Both Familial Parkinson’s Disease Mutations Accelerate α-Synuclein Aggregation*

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Parkinson’s disease (PD) is a neurodegenerative disorder that is pathologically characterized by the presence of intracytoplasmic Lewy bodies, the major component of which are filaments consisting of α-synuclein. Two recently identified point mutations in α-synuclein are the only known genetic causes of PD, but their pathogenetic mechanism is not understood.

Here we show that both wild type and mutant α-synuclein form insoluble fibrillar aggregates with antiparallel β-sheet structure upon incubation at physiological temperature in vitro. Importantly, aggregate formation is accelerated by both PD-linked mutations. Under the experimental conditions, the lag time for the formation of precipitable aggregates is about 280 h for the wild type, 180 h for the A30P mutant, and only 100 h for the A53T mutant protein. These data suggest that the formation of α-synuclein aggregates could be a critical step in PD pathogenesis, which is accelerated by the PD-linked mutations.

Parkinson’s disease is a neurodegenerative disorder that predominantly affects dopaminergic neurons in the nigrostriatal system but also several other regions of the brain. Two dominant mutations, A53T and A30P, in α-synuclein cause familial early onset PD (1, 2). The function of α-synuclein and the pathogenic mechanism of these mutations is unknown, but α-synuclein has been detected in Lewy bodies (3–5) and shown to be their major filamentous component (6). Lewy bodies are a pathological hallmark of PD (7–9), and we therefore hypothesized that the PD mutations would cause or enhance α-synuclein aggregation. Indeed, a very recent publication demonstrated in vitro fibrillization of A53T mutant but not A30P mutant or wild type α-synuclein (10). Here we demonstrate aggregation of all forms of α-synuclein. In a complete aggregation time course, we show that there is an aggregation continuum; although all forms of α-synuclein do aggregate, aggregation is accelerated for both mutants; A30P aggregates slightly faster than wild type, and A53T aggregates much faster. Because both mutant forms enhance the aggregation tendency observed in the wild type, we hypothesize that aggregation of α-synuclein may be important in all forms of PD.¹

EXPRESSON PROCEDURES

Cloning, Bacterial Expression, and Purification of α-Synuclein—A 536-bp human α-synuclein cDNA was obtained by polymerase chain reaction (PCR) amplification from Q-Sepharose FF (Amersham Pharmacia Biotech), equilibrated in 20 mM Tris, pH 7.5, with protease inhibitor mixture Complete (Boehringer Mannheim). Cells in suspension were broken by passing through a Microfluidizer, and a clarified lysate supernatant was collected after centrifugation at 18,000 × g for 45 min. E. coli containing proteins were precipitated by acid precipitation of the lysate supernatant. The pH was adjusted to 3.5, and after stirring for 20 min, the mixture was centrifuged for 1 h at 27,000 × g. After adjusting the pH of the resulting supernatant to 7.5, the sample was applied to Q-Sepharose FF (Amersham Pharmacia Biotech), equilibrated in 20 mM Tris, pH 7.5, and eluted with a NaCl gradient in equilibration buffer. α-Synuclein-containing fractions were identified by SDS-polyacrylamide gel electrophoresis and are >99% pure. The concentration of α-synuclein was determined by measuring absorbance at 280 nm and employing ε₂₈₀ of 0.354, determined by using Genetics Computer Group software.

Aggregation of α-Synuclein—Purified α-synuclein samples were concentrated to 7 mg/ml using Centricon-3 spin filters (Amicon). After concentration, the samples were centrifuged for 10 min at 100,000 × g to remove any aggregates that could have formed during the concentration step. The supernatants were all adjusted to a final concentration of 7 mg/ml using Tris-buffered saline, which consists of 20 mM Tris, pH 7.5, and 0.2 mM NaCl. The samples were then dispensed into 1.5 ml of Beckman ultracentrifuge microtubes and incubated at 37 °C. At various time points, the samples were centrifuged at 100,000 × g for 10 min, and 11 μl of their supernatants were removed and diluted to 110 μl with Tris-buffered saline. These dilutions were then analyzed by their absorbance at 280 nm. Finally, the remainder of the incubations were vortexed for 30 s to resuspend pelletted material and allowed to continue incubating.

Circular Dichroism—CD spectra were determined at 20 °C on a Jasco J-715 Spectropolarimeter using water-jacketed cuvettes with a path length of either 0.01 cm (for the far UV region, 250–190 nm, secondary structure) or 1 cm (for the near UV region, 340–240 nm, tertiary structure). Molar ellipticity was calculated using the protein concentration determined as above and a mean residue weight of 103.

FTIR Measurement and Analysis—FTIR spectra of aqueous protein solutions and dried aggregates were recorded at 25 °C with a Nicolet Magna 550 Fourier transform infrared spectrometer equipped with a deuterated triglycine sulfate detector. Protein solutions were prepared for infrared measurement in a sample cell (Spectra-Tech FT04–036) that employed CaF₂ windows separated by a 6-μm spacer. α-Synuclein aggregates were centrifuged at 13000 rpm for 10 min, spread on a 3-M

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³ The abbreviations used are: PD, Parkinson’s disease; FTIR, Fourier transform infrared spectroscopy.
A disposable IR card, and air-dried, and the infrared spectra were recorded. The final protein spectrum was smoothed with a 7-point Savitsky-Golay smooth function to remove the white noise. Second derivative spectra were calculated with the derivative function of the Nicolet Omnic software. To quantitate the secondary structure from the second derivative spectra, the spectra were inverted by multiplication by \( e^{1/2} \) and the curve fit (SpectraCalc Software from Galactic Industries) with Gaussian band profiles (13).

**Atomic Force Microscopy—** Aggregated \( \alpha \)-synuclein was resuspended in phosphate-buffered saline, and this suspension was vortexed for 10 s. 40 \( \mu l \) of this suspension was incubated on a circular piece of mica for about 3 min. Excess liquid was removed, and the sample on the mica was then imaged under 40 \( \mu l \) of phosphate-buffered saline using a Digital Instruments Nanoscope III atomic force microscope. The probe used for imaging was an oxide-sharpened silicon nitride twin tip with a nominal spring constant of 0.58 newton/m. The image was obtained in tapping mode in fluid using a drive frequency of 8.67 kHz, a drive amplitude of 200 mV, and a set point voltage of 0.252 V.

**Electron Microscopy—** Formvar-coated 300-mesh copper grids were inverted over 20-\( \mu l \) drops of prepared \( \alpha \)-synuclein aggregate suspensions for 10 min. The grids were then rinsed in ultrapure water to remove excess, nonadherent material and allowed to dry at room temperature. The grids were placed sample down on 2% aqueous uranyl acetate for 30 min, rinsed in water, and allowed to dry. The grids were examined at 120 kV, and representative fields were photographed at 45,000 diameters' magnification on a Philips CM120 transmission electron microscope.

**RESULTS**

To test the hypothesis that both PD mutations would cause or enhance aggregation of \( \alpha \)-synuclein and that this effect would be detectable in vitro, we cloned human \( \alpha \)-synuclein cDNA (11) and generated bacterial expression constructs for the wild type protein, the two PD-linked variants, and a form containing both PD mutations within the same molecule. After purification, all 4 proteins run indistinguishably as a single band on SDS-polyacrylamide gel electrophoresis at 19 kDa, and no differences in electrophoretic behavior were observed on native and nondenaturing gels (data not shown). To address whether the wild type and the mutants differ in their conformation, we performed CD and FTIR spectroscopy on solutions of all four species. Fresh solutions of all four proteins showed the natively unfolded structure previously described for the wild type protein (14), with identical near and far UV CD spectra (Fig. 1). The FTIR spectra of these molecules were also indistinguishable and indicated that they contain primarily random coil structure. Thus, the initial conformation of the wild type \( \alpha \)-synuclein and the mutants is identical.

Because \( \alpha \)-synuclein aggregation could be slow, we chose to analyze solutions of \( \alpha \)-synuclein over several days at three different temperatures. During the time frame of the experiment no aggregates formed when incubated at 4 °C or room temperature (data not shown). After incubation for several days at 37 °C, all four proteins began to form insoluble aggregates that could be precipitated by ultracentrifugation. This aggregation proceeded until most of the material had fallen out of solution. This process could be further accelerated by continuously shaking the solution (data not shown). Importantly, in all experiments, aggregate formation was faster for the PD-linked mutations than for the wild type, and the A53T mutation had a more dramatic effect than the A30P mutation. This is most clearly seen when the data are converted into approximate lag times before precipitable aggregates are detected. The lag time for the wild type protein was about 280 h, that of the A30P mutant was about 180 h, and that of the A53T...
mutant was only about 100 h. An example of such an aggregation time course is shown in Fig. 2. At 11 distinct time points we separated the soluble and insoluble material using ultracentrifugation. The secondary structure of the protein that remained soluble was analyzed by CD and did not change for any of the four proteins throughout the course of the experiment (data not shown). The structure of the protein that was in the pellet was analyzed by FTIR. As Fig. 3 clearly shows, aggregation and precipitation of α-synuclein is accompanied by a dramatic change in secondary structure, from the initial primarily random coil seen when the α-synuclein is in solution structure (1650 cm⁻¹ band, Fig. 3, top panel) to the final antiparallel β-sheet structure present in the α-synuclein pellet (1629 cm⁻¹, Fig. 3, bottom panel). This structure is commonly observed in protein aggregates. The spectra of the aggregate of all four forms are indistinguishable. To address whether the precipitates contain only amorphous aggregates or distinct fibrillar structures, we analyzed them by electron and atomic force microscopy (Fig. 4). Fibrils of the wild type and mutant proteins were readily detected in the precipitates by electron microscopy with positive staining. The diameter of the fibrils is around 12 nm. Fluid phase atomic force microscopy clearly demonstrates the presence of fibrils in their native aqueous environment.

**DISCUSSION**

Here we have shown that wild type and both mutant forms of α-synuclein can spontaneously form fibrillar β-sheet aggregates. The generation of such aggregates could be a critical step in the formation of Lewy bodies, which contain full-length α-synuclein as their major fibrillar component (6). Future studies will address in more detail the kinetics of α-synuclein aggregation and fibril morphology. The lag phase preceding α-synuclein fibril formation (Fig. 2) could be indicative of a nucleation-dependent polymerization, and the fact that stirring or shaking dramatically increases the aggregation rate is also supportive of such a mechanism (for review, see Ref. 15). Interestingly, the α-synuclein mutants studied here all show a

**FIG. 3.** Aggregate formation is accompanied by a change in secondary structure from random coil to antiparallel β-sheet. The top panel is the second derivative FTIR spectrum of the initial solution of wild type protein. This spectrum shows primarily random coil when the α-synuclein is in its solution structure (1650 cm⁻¹ band). The bottom panel is the second derivative FTIR spectrum of the wild type α-synuclein aggregate. This spectrum shows the final antiparallel β-sheet structure present in the α-synuclein pellet (1629 cm⁻¹).

**FIG. 4.** Wild type and mutant α-synuclein form fibrils. A, electron microscopy of wild type α-synuclein aggregates demonstrates the fibrillar nature of the precipitate. Fibers of various lengths are observed in this representative field. The diameter of the fibrils is around 12 nm. The bar represents 200 nm. B, atomic force microscopy of A53T mutant α-synuclein demonstrates the presence of hydrated fibers in their native aqueous environment. The image is a topographical height image of a 1-μm square area. Areas of increasing brightness represent areas of increasing height. The bar represents 200 nm.
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reduced lag phase with respect to wild type. Under our conditions the wild type lag phase is about 280 h, that of the A30P mutant is about 180 h, and that of the A53T mutant is about 100 h. The actual concentration of α-synuclein within cells forming Lewy bodies is unknown, but it is very likely to be much lower than in this experimental setup. However, it is not unreasonable to assume that in vivo, nuclei formation could be influenced by seeding cofactors, thus allowing α-synuclein to aggregate at lower concentrations than in our experiments. Furthermore, the formation of fibrils may take years in vivo but happens within weeks in our system. It will also be interesting to compare α-synuclein fibrils found in Lewy bodies (6) with our synthetic α-synuclein fibrils in the same experimental set up.

Most importantly, our results show that both point mutations causing familial PD enhance the aggregation tendency observed in the wild type protein. This is critical, because a pathogenesis model that can cover all known mutants plus the wild type situation is less likely be based on non-disease-relevant effects. For example, in the Alzheimer’s disease field, the key evidence for the amyloid hypothesis is the finding that all known early onset familial Alzheimer’s disease mutations have one common effect: they increase Aβ production (reviewed in Ref. 16). Our finding that all forms of α-synuclein aggregate, but both mutants aggregate faster, immediately suggests that the aggregation is relevant for the pathogenesis of PD and may explain the early disease onset in these families. Although the concentrations used here are nonphysiological, the data show an effect that mirrors the biology in an assay that accomplishes aggregation of all forms of α-synuclein, including wild type. This is important, because most Lewy body diseases seem to involve aggregation of wild type α-synuclein.

Our in vitro assay can be exploited for various applications. The observation that the two mutants enhance aggregation to different extents suggests that one could design additional mutations that would enhance β-sheet formation and further accelerate aggregation in vitro. As a first example, we tested the A53T/A30P double mutant; in some experiments this ana-

log aggregated faster than each of the original mutants; however, this effect was not always observed. Very rapidly aggregating mutant α-synuclein could be critical in the generation of a transgenic model of Lewy body formation. It will be important to see whether such mice develop Parkinsonian symptoms. Finally, the system we have established here can be readily adapted to high throughput screening for compounds that block α-synuclein aggregation. Such inhibitors could be useful as PD therapeutics if aggregation of α-synuclein is a critical step in all forms of PD.

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Page 9843, 5th sentence in the introduction: The words “but not A30P mutant or” should be deleted. The correct sentence should read: “Indeed, a very recent publication demonstrated accelerated in vitro fibrillization of A53T mutant versus wild type α-synuclein (10).”

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