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Solution conditions define morphological homogeneity of α-synuclein fibrils

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ABSTRACT

The intrinsically disordered human α-synuclein (αSyn) protein exhibits considerable heterogeneity in *in vitro* fibrillization reactions. Using atomic force microscopy (AFM) we show that depending on the solvent conditions, A140C mutant and wild-type αSyn can be directed to reproducibly form homogeneous populations of fibrils exhibiting regular periodicity. Results from Thioflavin-T fluorescence assays, determination of residual monomer concentrations and native polyacrylamide gel electrophoresis reveal that solvent conditions including EDTA facilitate incorporation of a larger fraction of monomers into fibrils. The fibrils formed in 10 mM Tris–HCl, 10 mM NaCl and 0.1 mM EDTA at pH 7.4 display a narrow distribution of periodicities with an average value of 102 ± 6 nm for the A140C mutant and 107 ± 9 nm for wt αSyn. The ability to produce a homogeneous fibril population can be instrumental in understanding the detailed structural features of fibrils and the fibril assembly process. Moreover, the availability of morphologically well-defined fibrils will enhance the potential for use of amyloids as biological nanomaterials.

1. Introduction

Self-assembled protein structures have been associated with several age related degenerative diseases like Parkinson's disease (PD), Alzheimer's disease (AD) and diabetes [1–3]. However, the propensity of a large number of proteins to form amyloid fibrils *in vitro* irrespective of their primary sequence has resulted in an extension of amyloid science from not only understanding mechanisms involved in fibrillization, diagnostics [4,5] and potential therapeutics [6–8] but also to studying their mechano-physical properties and designing functionalized fibrils *via* biochemical modifications for custom applications [9,10]. In the past decade, functionalities of amyloids have been extensively explored for use as nanomaterials. Fibrillar amyloids have been shown to be promising candidates as bio-templates for tissue engineering and bio-mineralization [11] and as metal or polyelectrolyte conjugated nanowires [12–15]. However, at present the applicability of the fibrils as bio-nanomaterials is considerably limited by the inherent heterogeneity in the *in vitro* fibrillization.

Fibrillization is a nucleation dependent polymerization process, in which nucleation is usually induced *de novo* by protein monomers but can also be circumvented by including pre-formed fibrils (seeded aggregation) [16,17]. The early phase of aggregation is suggested to be a complex molecular mix, where at different time points, alternative competing reaction pathways like fragmentation or surface catalyzed nucleation (secondary nucleation) may be favored [18–22]. The plateau phase of the aggregation reaction is an assortment of monomers, oligomers and fibrils with the morphology of the mature fibrils exhibiting appreciable heterogeneity [21,23–25]. Morphological heterogeneity or structural polymorphism can be inherent as seen with proteins like Amyloid β [26], αSyn [21,27,28], Ig light chains [29], ovalbumin [30], β-lactoglobulin and lysozyme [31], which form morphologically distinct assemblies under the same solvent conditions. Also, specific solvent conditions may favor certain morphology. Numerous reports have shown the critical role of solvent conditions like ionic strength, pH, temperature, inclusion of metal ions, and other small molecules on the aggregation kinetics and morphology of the fibrils [6–8,32–41]. In general, factors favoring faster fibrillization like higher salt, higher temperature and low pH are shown to result in more heterogeneous aggregations [42].

Features like height, periodicity and length are typically used to describe the morphology of amyloid fibrils. Of these, height and periodicity are shown to be dependent on the number of protofilaments in the fibrils and can be determined accurately by AFM at single fibril level in a direct and model free regime [34,35,37,38,40,43]. Length on the other

Abbreviations: αSyn, α-synuclein; AFM, Atomic Force Microscopy; A140C αSyn, substitution mutant of αSyn with residue 140 mutated from alanine to cysteine; EDTA, ethylenediaminetetraacetic acid; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; DTT, dithiothreitol; ThT, thioflavin-T; DTNB, [5,5′-dithiobis-(2-nitrobenzoic acid)]

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hand is critically affected by agitation during the aggregation reaction and stochastic shear forces inherent with handling, both of which favor fragmentation [33,39]. Moreover, length analysis of the fibrils deposited on the mica surface is reported to have adsorption (during sample preparation) and detection (during analysis) bias towards shorter or longer fibrils [36,44].

In the present study we use αSyn as a model protein and report the formation of mature αSyn fibrils with homogeneous morphology. αSyn is a 140 amino acid predominantly neuronal protein implicated in the pathogenesis of Parkinson's disease and other synucleopathies [1,2]. We systematically imaged a large number of single fibrils at high resolution (3.3 nm/pixel) with AFM and quantified the fibril morphology with respect to their height and periodicity. αSyn, like other amyloidogenic proteins assembles into fibrillar species *in vitro* under a range of solvent conditions and displays highly polymorphic mature fibrils [21,24,33,42,45]. Furthermore, aggregation kinetics of αSyn (wt and mutants) is known to be highly irreproducible and recently a number of studies have reported on new approaches to improve reproducibility of *in vitro* fibrillization assays [32,46,47].

In our study, the mutant carrying a cysteine at position 140 instead of an alanine (A140C) and wild type (wt) protein were used. Given the relative ease of functionalization of cysteine through the –SH group, replacement of alanine by cysteine facilitates biochemical modifications and makes this mutant an important sequence polymorph for functionalization studies. Also being at the carboxyl terminus, the residue is exposed to the solvent in the mature fibril and as such is less likely to interfere in the process of fibrillization [43,48,49]. Incubation of A140C αSyn in solvent conditions with 10 mM NaCl including 2 mM DTT (dithiothreitol) and 0.1 mM EDTA (ethylenediaminetetraacetic acid) at pH 7.4 for fibrillization followed by AFM imaging and subsequent image analysis revealed the formation of a homogeneous pool of fibrils with an average periodicity of 102 ± 6 nm and height of 6.4 ± 0.4 nm. EDTA was included in the reaction to enhance the half-life of DTT (used to prevent dimerization of A140C by intermolecular disulfide bond formation). Similar experiments using wt αSyn also showed that inclusion of EDTA influences the fibrillization reaction leading to formation of fibrils exhibiting uniform periodicities with an average value of 107 ± 9 nm and fibril height of 6.9 ± 0.6 nm. Together, these results show that the inclusion of EDTA in the given fibrillization reaction conditions induces the formation of fibrils with uniform heights and periodicities.

2. Materials and methods

2.1. Expression and purification of αSyn

wt and A140C αSyn were used in the present study. *Escherichia coli* BL21(DE3) cells transformed with the pT7-7 plasmid carrying the wt αSyn gene were cultured in 1 liter of LB medium with 100 μg/ml ampicillin. At an OD of 0.6–0.7 the T-7 promoter was induced by 1 mM IPTG for 4 hours. Cells were harvested by centrifugation at 6000 × *g* for 10 min. The cell pellet was resuspended in 1/10th of the culture volume in 10 mM Tris–HCl, pH 8.0, 1 mM EDTA and 1 mM PMSF, and stirred for 1 hour at 4 °C. Cells were lysed by sonication for 2 min. Cellular debris was removed by centrifugation at 10,000 × *g* for 20 min at 4 °C. Nucleic acids were removed from the lysate by adding 1% (w/v) of streptomycin sulfate and stirring for 15 min at 4 °C, followed by centrifugation at 13,500 × *g* for 30 min at 4 °C. αSyn was salted-out from the solution by slow addition of 0.295 g/ml of ammonium sulfate and mild stirring for 1 hour at 4 °C. Precipitated protein was collected by centrifugation at 13,500 × *g* for 30 min at 4 °C. The ammonium sulfate pellet was gently resuspended in 1/20th of the culture volume in 10 mM Tris–HCl, pH 7.4 and filtered through a 0.22 μm filter. The solution was loaded onto a 6 ml ResourceQ column using an Åkta Purifier system (GE Healthcare). αSyn was eluted using a linear gradient of NaCl (0–500 mM) in 10 mM Tris–HCl, pH 7.4 at a flow-rate of 3 ml/min over 20 column volumes and 1 ml fractions were collected. Fractions were checked for αSyn using SDS–PAGE and pooled. The pooled sample was concentrated (Vivaspin-20, 10 kDa; GE Healthcare) to a volume of <2.5 ml. The sample was desalted with a PD-10 column (GE Healthcare) using 10 mM Tris–HCl pH 7.4. The volume was adjusted with Tris–HCl, pH 7.4 to a concentration of 250 μM, and divided in aliquots of 0.5 ml and stored at −80 °C. The A140C mutant construct was generated by site directed mutagenesis using a QuikChange II Site-Directed Mutagenesis Kit (Stratagene). A140C protein was expressed and purified according to the same protocol as wt αSyn, with additional inclusion of 1 mM DTT in all buffers.

2.2. Fibrillization reaction

Two hundred fifty micromolar monomeric stocks of wt and A140C mutant αSyn frozen at −80 °C were thawed and filtered through 0.02 μm, 10 mm Anotop10 Whatman syringe filters. Aggregation reactions were set up with 100 μM A140C αSyn, 10 mM Tris–HCl, 1 or 10 mM NaCl, (+/-) 0.1 mM EDTA, 2 mM fresh DTT at pH 7.4 and with 100 μM wt αSyn, 10 mM Tris–HCl, 1 or 10 mM NaCl, (+/-) 0.1 mM EDTA at pH 7.4. All reactions were prepared in triplicate with volumes of 400 μl each in 2 ml Lo-Bind round bottom Eppendorf centrifuge tubes and were incubated at 37 °C with 500 rpm orbital shaking in an Eppendorf Thermo-mixer comfort. Fibrillization using Tris(2-carboxyethyl)phosphine hydrochloride (TCEP-Fluka BioChemika) was done in a similar manner as with DTT with minor differences. The protein aliquots from –80 °C were desalted using a Zeba spin desalting column (7 k MWCO; Pierce Biotechnology) to remove residual DTT and 100 μM A140C αSyn solutions were prepared in 10 mM Tris–HCl, 10 mM NaCl, 2 mM TCEP (final concentrations) at pH 7.4.

2.3. Thioflavin-T assay

Progress of fibrillization was followed by a Thioflavin-T (ThT) fluorescence assay. A stock solution of 1 mM ThT was prepared in 50 mM glycine–NaOH buffer, pH 8.2 and filtered through a 0.22 μm syringe filter. At each time point (every 24 hours), 5 μl aliquots of sample were drawn and diluted in 2 ml of 5 μM ThT working solution diluted in glycine–NaOH buffer, pH 8.2. Fluorescence intensity was measured in triplicate on a Cary Eclipse fluorescence spectrophotometer (Varian Inc., Palo Alto, CA, USA), with excitation at 457 nm and emission detection from 475 to 600 nm using slit widths of 10 nm. ThT curves were prepared by plotting the emission intensity readings at 485 nm *versus* aggregation time; triplicate values were averaged and blank subtracted.

2.4. Residual monomer concentration (RMC)

The amount of monomers left in the aggregation reaction after attaining the plateau phase in a ThT assay was determined by centrifugation of 200 μl aliquots of aggregation reactions at 21,000 × *g* for 1 hour in an IEC Micromax microcentrifuge (Thermo Fisher Scientific Holding B.V., Breda, The Netherlands). The supernatant recovered from the centrifuged samples was filtered through 0.02 μm, 10 mm Anotop10 Whatman syringe filters and the absorbance was measured at 280 nm and 330 nm in a Shimadzu UV-2401 PC spectrophotometer (Shimadzu Benelux B.V., 's-Hertogenbosch, The Netherlands). The absorption values at 280 nm were corrected for scattering contributions before calculating the residual monomer concentration [50].

2.5. Atomic force microscopy — fibril morphology analysis

AFM samples were prepared by adsorbing 20 μl of fibril sample, 5 to 10 times diluted in 10 mM Tris–HCl, 10 mM NaCl at pH 7.4 on freshly cleaved mica (Muscovite mica, V-1 quality, EMS) for 4 min, followed by 2 gentle washes with 100 μl of fresh Milli-Q water and drying in a