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High-Pressure-Driven Reversible Dissociation of α-Synuclein Fibrils Reveals Structural Hierarchy

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ABSTRACT The analysis of the α-synuclein (aS) aggregation process, which is involved in Parkinson's disease etiopathogenesis, and of the structural feature of the resulting amyloid fibrils may shed light on the relationship between the structure of aS aggregates and their toxicity. This may be considered a paradigm of the ground work needed to tackle the molecular basis of all the protein-aggregation-related diseases. With this aim, we used chemical and physical dissociation methods to explore the structural organization of wild-type aS fibrils. High pressure (in the kbar range) and alkaline pH were used to disassemble fibrils to collect information on the hierarchic pathway by which distinct β-sheets sequentially unfold using the unique possibility offered by high-pressure Fourier transform infrared spectroscopy. The results point toward the formation of kinetic traps in the energy landscape of aS fibril disassembly and the presence of transient partially folded species during the process. Since we found that the dissociation of wild-type aS fibrils by high pressure is reversible upon pressure release, the disassembled molecules likely retain structural information that favors fibril reformation. To deconstruct the role of the different regions of aS sequence in this process, we measured the high-pressure dissociation of amyloids formed by covalent chimeric dimers of aS (syn-syn) and by the aS deletion mutant that lacks the C-terminus, i.e., aS (1–99). The results allowed us to single out the role of dimerization and that of the C-terminus in the complete maturation of fibrillar aS.

INTRODUCTION

α-synuclein (aS) is a 140-amino-acid (aa) protein mainly expressed at presynaptic terminals in the mammalian central nervous system. It can be divided into three domains: the amphipathic N-terminal region (1–60 aa), which acquires an α-helical structure when bound to lipid membranes (1); the non-β-amyloid component (NAC) (61–95 aa), which is highly hydrophobic and involved in aS aggregation (2); and the C-terminus (96–140 aa), which is highly acidic and has no distinct structural features. aS monomers were first described as a natively unfolded protein in solution, even though tetrameric α-helical forms were observed (3). It has been also shown that the structure of monomeric aS in solution is more compact than expected for a completely unfolded polypeptide. In fact, long-range interactions between the C-terminal region and the hydrophobic NAC domain have been proposed to stabilize aS in the monomeric state in vitro (4) and in mammalian cells (5).

The physiological functions of aS are not fully understood, but several studies suggest it plays a role in synaptic vesicle recycling (6,7), SNARE complex assembly (8), and neuronal plasticity (9). Great efforts have been made to characterize structural and functional properties of aS after the discovery of the link between aS aggregation and Parkinson's disease (10). A β-sheet-rich fibrillar form of aS is the major component of Lewy bodies (11), which are intracellular deposits of proteins and lipids found within surviving neurons in brains of patients affected by Parkinson's disease or other synucleinopathies (12). The structure of aS amyloid fibrils was studied by x-ray diffraction, solid-state NMR, and cryo-electron microscopy (13–15), and by micro-electron diffraction (16) down to atomic resolution. The results suggested a cross-β structure, similar to other amyloid fibrils, in which the β-sheet motifs are oriented perpendicular to the fibril axis. However, the structural

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details of fibrillar aS represent the ground on which to construct a classification of the divers strains reported in the recent literature (17) and associated with different phenotype traits.

The early stage of aS aggregation is an oligomerization process, where few monomers assemble to form transient and heterogeneous oligomeric species. Fibrillar structures are then formed through successive additions of monomers or other oligomeric species to the growing aggregates. During this process, several distinct transient oligomeric and protofibrillar species seem to populate the system (for a review of the aS aggregation process, see Plotegher et al. (18)). The aS oligomers can be conceived either as on- or off-pathway intermediates in fibril formation. In an attempt to define the toxic forms of aS, oligomers were shown to be responsible for neuronal death (19). However, neuronal death was also associated with the presence of Lewy bodies, whereas neurons with no inclusions seem to be less vulnerable (20). Given the heterogeneity of the different aS aggregates and the proposed toxic effects assigned to both aS oligomers and fibrils, the molecular mechanisms of cytotoxicity of aS aggregates remain as elusive as the structural features that govern them.

Therefore, despite their relevance in neurodegeneration, the structural information on the several aggregation intermediates of aS is still scanty, and difficulties arise when trying to identify those that are relevant for the in vivo aggregation process and for the pathology. This can be rationalized by considering several technical limitations due to the heterogeneous and transient nature of oligomeric intermediates, which hinders isolation of the individual components. However, ensemble measurements, such as circular dichroism (CD) and infrared (IR) spectroscopy, indicate that most of the on-pathway aS oligomeric intermediates contain substantial β-sheet structure (21).

An alternative avenue of investigation starts with analysis of the end products, i.e., the mature aS amyloid fibrils. These structures show a substantial thermodynamic stability, and previous studies associated fibril structural inertia with the involvement of several weak interactions, such as the hydrophobic effect, π-π interactions, and ion pairing among aa side chains. It is now well accepted that amyloid fibrils can be destabilized or even completely dissociated by tuning the net charge of the polypeptide chain (22–25). For example, aS fibrils disassemble into monomeric units in a very alkaline environment (25) or when subjected to low temperature, which weakens electrostatic interactions and hydrophobic effects (23,24).

An alternative strategy to obtain a controlled reversible dissociation of amyloid fibrils, based on the fine-tuning of weak interactions, is high hydrostatic pressure (HP) (26–29). It was observed that pressures of ~2 kbar dissociate aS fibrils (26,29), by forcing the equilibrium toward dissociation. HP shifts the system toward a state that occupies the minimal achievable volume, which is reached by solvent electrostriction around charged groups and is associated with important water density changes. The latter affects the intensity of the hydrophobic effect (30), ion-pair dissociation, and exposure of the hydrophobic surface to solvent. Therefore, using kbar pressures, it is feasible to finely tune weak interactions, inducing partial or total disassembly of the tertiary structure and even a cooperative unfolding of the H-bond wires of the protein secondary structure.

Here, we present a Fourier transform IR (FTIR) and Raman characterization of the HP- (in the kbar range) and pH-driven dissociation of wild-type (wt) aS fibrils with the goal of providing a structural characterization of aggregation intermediates and the processes that may be relevant in defining new methods to interfere with the aggregation and its associated toxicity. The disassembly seems to follow a hierarchic pathway where distinct β-sheets unfold sequentially. The results point toward the formation of kinetic traps in the energy landscape of aS fibril disassembly, explored using the unique possibility offered by HP FTIR to characterize transient partially folded species. In an attempt to deconstruct the role of the different regions of aS in fibril stability, we measured the HP dissociation of amyloids formed by covalent chimeric dimers of aS (syn-syn) (31) and by the aS deletion mutant lacking the C-terminus, i.e., aS (1–99).

MATERIALS AND METHODS

Protein purification

The syn-syn dimer, constituted by aa 1–104 and 29–140 of aS cloned in tandem, wt aS, and C-terminal depleted aS (1–99) were all expressed and purified as described elsewhere (31–33). Briefly, proteins were expressed in *Escherichia coli* strain BL21(DE3). For wt aS and the syn-syn dimer, cells were grown at 37°C in lysogeny broth to $OD_{600} = 0.3$–0.4 and induced with 0.1 mM isopropyl β-thiogalactopyranoside for 5 h. Cells were then collected and the recombinant proteins were recovered from the periplasm by osmotic shock. The periplasmic homogenate was then boiled for 15 min and the soluble aS-containing fraction was subjected to a two-step (35 and 55%) ammonium sulfate precipitation. The pellet was resuspended, dialyzed against 20 mM Tris-HCl (pH 8.0), loaded into a 6 mL Resource Q column (Amersham Biosciences, Little Chalfont, United Kingdom), and eluted with a 0–500 mM gradient of NaCl.

aS (1–99) overexpressing cells were grown at 37°C in lysogeny broth to $OD_{600} = 0.8$ and induced with 0.3 mM isopropyl β-thiogalactopyranoside. After 4 h, homogenized cells were boiled for 15 min and ammonium sulfate was added to the soluble fraction in two steps (35 and 55%). The resuspended pellet was dialyzed and loaded into a 6 mL Resource S column (Amersham Biosciences) and eluted with a 0–500 mM NaCl gradient. Finally, all the proteins were dialyzed against water, lyophilized, and stored at −20°C.

Fibril preparation

Proteins were resuspended into deuterated phosphate-buffered saline (PBS; pH 7.4) and filtered with a 0.22 μm filter to eliminate residual aggregates due to lyophilization. Soluble proteins were quantified using an Agilent 8453 ultraviolet-visible diode array (Agilent, Santa Clara, CA); absorbance was measured at 280 nm and protein concentration was calculated using protein molar extinction coefficients corresponding to ε(aS) = 5960 M^{-1} cm^{-1}, ε(syn-syn) = 7450 M^{-1} cm^{-1}, and ε(aS (1–99)) = 1490 M^{-1} cm^{-1}. Fibrils were obtained by aggregating the solubilized proteins at a