the critical concentration. However, extensive ultrasonic irradiation transformed preformed fibrils into amorphous aggregates even above the critical concentration. Exposing preformed fibrils to the hydrophobic air–water interface of cavitation bubbles may have destabilized the fibrils and stabilized amorphous aggregates. Upon extensive ultrasonic irradiation, the accompanying decomposition of chemical structures was suggested when monitored by analytical ultracentrifugation. Amorphous aggregates produced by extensive ultrasonication showed higher cytotoxicity, suggesting that, although ultrasonication might be a useful approach for inactivating amyloid fibrils, potential cytotoxicity of amorphous aggregates should be considered.

1. Introduction

Amyloid fibrils, highly-ordered filamentous aggregates approximately 10 nm in diameter and several μm in length, are now attracting increasing attention [1–6]. Their deposition is associated with the pathology of more than 30 serious disorders such as Parkinson's disease and dialysis-related amyloidosis [7]. Various peptides and proteins not directly related to diseases have also been shown to form amyloid fibrils in vitro under certain conditions, which suggests that the formation of amyloid fibrils (i.e. amyloid fibrillation) is a generic property of relatively short polypeptides. Thus, clarifying the mechanism of amyloid fibrillation is essential to improve our understanding of proteins and advance therapeutic strategies against protein misfolding diseases.

Although it is sometimes practically difficult to dissolve preformed amyloid deposits, amyloid fibrils are considered to be in equilibrium with monomers [3,8–10]. Based on this finding, amyloid fibrillation is similar to the crystallization of substances, which only occurs when the protein concentration is above the critical concentration or equilibrium solubility [3,11–13]. Kinetically, amyloid fibrillation comprises of primary nucleation and growth processes. In addition, secondary nucleation by the fragmentation of preformed fibrils accelerates the overall rate of fibrillation. After fibrillation is completed, fibrils are in equilibrium with monomers at the critical concentration. However, various amyloidogenic proteins remain monomeric even above the critical concentration because of "supersaturation". Supersaturation refers to a kinetically trapped state in which solutes retain solubility for an extended period or indefinitely even though their concentration is higher than thermodynamic solubility. Supersaturation can be broken by seeding or various kinds of agitations that create nuclei [13–15]. Taken together, we revisited a classical model of solubility- and supersaturation-limited amyloid fibrillation [3,11,16], in which, although fibrillation is determined by the equilibrium solubility, supersaturation prevents phase transition [13,15,17].

Ultrasonication is routinely used to prepare short fibril seeds from preformed long fibrils [18–20], and was found to be useful for accelerating spontaneous fibrillation from monomers. Stathopulos et al. [21] first showed that ultrasonication triggered the formation of the amyloid-like aggregates of various proteins. We then demonstrated that amyloid fibrillation was accelerated when ultrasonication pulses were applied to the monomers of β_2-microglobulin (β2-m) [22], a causative protein of dialysis-related amyloidosis [23]. The ultrasonication-dependent fragmentation and production of amyloid fibrils are now widely used to characterize fibrils [9,24–27]. We recently developed a unique

Abbreviations: AFM, atomic force microscopy; β2-m, β_2-microglobulin; CD, circular dichroism; TEM, transmission electron microscopy; ThT, thioflavin T.

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method to verify the amyloidogenicity of proteins that combines an ultrasonicator to induce the formation of fibrils and a microplate reader to monitor amyloid-specific thioflavin T (ThT) fluorescence [14,17,28].

α-Synuclein, an intrinsically disordered protein associated with Parkinson's disease [29,30], is one of the most important targets in the study of protein misfolding [31,32]. Although we previously reported the ultrasonication-dependent fibrillation of α-synuclein [33], its mechanism had not yet been evaluated in detail. In the present study, we examined the factors determining α-synuclein fibrillation by a microplate reader combined with the ThT assay and ultrasonication. The results obtained suggest that, in addition to effectively inducing the formation of fibrils by disrupting supersaturation, extensive ultrasonic irradiation is useful for transforming preformed fibrils into non-amyloidogenic aggregates. We recently reported the similar effects of ultrasonication on the fibrils of the amyloid β(1–40) peptide [34]. The present results with α-synuclein demonstrate that the effects of ultrasonication on the induction and inactivation of amyloid fibrils are common to various amyloidogenic proteins. Importantly, cytotoxicity of extensively ultrasonicated α-synuclein fibrils was higher than that of mature amyloid fibrils.

2. Materials and methods

2.1. Expression of α-synuclein

α-Synuclein was expressed in *Escherichia coli* BLR(DE3) or BL21(DE3) (Novagen) and purified as described [35,36]. Briefly, α-synuclein in fractured cells with ultrasonication was precipitated by ammonium sulfate at 75% (v/v). After centrifugation and dialysis, the solution containing α-synuclein was applied to a Resource-Q column (GE Healthcare), and then further purified by a COSMOSIL Protein R column (Nacalai Tesque, Kyoto, Japan). The purity of the solution was confirmed to be more than 95% by SDS-PAGE and MALDI mass spectroscopy.

2.2. Fibril formation and degradation by ultrasonication

As standard conditions, α-synuclein was dissolved at 1.0 mg/ml (70 μM) in 20 mM sodium phosphate buffer (pH 7.4) containing 0.1 M NaCl. The NaCl concentrations used were 1.0, 0.5, and 0.1 M and the protein concentrations were 1.0, 0.5, 0.25, and 0.1 mg/ml.

A total of 0.2 ml of the solution was applied to at least three wells of a 96-well microplate. The plate was placed on a water bath-type ultrasonic transmitter with a temperature controller (ELESTEIN SP070-PG-M, Elekon, Chiba, Japan), which can apply repetitive ultrasonication pulses to samples from three directions [14]. The frequency of the ultrasonic waves was 17–20 kHz and the power output was set to 350 W. The temperature was set to 37 °C. The periods under ultrasonication and quiescence were set as 1 min and 9 min, respectively. To examine the dependence of fibrillation on ultrasonic energy, the periods of ultrasonication and quiescence used were varied at 15 min/5 min, 7 min/3 min, and 5 min/15 min, respectively. We also examined the dependence on the protein concentration in cycles of 15 min ultrasonication and 5 min quiescence. Ultrasonic irradiation increased the temperature of sample solutions [27,37] up to 3 °C from the setting temperature of 37 °C. Fibril formation was monitored by ThT fluorescence with an MTP-810 microplate reader (Corona Electric Co., Tokyo, Japan). The fluorescence intensity of ThT at 490 nm was measured at an excitation of 450 nm at 37 °C. Measurements were conducted with the multipoint measurement mode, and fluorescence values at 25 points in each well were averaged.

To examine the effects of ultrasonication on preformed fibrils, fibrils were formed at a concentration of 0.5 mg/ml (35 μM) α-synuclein in 20 mM sodium phosphate buffer (pH 7.4) containing 5 μM ThT and 100 mM NaCl. Cycles of 15 min ultrasonication and 5 min quiescence were used. Preformed fibrils at various concentrations were then treated with ultrasonic pulses in microplate cells, to which 200 μl each was added.

2.3. AFM, TEM, CD, and analytical centrifugation measurements

Transmission electron microscopy (TEM) images were acquired using a transmission electron microscope (H-7650, HITACHI, Tokyo, Japan) at 80 kV as described [25,38]. Atomic force microscopy (AFM) images were acquired using a Digital Instruments Nanoscope IIIa scanning microscope (Bruker AXS, Tokyo, Japan) at 25 °C as described [25,38].

Far-UV CD spectra were measured with a Jasco 820 CD spectrophotometer as described previously [25,38]. Measurements were performed at 25 °C using a quartz cuvette with a 1 mm path length, and the results were expressed as mean residue ellipticity [θ].

To estimate the fractions of monomers and aggregates, sedimentation velocity measurements were performed at 4 °C using a Beckman-

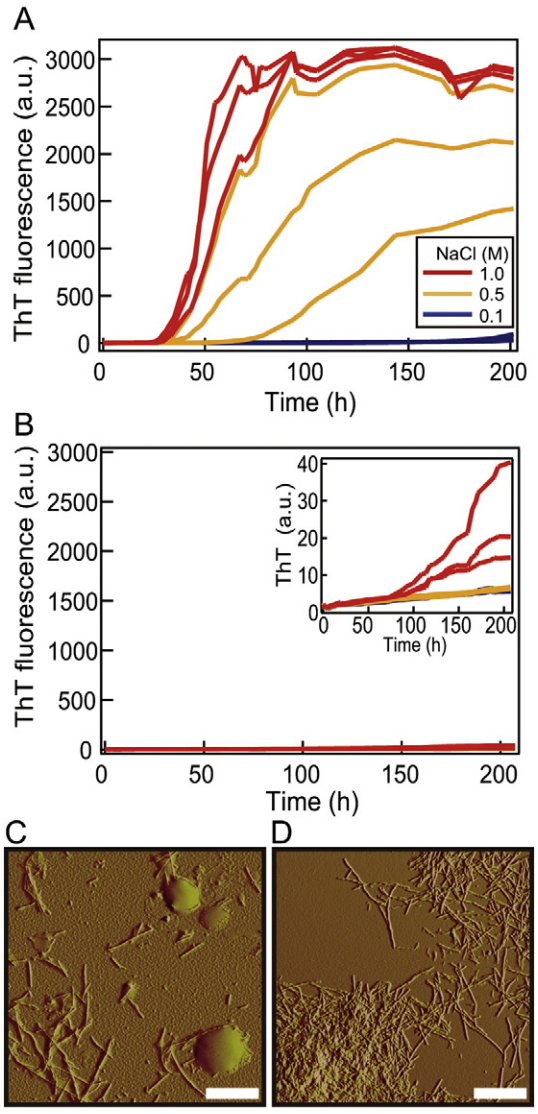

Fig. 1. Formation of α-synuclein fibrils with (A) and without (B) ultrasonication. Fibrillation of α-synuclein at 1.0 mg/ml (70 μM) in the presence of 1.0 (red), 0.5 (orange), and 0.1 M (blue) NaCl was monitored with ThT fluorescence. Cycles of 1 min ultrasonication and 9 min quiescence were used. Representative time courses are shown for the respective conditions. C, D, AFM images of the ultrasonication-induced fibrils formed in the presence of 1.0 (C) or 0.5 (D) M NaCl are shown. The scale bars represent 1 μm.