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Cellular Polyamines Promote the Aggregation of α-Synuclein*

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The cellular polyamines putrescine, spermidine, and spermine accelerate the aggregation and fibrillization of α-synuclein, the major protein component of Lewy bodies associated with Parkinson's disease. Circular dichroism and fluorometric thioflavin T kinetic studies showed a transition of α-synuclein from unaggregated to highly aggregated states, characterized by lag and transition phases. In the presence of polyamines, both the lag and transition times were significantly shorter. All three polyamines accelerated the aggregation and fibrillization of α-synuclein to a degree that increased with the total charge, length, and concentration of the polyamine. Electron and scanning force microscopy of the reaction products after the lag phase revealed the presence of aggregated particles (protofibrils) and small fibrils. At the end of the transition phase, α-synuclein formed long fibrils in all cases, although some morphological variations were apparent. In the presence of polyamines, fibrils formed large networks leading ultimately to condensed aggregates. In the absence of polyamines, fibrils were mostly isolated. We conclude that the polyamines at physiological concentrations can modulate the propensity of α-synuclein to form fibrils and may hence play a role in the formation of cytosolic α-synuclein aggregates.

Alpha-synuclein, a 14-kDa protein abundantly expressed in various parts of the brain, is the major component of Lewy bodies, the fibrillar proteinaceous cytosolic inclusions associated with Parkinson's disease (PD).[1] α-Synuclein has also been implicated in the pathogenesis of Alzheimer's disease (AD), multiple system atrophy, diffuse Lewy body disease, and amyotrophic lateral disease (1–5). The protein is "natively unfolded" (6) but undergoes aggregation leading to fibrillar structures, in which it adopts a β-sheet secondary structure. The conformational transition of α-synuclein into a β-sheet structure and the fibrillization process are believed to occur concurrently (7), although the mechanism is as yet unclear. The formation of fibrils with an ordered β-sheet structure from monomeric protein components is associated with many other diseases, including AD, Huntington's disease, diabetes, and prion diseases (8–12). Therefore, understanding the mechanism of fibrillization and the factors modulating the aggregation process is essential for devising therapeutic strategies against these diseases. Depending on the structure of the aggregates formed, they are classified either as amorphous, lacking ordered structures, or as fibrils, exhibiting a β-sheet secondary structure. Both types of aggregates occur in Lewy bodies (13, 14), and their morphology is influenced significantly by the solution conditions (13, 15).

Based on the amino acid sequence, three distinct domains have been identified in α-synuclein: (i) the N-terminal amphipathic region, rich in amino acids with a high propensity for α-helix formation, and known to be involved in binding to cell membranes and lipids, (ii) the central hydrophobic region (amino acids 61- 95), and (iii) the acidic C-terminal region in which most of the negatively charged amino acids are located (16, 17). The aggregation of α-synuclein is promoted by a variety of agents, including metal ions, lipids, pesticides, and conditions that generate oxidative stress (18–22). Two mutations in the α-synuclein gene (A53T and A30P) occur in about 10% of familial PD cases and have been shown to accelerate the fibrillization of the protein (23–25). α-Synuclein aggregation is accelerated by cationic molecules, such as glycosyl amines and polylysine, and by the multivalent metal ions Cu^{2+}, Fe^{2+}, Fe^{3+}, Zn^{2+}, and Al^{3+} (13, 18, 26). These metal ions interact with the protein and may act by inducing conformational transitions (18). The recent identification of dopamine and its structural analogs as ligands for α-synuclein that inhibit the formation of mature fibrils (27) offers new perspectives for the utilization of small molecules to control the process of α-synuclein aggregation/fibrillization.

In this work we studied the aggregation of α-synuclein in the presence of the biogenic polyamines putrescine ($H_2N(CH_2)_4NH_2$), spermidine ($H_2N(CH_2)_3NH(CH_2)_4NH_2$), and spermine ($H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$). Polyamines are naturally occurring organic cations involved in a large number of cellular functions, including DNA replication, transcription, and protein synthesis (28, 29). The cellular concentration of spermine is ~1 mM, with higher concentrations reported for putrescine and spermidine (30–32). Due to their cationic nature, polyamines interact with polyanionic molecules such as DNA and RNA and induce structural changes dependent on the nucleotide sequence. In addition to their positive charge, the hydrophobicity of the polyamines is another important factor modulating their interaction with other macromolecules.

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[1] The abbreviations used are: PD, Parkinson's disease; AD, Alzheimer's disease; CD, circular dichroism; EM, electron microscopy; SFM, scanning force microscopy; thio T, thioflavin T.

Polyamines are present in neuronal cells (33, 34). Although systematic function studies of their role are lacking, they are known to play a role in the interaction with neurotransmitter receptors, *e.g.* the *N*-methyl-D-aspartate receptor, and ion channels, *e.g.* K^+ (35, 36). The polyamine levels in the substantia nigra of diseased human brains of individuals exhibiting neurological disorders, including PD, are not lowered, suggesting that the regulation of the substances is well maintained even in degenerating cells (37). Spermine may serve to protect neuronal cells from oxidative stress (38), a condition known to induce aggregation of the amyloid Aβ peptide, the major component of amyloid deposits associated with Alzheimer's disease. It was also shown in cultured neuronal cells that the Aβ peptide combined with spermine is more toxic than the peptide alone (39). A possible role for these cations in the aggregation of α-synuclein has not been proposed. Although α-synuclein is present both in the nucleus and cytoplasm of neuronal cells, insoluble protein aggregates form exclusively in the cytoplasm (40, 41). Polyamines are present in the cytoplasm at concentrations regulated by biosynthesis and by uptake mediated by the polyamine transport proteins (42–44). Thus, an intriguing possibility is that the subcellular co-localization of α-synuclein and polyamines in the cytoplasm may facilitate their interaction. Indeed, our results suggest that polyamines at physiologically relevant concentrations may serve to modulate the aggregation and fibrillization of α-synuclein *in vivo*.

EXPERIMENTAL PROCEDURES

Expression and Purification of α-Synuclein—The recombinant plasmid pT7-7, encoding α-synuclein, was kindly provided by the laboratory of Peter Lansbury. The plasmid was transformed into *Escherichia coli* BL21(DE3), and α-synuclein expression was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside. The cell pellet was collected by centrifugation at 4500 × *g*, resuspended in lysis buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol), freeze-thawed three times, and sonicated. DNA was removed by precipitation with streptomycin (10 mg/ml) and centrifugation at 22,000 × *g* (rotor JA-20, Beckman Avanti J-25 centrifuge) for 30 min at 4 °C. The supernatant was collected, incubated in a boiling water bath for 20 min, and centrifuged at 22,000 × *g*. α-Synuclein was precipitated by adding ammonium sulfate to the supernatant (final concentration, 361 mg/ml) and centrifuged at 22,000 × *g*. The precipitate was resuspended in 25 mM Tris-HCl, pH 8.0, applied to a Poros HQ column of a Biocad gel perfusion chromatographic system (Applied Biosystems), and eluted with a NaCl gradient (final concentration, 300 mM) in the same buffer. The protein fractions were collected, dialyzed against 10 mM Tris-HCl, pH 8.0, and concentrated with Millipore Centricon filters. The purity was >95% according to polyacrylamide gel electrophoresis, electrospray ionization-mass spectrometry, and analytical gel filtration. The protein was quantitated spectroscopically using a molar extinction coefficient at 274 nm of 5600 M^{-1} cm^{-1} (6).

Aggregation Studies—α-Synuclein (70 μM) was incubated in 25 mM Tris-HCl, pH 7.5, at 37 °C with vigorous stirring (magnetic bar) in glass vials. The polyamines putrescine-2HCl, spermidine-3HCl, and spermine-4HCl were purchased from Sigma. Concentrated stock solutions were prepared in distilled water and diluted into the protein solutions in 25 mM Tris-HCl, pH 7.5. The pH of the solution was measured after polyamine addition, and no significant change was observed. Aliquots were removed from the incubation mix at different time intervals and diluted to appropriate concentrations for CD and fluorescence measurements.

CD Spectroscopy—CD spectra were recorded on a Jasco 720 spectropolarimeter equipped with a Peltier temperature controller. 15 μl of the protein solution, incubated with stirring in buffer alone or in buffer supplemented with polyamines at 37 °C, was diluted in 10 mM Tris-HCl, pH 8.0, to a final volume of 200 μl for CD measurements in cuvettes of 1-mm path length. These diluted samples were not stirred further. The spectra were buffer-subtracted, and three scans were averaged.

Fluorescence Measurements (Thioflavin T Binding Assay)—After incubation, protein solutions were diluted 40-fold with 20 μM thioflavin T (thio T) (Sigma) in 10 mM Tris-HCl, pH 8.0. Fluorescence was measured on a Varian Cary Eclipse spectrofluorometer in 1-cm path length quartz cuvettes. Emission spectra (470–650 nm) were recorded for excitation at 450 nm, using a 5-nm band-pass for both excitation and emission. The contribution of unbound thio T to the fluorescence was measured on a thio T sample at the same concentration but in the absence of α-synuclein. The 40-fold diluted concentrations of polyamines in the fluorescence assay did not exert an effect on the fluorescence intensity of free thio T.

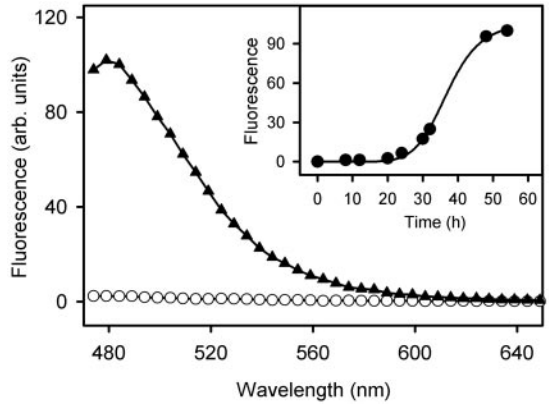

FIG. 1. **Fluorescence emission spectra of thio T in presence of native (○) and aggregated (▲) α-synuclein (52-h incubation).** The excitation wavelength was 450 nm. *Inset*, the variation of fluorescence emission intensity at 480 nm with incubation time. 70 μM α-synuclein was incubated in 25 mM Tris-HCl, pH 7.5, at 37 °C and diluted to 1.75 μM in 10 mM Tris-HCl, pH 8, for fluorescence measurements.

Electron Microscopy—An aliquot was withdrawn from the incubation mixture and placed onto a glow-discharged carbon film attached to an EM grid. Carbon films, 3- to 4-nm thick, were pretreated by glow discharge in the presence of pentylamine vapor (residual pressure, ~150 millitorr; discharge current, 2–3 mA; duration of discharge, 30 s) as described elsewhere (45). The adsorption continued for 1–2 min, after which the grids were rinsed with a few drops of 2% aqueous uranyl acetate, blotted with filter paper, and dried. The samples were examined with a Philips CM12 electron microscope. The negatives were scanned with a DuoScan T2500 scanner (Agfa) at 1200 dots per inch. Micrographs were measured using Image software (National Institutes of Health) modified for Windows. For printing, images were flattened using a high pass filter with a radius of 250 pixels and subsequently adjusted for contrast/brightness using Adobe Photoshop.

Scanning Force Microscopy—SFM images were acquired on a Digital Instruments Nanoscope III microscope. A 2.5 μM solution of α-synuclein in 10 mM Tris-HCl, pH 8.0, was deposited onto a freshly cleaved mica surface. The SFM head equipped with a fluid cell was placed on the top of the J-Scanner, and more protein solution was added to the fluid cell. Imaging was performed in liquid in tapping mode. Cantilevers (NP-S, Digital Instruments) with a nominal spring constant of 0.32 newtons/m were used at an oscillation frequency of ~9 kHz. For the imaging of α-synuclein fibrils, 0.5 mM $MgCl_2$ was added to the incubation buffer before imaging. Samples containing polyamines were imaged without added magnesium.

RESULTS

Thioflavin T Assay—Thioflavin T is a weakly fluorescent dye in the free state but strongly fluorescent when bound to amyloidogenic proteins in their aggregated state (25, 46). The fluorescence changes accompanying the binding of thio T to α-synuclein before and after aggregation are depicted in Fig. 1. Thio T fluorescence was very weak when the assay was performed immediately upon initiating the incubation. After a lag time of ~22 h, the intensity increased and reached a limiting value after ~52 h (*inset*).

The variation of fluorescence emission intensity of thio T in the presence of α-synuclein incubated with putrescine, spermidine, and spermine is depicted in Fig. 2, *A*, *B*, and *C*, respectively. Putrescine was used at concentrations of 1, 5, and 10 mM. In the presence of 1 mM putrescine, the increase in initial intensity occurred after 10 h and reached a plateau at ~18 h. At higher putrescine concentration, the kinetics of fibrillization was faster, reflected in a decrease in the lag time for the thio T intensity

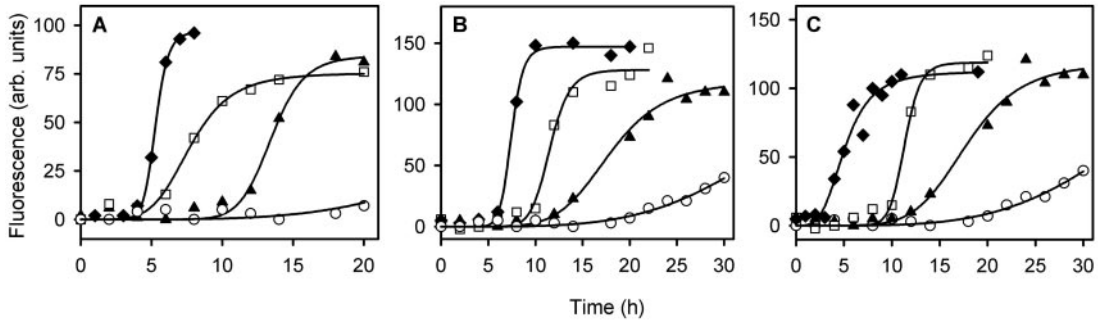

FIG. 2. **Variation of fluorescence emission intensity of thio T at 480 nm in presence of α-synuclein incubated with putrescine (*A*), spermidine (*B*), and spermine (*C*).** The concentration of polyamines were, putrescine: 0 (○), 1 (▲), 5 (□), and 10 (♦) mM, spermidine: 0 (○), 100 μM (▲), 500 μM (□), and 2 mM (♦) and spermine: 0 (○), 10 (▲), 25 (□), and 100 (♦) μM. The excitation wavelength was 450 nm. The concentrations of α-synuclein and thio T were 1.75 and 20 μM, respectively.

signal (*e.g.* to 4 h for 10 mM putrescine). Spermidine was more efficient than putrescine in promoting α-synuclein aggregation. An enhanced aggregation rate was observed at a spermidine concentration as low as 100 μM (Fig. 2*B*). The lag time decreased with increasing polyamine concentration. At 2 mM spermidine, the lag time was 6 h, and saturation in the binding profile occurred after 9 h with the development of visible turbidity. Spermine was the most effective of the polyamines in inducing the aggregation of α-synuclein (Fig. 2*C*). This tetra-cationic polyamine promoted aggregation at a very low concentration (10 μM). For α-synuclein incubated with 100 μM spermine, the lag time was ~2 h and saturation in the binding profile occurred after 9 h. Aggregation was visible at spermine concentrations ≥100 μM.

A qualitative comparison of the efficiency of different polyamines to promote α-synuclein aggregation, characterized by the lag time t_1, the time at which initial increase in thio T intensity was observed, and transition time t_2, the incubation time at which the kinetic profile was saturated, is given in Table I. Because the plateau values in the kinetic curves of α-synuclein aggregated under different conditions were different, t_2 was calculated from individual saturation values. In the absence of polyamines, the increase in thio T fluorescence was complete at ~52 h with the inception of aggregation at ~22 h. In the presence of 2 mM spermidine and 100 μM spermine, the lag time decreased to ~6 and 2 h, respectively, with saturation in the fluorescence signal at ~9 h.

Circular Dichroism Spectroscopy—The CD spectrum of native α-synuclein was characterized by a strong negative CD band in the 195- to 200-nm region, indicative of a disordered structure. In contrast, the aggregated form had a positive band at ~200 nm, and a negative band at ~220 nm corresponding to a β-sheet structure (Fig. 3*A*). This transition in secondary structure to a β-sheet is a characteristic structural feature of α-synuclein aggregation and fibrillization (6, 25). CD studies of native α-synuclein incubated with 10 mM putrescine (Fig. 3*B*), 2 mM spermidine, or 100 μM spermine (Fig. 3*C*) were carried out at three time points (0, 8, and 24 h). In all three cases, CD spectra measured immediately after addition of the polyamine to the monomeric protein (t = 0 data point) were indistinguishable from the CD spectrum of the protein alone.

After 8-h incubation with 10 mM putrescine, the sign of the ellipticity of the band in the 200-nm region changed from negative to positive, with a concurrent increase in negative ellipticity at 220 nm, indicating that a significant fraction of the protein was converted to a β-sheet configuration. After 24 h, the positive ellipticity at 200 nm and the negative ellipticity at 220 nm increased further. No additional CD spectral changes were detected at later times.

Incubation of α-synuclein with 2 mM spermidine for 8 h at 37 °C yielded a turbid solution. The CD signal was very weak and noisy, suggesting that the protein had precipitated (data not shown). The spectral properties of the solution after 24 h were similar.

TABLE I
Effect of increasing concentrations of polyamines on the aggregation kinetics of α-synuclein

t_1 and t_2 are the lag and transition times, respectively, determined from the initial increase and saturation of the thio T kinetic profile.

Polyamine	Concentration	t_1	t_2
	mM	*h*	
None		~22	~52
Putrescine	1	~10	~18
	5	~5	~14
	10	~4	~8
Spermidine	0.1	~10	~26
	0.5	~8	~16
	2	~6	~9
Spermine	0.01	~10	~26
	0.025	~9	~15
	0.1	~2	~9

The CD spectral changes associated with the aggregation of α-synuclein in the presence of 100 μM spermine are depicted in Fig. 3*C*. After incubation for 8 h, the sample was visibly turbid, but the circular dichroism spectrum was still characteristic of a disordered structure, albeit of lower intensity. However, the CD spectrum measured after 24 h showed a spectral pattern characteristic of a β-sheet structure, but with a decreased absolute ellipticity compared with that of the β-sheet conformation achieved in the absence of polyamines, suggesting an increased solubility of the aggregates with time. We conclude that visible aggregates formed at earlier time points may have acted as nucleation centers for fibril formation or, alternatively, that the formation of ordered structures from aggregates proceeded very slowly.

Electron Microscopy—The structures of α-synuclein aggregates formed upon incubation with and without polyamines for 6 h, 3 days, and 7 days were examined by EM. Aggregates formed in the absence of any polyamines after a 3-day incubation (Fig. 4*A*) were seen as long fibrils (width ~13 nm), often forming large networks, and distributed rather uniformly on the surface of the carbon film. In the presence of polyamines, the structure of aggregates varied with polyamine nature and incubation time. Fig. 4 (*B* and *C*) shows EM images of α-synuclein after a 6-h incubation with 1 mM spermidine and 0.5 mM spermine, respectively. In the presence of spermidine, α-synuclein formed small aggregates of variable size, whereas in the presence of spermine small aggregates co-existed with short fibrils. At long incubation time (3 days), the samples displayed a fibrillar structure regardless of the nature of the polyamine; this structure was qualitatively similar to that

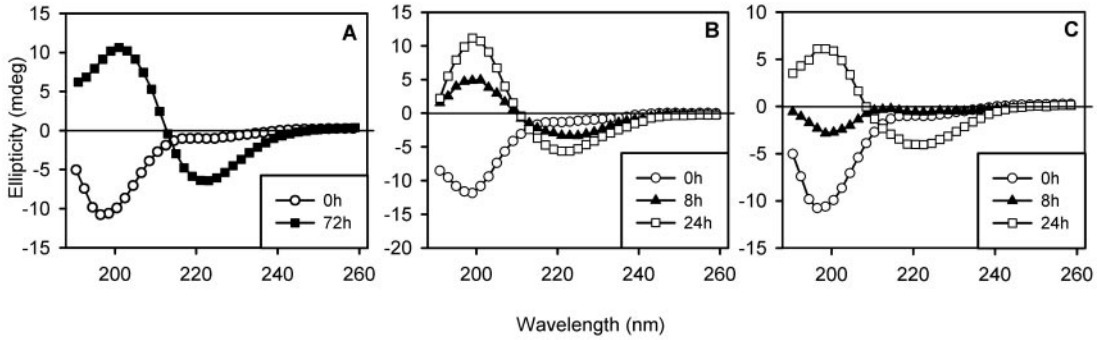

FIG. 3. **Time-dependent CD spectra of α-synuclein alone (*A*) and in presence of 10 mM putrescine (*B*) and 100 μM spermine (*C*), measured at different incubation times.** CD spectra were measured immediately after initiation of incubation of protein ($t = 0$) in buffer alone (*A*) or in buffer supplemented with polyamines (*B* and *C*). The incubations were continued and further spectra were acquired at the time points indicated in the figures.

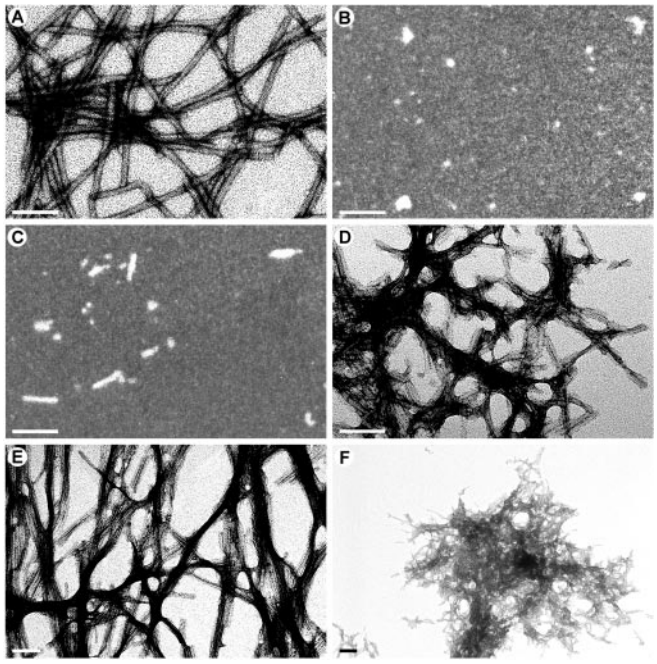

FIG. 4. **Electron microscopy images of α-synuclein aggregated in the presence and absence of polyamines, at different time points.** *A*, α-synuclein alone, 3 days; *B*, 1 mM spermidine, 6 h (*dark field image*); *C*, 500 μM spermine, 6 h (*dark field image*); *D*, 5 mM putrescine, 3 days; *E*, 10 μM spermine, 3 days; and *F*, 1 mM spermidine, 12 days. *Scale bars*, 100 nm.

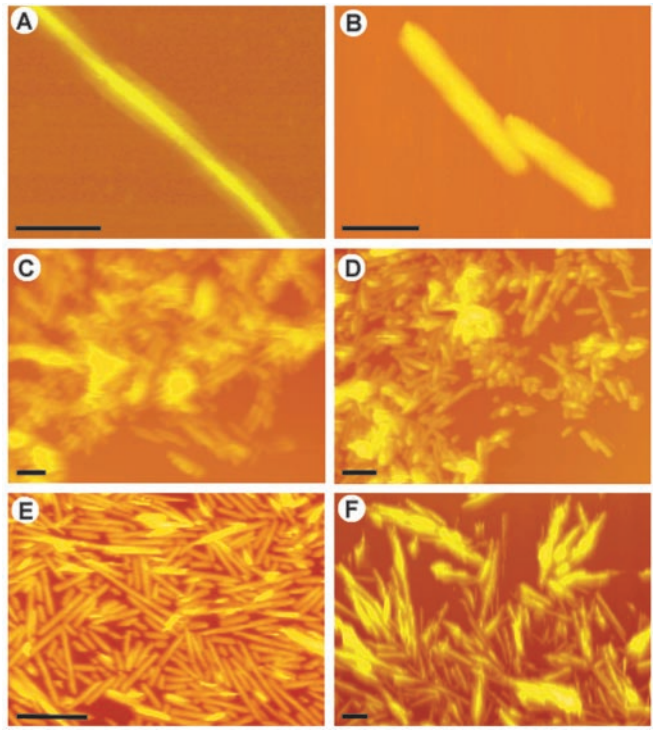

FIG. 5. **Scanning force microscopy images of α-synuclein aggregated in the presence and absence of polyamines, at different time points.** *A*, α-synuclein alone, 48 h; *B*, 1 mM spermidine, 4 h; *C*, 100 μM spermine, 4 h; *D*, 1 mM spermidine, 24 h; *E*, 5 mM putrescine, 24 h; and *F*, 5 mM putrescine, 4 days. *Scale bars*: 100 nm (*A* and *B*); 200 nm (*C–F*).

formed in the absence of polyamines. However, the nets were slightly larger and more condensed, possibly indicative of an increased adhesiveness of the fibrils. Two examples are shown in Fig. 4 (*D* and *E*) corresponding to α-synuclein incubated with 5 mM putrescine and 10 μM spermine, respectively. Incubation for 12 days in 1 mM spermidine led to highly condensed aggregates, unevenly distributed over the carbon surface (Fig. 4*F*). We speculate that these aggregates represented stacks of individual fibrils.

Scanning Force Microscopy—The structure of protein fibrils is associated with a range of morphologies differing in structural parameters such as fibril length, width, height, twist, and helical periodicity (8, 47, 48). SFM constitutes a powerful tool for visualizing and studying fibril morphology (47, 49). In the absence of polyamines and after a long incubation time (48 h), α-synuclein formed isolated fibrils ~12 nm in height (Fig. 5*A*). In samples incubated with polyamines, protein aggregates were seen at time points as early as 4 h. The fibrils formed in the presence of spermidine were isolated, short entities with mean heights of ~12 nm (Fig. 5*B*), whereas spermine induced formation of large aggregates displaying a highly diffuse structure (Fig. 5*C*). Similar aggregates formed in the presence of 1 mM spermidine but required longer (24 h) incubation (Fig. 5*D*). In 5 mM putrescine α-synuclein formed large aggregates consisting of clearly visible individual short fibrils (Fig. 5, *E* and *F*, for 24-h and 4-day incubations, respectively).

DISCUSSION

Polyamines Modulate the Kinetics of α-Synuclein Aggregation—The aggregation of α-synuclein into ordered fibrils is a kinetically slow process involving the formation of a β-sheet structure by the natively unfolded protein (50). Our studies suggest that biogenic polyamines may play a role in accelerating this process. α-Synuclein has 24 negatively charged residues, the majority of which are located in the C-terminal domain. The presence of this acidic region and the hydrophobic region located between the C and N termini provide binding targets for cationic and hydrophobic molecules that could en-

hance the aggregation process. The polyamines are multivalent cations with aliphatic hydrocarbon chains separating the charges, and thus could potentially bind to α-synuclein via both hydrophobic and electrostatic interactions. The potency with which polyamines facilitate aggregation correlates with their cationic charge and the number of aliphatic carbon chains between the amino/imino groups. Thus, the tetracationic spermine with three hydrocarbon chains is more potent than the lower homologs spermidine and putrescine. Presumably, the greater number of charges located further apart by flexible linkers permit more effective interactions with different regions of the same protein or between different proteins. Because hydrophobic interactions are important for holding different β-sheets together and are thus essential features of the aggregation process, molecules that can bind to the hydrophobic region but do not form β-sheets by themselves are being developed and screened as drugs against diseases characterized by the formation of protein aggregates (51).

The model suggested by Uversky *et al.* (18) for metal ion-induced fibrillization of α-synuclein is relevant to the present study. As in the case of multivalent metal ions, polyamines may act by bridging the carboxylate groups from the same protein or from different proteins, thereby promoting aggregation. However, the aggregation pathways may be different because the metal ions induce a partially folded conformation of α-synuclein, whereas no such secondary structural transition is observed in the presence of polyamines. The ability of spermine to induce the aggregation, at a concentration of about 1/10 that of spermidine and 1/100 that of putrescine, cannot be explained by simple electrostatic considerations. As discussed above, hydrophobic interactions may be responsible for the higher potential of spermine to promote α-synuclein aggregation at physiologically relevant concentrations.

α-Synuclein aggregation is a cooperative process characterized by a significant lag time. In addition to reducing the lag time, polyamines also enhance the cooperativity of the transition. Saturation in the kinetic profile is reached ~7 h after inception for α-synuclein incubated with 2 mM spermidine or 100 μM spermine, compared with ~30 h for α-synuclein incubated alone (Fig. 2 and Table I). The enhancement of the aggregation rate in the presence of polyamines reported here may serve to devise faster aggregation assays, a major goal in the screening of large numbers of potential drug molecules for the prevention or inhibition of fibrillization. A competition assay in the presence of a ligand like spermine could be carried out in hours rather than the many days currently required for α-synuclein aggregation in the absence of added ligands.

Formation of Amorphous Aggregates and Protofibrils Precedes α-Synuclein Fibrillization—The formation of fibrils from natively unfolded protein is presumed to involve at least four species in the aggregation pathway, which follows the scheme,

$$U_N \rightarrow I \rightarrow P \rightarrow F$$

SCHEME 1

where U_N, I, P, and F represent the initial unfolded state, initial nucleation sites containing proteins in a conformation suitable for protein-protein interactions, protofibrils, and the mature fibrils, respectively (52, 53). The protofibrils are intermediate structures in the protein fibrillogenesis, exhibited by many proteins, and assume different shapes (spherical, annular, and chain protofibrils) often with lengths extending up to 200 nm and 1–10 nm in diameter (8, 47, 54). Mature fibrils are formed by the association of protofibrils or by further growth of protofibrils by the attachment of monomeric molecules (48, 55). The initial lag phase, the time required to form the nucleation centers, and the propagation phase that leads to the formation of the protofibrils, were affected by the interaction with polyamines. Work on the development of a kinetic scheme incorporating both amorphous and fibrillar aggregates is in progress.

The increase in thio T fluorescence and the CD spectral changes corresponding to the transition to the β-sheet secondary conformation occurred simultaneously during α-synuclein aggregation in the absence and presence of putrescine. However, it was unclear from CD spectra whether the aggregates formed in the presence of spermidine or spermine at high concentration possessed a β-sheet structure. The development of turbidity in the sample, and the decrease in CD signal intensity were indicative of precipitation of the protein. Fibrillar structures were seen by SFM as early as 4 h, in the presence of high concentrations of polyamines (Fig. 5, *B* and *C*). Examination by EM of the samples obtained under similar conditions after 6 h revealed a significant fraction of aggregates corresponding to that of protofibrils (Fig. 4, *B* and *C*). Only larger sized fibrils were present after 3 days of incubation, implying that most of the protofibrils aggregated into mature fibrils. However, the thio T fluorescence did not increase after 8–10 h of incubation, revealing that the transition from protofibrils to fibrils was not associated with structural changes leading to additional thio T binding. A logical conclusion is that the fibrils probably formed by association of protofibrils.

Alternatively, the rapid aggregation at high polyamine concentration may reflect the formation of insoluble small amorphous aggregates and the corresponding turbidity at early time points (8 h) as observed for α-synuclein incubated with 100 μM spermine (Fig. 3*C*). Upon further incubation (24 h), a substantial fraction of the protein adopts a more soluble β-sheet conformation, giving rise to the characteristic CD spectra. In the case of the amyloid Aβ aggregation, the conversion of amorphous to fibrillar structures and the generation of nucleation sites from amorphous aggregates for the formation of structured fibrils by addition of monomeric Aβ have been proposed (56).

Morphological Difference in the Fibrillar/Amorphous Aggregates of α-Synuclein Formed in the Absence and Presence of Polyamines—Electron microscopy and SFM revealed differences in the α-synuclein aggregates formed in the presence or absence of polyamines. In the EM images, the fibrils of α-synuclein formed in the absence of polyamines were evenly distributed on the surface of carbon films, forming networks of individual fibrils, many of which were twisted (Fig. 4). In contrast, the polyamine-mediated aggregates comprised a multitude of fibrils, containing many filaments assembled as large aggregates. The aggregate morphology may be important in toxicity as shown for α-synuclein (13) and other amyloid aggregates (57–59).

Polyamines in Neurodegeneration and Neuroprotection—The concentrations of polyamines are maintained during neurodegeneration associated with PD and AD (37), although the activities of ornithine decarboxylase and spermidine/spermidine acetyl transferase, the key enzymes in the biosynthetic pathway of polyamines, increase significantly (39, 60). Polyamines are scavengers for free radicals and protect cells from free radical-induced oxidative damage, a process that promotes aggregation of α-synuclein (21, 61). Paradoxically, our observation that the polyamines accelerate aggregation suggests that they may also facilitate neurodegeneration. A similar effect has been reported for dopamine, which promotes the formation of α-synuclein protofibrils, considered by some (27) to be more neurotoxic than the mature fibrils. In contrast, Levadopa, the precursor of dopamine, is a major drug used against PD (62). An intriguing possibility is that a balance is maintained between the cell protective and degenerative functions of these

molecules. This balance would be disrupted upon degeneration by the failure or dysfunction of regulatory mechanisms such as the degradation of aggregates or binding of α-synuclein to membranes, a state stabilizing the protein in an α-helical structure (63).

We have demonstrated in this study that small molecules like polyamines can modulate the kinetics of fibrillization of α-synuclein. This finding may be important for the development of more efficient bioassays and the identification of drug candidates that inhibit, delay, or reverse aggregation. More fundamental is the possibility that these ubiquitous cations are directly involved in the development of Parkinson's disease.

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