Forcing Nonamyloidogenic β-Synuclein To Fibrillate†

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ABSTRACT: The fibrillation and aggregation of α-synuclein is a key process in the formation of intracellular inclusions, Lewy bodies, in substantia nigral neurons and, potentially, in the pathology of Parkinson's disease and several other neurodegenerative disorders. α-Synuclein and its homologue β-synuclein are both natively unfolded proteins that colocalize in presynaptic terminals of neurons in many regions of the brain, including those of dopamine-producing cells of the *substantia nigra*. Unlike its homologue, β-synuclein does not form fibrils and has been shown to inhibit the fibrillation of α-synuclein. In this study, we demonstrate that fast and efficient aggregation and fibrillation of β-synuclein can be induced in the presence of a variety of factors. Certain metals (Zn^{2+}, Pb^{2+}, and Cu^{2+}) induce a partially folded conformation of β-synuclein that triggers rapid fibrillation. In the presence of these metals, mixtures of α- and β-synucleins exhibited rapid fibrillation. The metal-induced fibrillation of β-synuclein was further accelerated by the addition of glycosaminoglycans or high concentrations of macromolecular crowding agents. β-Synuclein also rapidly formed soluble oligomers and fibrils in the presence of pesticides, whereas the addition of low concentrations of organic solvents induced formation of amorphous aggregates. These new findings demonstrate the potential effect of environmental pollutants in generating an amyloidogenic, and potentially neurotoxic, conformation, in an otherwise benign protein.

The unprecedented increase in the average longevity of humans in the past half-century has led to increasing prevalence of age-related neurodegenerative disorders, such as Parkinson's disease (PD)[1] and Alzheimer's disease. As the second most prevalent neurodegenerative disorder, PD afflicts nearly 5 million people worldwide according to the World Health Organization and predominantly targets those over the age of 50. PD is a chronic disease, which results from the death of dopaminergic neurons in the *substantia nigra* and consequent depletion of dopamine, a neurotransmitter necessary for motor function. Although the exact cause of the cytotoxicity is unknown, growing evidence suggests multiple causative pathways, which include influences from environmental and genetic factors among others (*1*, *2*).

For surviving dopaminergic neurons, a pathological hallmark of the disease involves proteinaceous fibrillar intracytoplasmic deposits known as Lewy bodies (LBs) and Lewy neurites (LNs) (*3*, *4*). The fibrillar form of α-synuclein, a presynaptic protein that is expressed as a 14 kDa polypeptide of 140 amino acid residues, constitutes a core of LBs and LNs (*5*). This protein has been estimated to account for as much as 1% of the total protein in soluble cytosolic brain fractions. The family of synucleins includes α-synuclein, also known as the nonamyloid component precursor protein, NACP, or synelfin (*6*−*9*); β-synuclein, also called phosphoneuroprotein 14 or PNP14 (*8*, *10*); and γ-synuclein, also known as breast cancer-specific gene 1 (BCSG1) and persyn (*11*, *12*). The primary structure of α-synuclein exhibits six imperfect repeats in the N-terminal region and a highly acidic C-terminal region, which are characteristic features of the entire synuclein family (*9*, *13*). Although the exact function of α-synuclein is unknown, a growing body of evidence suggests that this protein potentially plays a role in synaptic vesicle trafficking and perhaps in neurotransmitter release (*8*) because of its ability to bind a wide range of natural brain vesicles (*9*, *14*) and its tentative affiliation with the presynaptic terminal.

α-Synuclein is a natively unfolded protein because of its combination of high net charge and low hydrophobicity (*15*). However, under appropriate conditions, α-synuclein adopts a partially folded conformation (*16*), which leads to self-association and amyloid-like fibrils, which possess morphologies and staining characteristics similar to those extracted from the brains of PD patients (*17*, *18*). The aggregation of α-synuclein has been implicated in the formation of LBs and LNs in PD and dementia with LBs, as well as formation of intraglial inclusions in multiple-system atrophy and Hallervorden-Spatz disease (*19*). Although the molecular basis for the neurodegeneration in PD and other synucleinopathies is as yet unknown, the accumulation of α-synuclein-derived fibrillar material represents a potential link

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[1] Abbreviations: PD, Parkinson's disease; ThT, thioflavin T; LB, Lewy body; LN, Lewy neurites; CD, circular dichroism; AFM, atomic force microscopy; GAGs, glycosaminoglycans; SEC, size-exclusion chromatography; ATR, attenuated total reflectance.

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between protein misfolding and the pathogenesis of these disorders.

Human β-synuclein is a 134-residue neuronal protein that is 78% homologous to α-synuclein. The α- and β-synucleins share a conserved C-terminus with three identically placed tyrosine residues. However, β-synuclein is missing 11 residues within the specific NAC region (*13*). The activity of β-synuclein may be regulated by phosphorylation (*10*). This protein, like α-synuclein, is expressed predominantly in the brain; however, in contrast to α-synuclein, β-synuclein is distributed more uniformly throughout the brain (*20*, *21*). β-Synuclein colocalizes with α-synuclein in the *substantia nigra* (*22*), but it is not generally found in LBs or LNs. However, it has recently been established that in addition to the traditional α-synuclein-containing LBs and LNs, the development of PD and dementia with LBs is accompanied by the appearance of novel α- and β-synuclein-positive lesions in hippocampus (*23*). Pathological vesicular-like lesions were costained by antibodies to α- and β-synucleins and were shown to localize at the presynaptic axon terminals in the hippocampal dentate, hilar, and CA2/3 regions (*23*). This broadens the concept of neurodegenerative "synucleinopathies" by implicating β-synuclein, in addition to α-synuclein, in the onset and progression of these two diseases. Other than this, there has been no other study clearly linking the potential involvement of β-synuclein in the pathogenesis of PD.

Recently, it has been reported that molar excesses of β-synuclein effectively inhibit α-synuclein fibrillation in vitro (*24*, *25*), and studies on transgenic mice brain and in transfected non-neuronal cells showed a comparable inhibitory effect in vivo (*26*). This indicates that β-synuclein may regulate α-synuclein fibrillation, perhaps acting as a chaperone to minimize the aggregation of α-synuclein. On the basis of these observations, it has been concluded that a decrease in the levels of β-synuclein should be considered as a possible factor in the PD etiology (*24*).

A growing body of evidence indicates PD may be an environmentally triggered disease. For example, several studies have implicated environmental factors such as pesticides and metals in the pathogenesis of PD (*27*−*30*). Farming, rural living, well-water drinking, exposure to agricultural chemicals, and welding are all conditions that have been associated with an increased risk for PD (*31*−*34*). Analysis of LBs bodies isolated from PD patient brains uncovered high levels of iron and the presence of aluminum (*35*, *36*). Further, it has been shown that different environmental contaminants, such as heavy metals (*2*, *37*), are able to stabilize the amyloidogenic partially folded conformation in α-synuclein and dramatically accelerate the fibrillation of this protein in vitro. Given the prevalence of heavy metals in the environment and their prospective role in the pathological aggregation of α-synuclein, we decided to investigate the structural and fibrillation properties of β-synuclein in the presence of several metals and pesticides. We provide evidence that certain metals and pesticides are able to induce amyloid-like fibrils, and potentially neurodegenerative states of β-synuclein.

MATERIALS AND METHODS

Expression and Purification of Human α- and β-Synuclein. Human recombinant α- and β-synucleins were expressed using the *Escherichia coli* BL21(DE3) cell line transfected with pRK172/α-synuclein or pRK172/β-synuclein plasmids (generously donated by M. Goedert, MRC, Cambridge, U.K.). Expression and purification of human recombinant α- and β-synucleins from *E. coli* were performed as previously described (*24*). Purity of proteins was determined by SDS−polyacrylamide gel electrophoresis, UV absorbance spectroscopy, and mass spectrometry.

Supplies and Chemicals. Thioflavin T (ThT) and PEG 10000 were obtained from Sigma. Heparin (100000) sodium salt was from Gibco BRL. $ZnSO_4$, $Pb(NO_3)_2$, and $CaCl_2$ (analytical grade) were from Fisher. Analytical grade $CuCl_2$ and $Hg(CH_3CO_2)_2$ were from Aldrich, whereas $AlCl_3$ and $FeSO_4$ were from Mallinckrodt Chemical Works. All other chemicals were analytical grade and from Fisher. All buffers and solutions were prepared with Nanopure water and stored in plastic vials.

Fibril Formation Assay. Fibril formation of β-synuclein in the presence of various metals was monitored using the thioflavin T (ThT) assay in a fluorescence plate reader (Fluoroskan Ascent) as described previously (*38*). Protein solutions contained 20 μM ThT and 1.0 mg/mL (70 μM) β- or α-synuclein in 50 mM Tris-HCl/100 mM NaCl buffer at pH 7.5. Multiple samples were assayed simultaneously on a 96-well plate (white plastic, clear bottom). A Teflon bead ($^1/_8$ in. diameter; McMaster-Carr, Los Angeles, CA) was added to each well. Samples were run in at least triplicate or quadruplicate at 37 °C with 120 rpm shaking and 20 mm diameter rotation. ThT fluorescence was excited at 450 nm, and the emission wavelength was 482 nm. The results of these measurements were plotted as a function of ThT fluorescence intensity versus time and fitted by a sigmoidal curve as previously described (*39*).

We have found that the intensity of ThT signals is not necessarily a reliable quantitative measure of the amount of fibrils: several factors can affect the signal, including competition between other compounds present for the ThT binding sites, quenching of ThT fluorescence by quenchers either in solution or bound to the fibrils, and different fibril morphology. Thus, to demonstrate differences in kinetics, we prefer to use normalized ThT intensities, in which the data are shown with the same final fluorescence intensity.

Lowry Assay. Aliquots of the 50 μL sample were centrifuged at 13 000 rpm for 20 min. The supernatant was removed and mixed with 50 μL of buffer, while the pellet was resuspended in 100 μL of buffer for each sample tested. The supernatant and resuspended pellet were added separately to a mixture of 2 mL of reagent A and 0.2 mL of 50% Folin reagent. After vigorous mixing, samples were allowed to sit for 2 h so the reaction could reach completion.

Circular Dichroism Measurements. CD spectra were recorded on an AVIV (Lakewood, NJ) 60DS spectrophotometer using β-synuclein concentrations of 1.0 mg/mL and a 0.1 mm path length cell. Spectra were recorded from 250 to 190 nm with a step size of 1.0 nm, a bandwidth of 1.5 nm, and an averaging time of 2 s. For all spectra, an average of five scans were obtained. CD spectra of the appropriate buffers were recorded and subtracted from the protein spectra.

FTIR Spectra. Data were collected on a ThermoNicolet 670 FTIR spectrometer equipped with a germanium ATR out-of-compartment internal reflection element. The hydrated

thin films were prepared and analyzed as described previously (*40*, *41*) with backgrounds of 10 mM HEPES buffer with corresponding metal spectra subtracted from protein spectra.

Size-Exclusion Chromatography (SEC). Size-exclusion measurements were performed on a TSK-GEL G2000SW$_{XL}$ size-exclusion column (7.8 mm inside diameter × 30 cm), calibrated with a set of native globular proteins with known molecular masses. The hydrodynamic dimensions (Stokes radii, R_S) for a protein in different associated forms were recalculated from apparent molecular masses, M_{app}, determined by gel-filtration chromatography, using the equations

$$\log(R_S) = 0.369 \times \log(M_{app}) - 0.254 \quad (1)$$

Hydrodynamic dimensions of the natively unfolded protein with a molecular mass of 14 460 (or 28 920) Da and the pre-molten globule-like partially folded protein with a molecular mass of 28 920 (or 14 460) Da were calculated from empirical equations

$$\log(R_S^{NU}) = -(0.551 \pm 0.032) + (0.493 \pm 0.008) \times \log(M) \quad (2)$$

$$\log(R_S^{PMG}) = -(0.239 \pm 0.055) + (0.403 \pm 0.012) \times \log(M) \quad (3)$$

where M is the molecular mass and R_S^{NU} and R_S^{PMG} are the Stokes radii of the natively unfolded (NU) and pre-molten globule-like (PMG) protein, respectively (*42*, *43*).

Protein (~0.1 mg/mL) was loaded onto the column, and the elution was carried out isocratically at a flow rate of 0.4 mL/min and monitored by the absorbance at 275 nm. Prior to measurement, solutions were filtered with a 0.1 μm Whatman Anodisc-13 filter. All measurements were taken at 25 °C.

Electron Microscopy. Transmission electron micrographs were collected using a JEOL JEM-100B microscope operating with an accelerating voltage of 80 kV. Typical nominal magnifications were 75 000 times. Samples were deposited on Formvar-coated 300-mesh copper grids (Ted Pella, Redding, CA). We prepared grids by incubating 2-fold diluted protein samples for 10 min, washing them three times with water, negative staining with 1% uranyl acetate for 5 min, and then washing them with water thrice more.

Atomic Force Microscopy Imaging. 1-(3-Aminopropyl)-silatrane (APS)-modified mica was used as an AFM substrate (*44*). Five microliters of the sample was placed on APS-mica for 2 min, rinsed with deionized water, and dried with argon as described previously (*44*). Images were acquired in air using MAC mode AFM (Molecular Imaging, Phoenix, AZ) and a MultiMode SPM NanoScope IIIa system (Veeco/Digital Instruments, Santa Barbara, CA) operating in tapping mode. Type II MAC levers (Molecular Imaging) with a spring constant of ~2.8 N/m were used with MAC mode AFM. Silicone cantilevers from Olympus (Asylum Research, Santa Barbara, CA) with a spring constant of ~42 N/m were used with the MultiMode system. The typical resonant frequency was 60−70 kHz for type II Mac tips and 360−400 kHz for the Olympus tips. The width and height measurements were ascertained from the AFM images using Femtoscan software (Advanced Technologies Center, Moscow, Russia). The diameters of spherical oligomers were estimated from the height measurements.

RESULTS

Effect of Different Metals on the Aggregation, Fibrillation, and Structural Properties of β-Synuclein

Zn^{2+}, Pb^{2+}, and Cu^{2+} Induce β-Synuclein Fibril Formation, whereas Other Metals Promote Oligomerization of the Protein. Metal cations have been shown to significantly accelerate α-synuclein fibrillation in vitro (*2*, *37*, *45*). Furthermore, although the fibrillation of α-synuclein at neutral pH is completely inhibited by methionine oxidation, the presence of certain metals (Ti^{3+}, Zn^{2+}, Al^{3+}, and Pb^{2+}) overcame this inhibition (*46*). Thus, we examined the effect of many of the same metals on β-synuclein.

The amyloid-specific fluorescent dye thioflavin T was used as a probe for the detection of fibrils (*47*). Although this dye is relatively specific for fibrils, it does interact with some aggregation intermediates, leading to small increases in fluorescence, for example, with oligomeric intermediates in the aggregation of the immunoglobulin light chain LEN (*48*).

As shown in Figure 1, Cu^{2+}, Zn^{2+}, and Pb^{2+} induced fibrillation of β-synuclein, Al^{3+}, Hg^{2+}, and Fe^{2+} promoted formation of aggregates with a low affinity for ThT (note the different ordinate scales in panels A and B of Figure 1), and Ca^{2+} had no visible effect (at least within the time scale analyzed). The data for the ThT intensities have been normalized to make the differences in kinetics more readily seen: the variation in the final intensity of the ThT signal varied over a maximum 2−3-fold range for the metals and pesticides (see below) that caused fibrillation. These results for β-synuclein are similar to those for the effect of metals on methionine-oxidized α-synuclein. After incubation at pH 7.5 and 37 °C for 60 h with stirring, the samples of β-synuclein were subjected to centrifugation and analysis of the supernatants and pellets by the Lowry protein assay. The results of this analysis are shown in Figure 2 as relative amounts of soluble and insoluble protein in each sample. This figure shows that most of the protein alone or in the presence of Al^{3+}, Hg^{2+}, and Ca^{2+} was in the supernatant, in contrast to the amount of protein in the presence of Zn^{2+} or Pb^{2+}, which was predominantly in the pellet. These results corroborate the ThT assay results, which show a dramatic increase in the ThT signal with Pb^{2+} and Zn^{2+}, but not with Al^{3+}.

Figure 3 represents the results of size-exclusion HPLC analysis of the soluble fraction of β-synuclein after its incubation for 60 h either alone or in the presence of different metals, and provides additional support that metals induced effective aggregation of β-synuclein. Figure 3 shows that nonincubated β-synuclein elutes as a single peak, whose elution volume corresponds to the monomeric protein, whereas incubated β-synuclein shows two major peaks, corresponding to monomers and dimers.

The conclusion that the new elution peak corresponds to the dimer is based on the observed R_S of 36.5 Å, which coincides with the expected dimensions of a pre-molten globule corresponding to the β-synuclein dimer [R_S = 36.0 Å (*24*)], and that the observed elution volume corresponds to that of the dimer of α-synuclein (*16*).

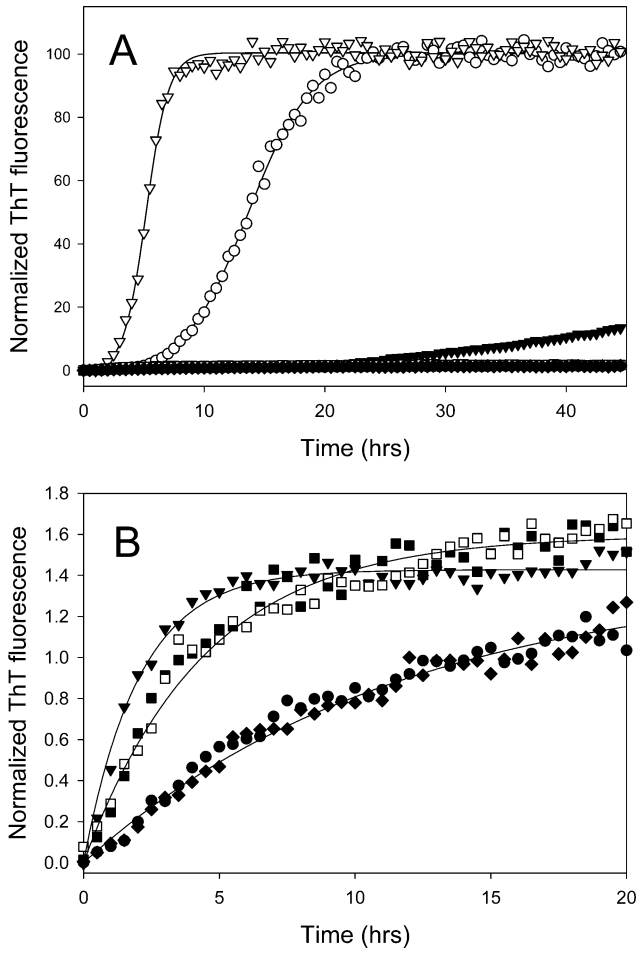

FIGURE 1: Some metals induce the fibrillation of β-synuclein in vitro. Fibril formation was monitored by ThT fluorescence. The symbols represent the ThT fluorescence intensities determined experimentally, and the lines are fitted according to ref *39*: control, no cation (●), Ca^{2+} (◆), Al^{3+} (■), Hg^{2+} (□), Cu^{2+} (▼), Pb^{2+} (○), and Zn^{2+} (▽). The cations are listed in order of increasing ability to promote aggregation and/or fibrillation. In panel A, the data are shown full scale; in panel B, the data are shown on an expanded ThT intensity scale. The protein concentration was 70 μM, and the cation concentrations were 5 mM. Samples were incubated at 37 °C with agitation.

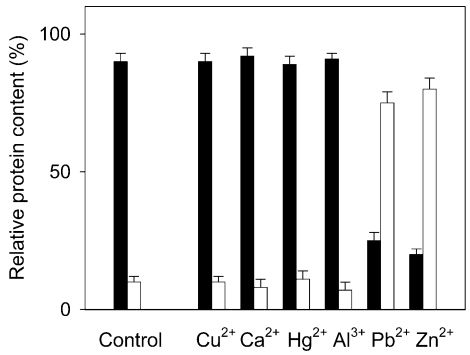

FIGURE 2: Zinc and lead induce aggregation and/or fibrillation of β-synuclein. Lowry analysis of soluble and insoluble products of β-synuclein incubation in the absence or presence of Cu^{2+}, Ca^{2+}, Al^{3+}, Hg^{2+}, Zn^{2+}, and Pb^{2+}. Black and white bars represent the relative protein concentration in the supernatant and pellet, respectively. The protein was incubated as described in the legend of Figure 1 for 60 h.

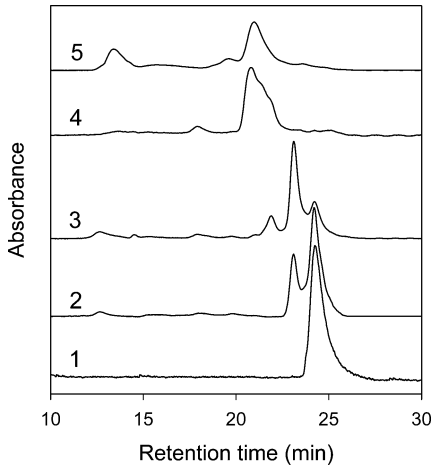

FIGURE 3: Incubation of β-synuclein leads to the formation of soluble oligomers. SEC HPLC analysis of the association state of β-synuclein before (trace 1) and after incubation (2) alone or after incubation in the presence of 500 μM Ca^{2+} (3), Al^{3+} (4), or Hg^{2+} (5). The protein was incubated at 37 °C for 60 h with agitation. Prior to chromatography, the solutions were filtered with a 0.1 μm Whatman Anodisc-13 filter. All measurements were carried out at 25 °C.

After incubation with Ca^{2+}, the population of the monomer decreases considerably, with a corresponding increase in the amount of dimer and higher-order oligomers. Profiles of β-synuclein incubated in the presence of Al^{3+} and Hg^{2+} do not show any peak corresponding to the monomer, and all the soluble protein is in the form of dimers and higher-order oligomers. Comparable data were observed for a sample incubated in the presence of Fe^{2+}. After incubation of β-synuclein in the presence of Zn^{2+}, Pb^{2+}, and Cu^{2+}, no soluble protein was detected by SEC. These observations confirm our conclusion that incubation with metals induces aggregation of β-synuclein either as insoluble aggregates (fibrils) or as soluble oligomers.

The effects of increasing concentrations of Zn^{2+} (Figure 4) and Pb^{2+} (Figure 5) on aggregation and fibrillation of β-synuclein were investigated. Both metals accelerate β-synuclein aggregation and fibrillation in a concentration-dependent manner, with low ion concentrations (<1 mM) favoring formation of soluble oligomers (see the inset of Figure 4A) and high metal concentrations (>1 mM) promoting fibrillation. Figures 4B and 5B show that the rates of the two key processes of β-synuclein fibrillation, nucleation and elongation, were both affected in a similar manner by an increase in the salt concentration. This means that Zn^{2+} and Pb^{2+} affect the formation of nuclei and the growth of β-synuclein fibrils in a correlated manner. The shape of the curves of rate against metal ion concentration suggests that Zn^{2+} and Pb^{2+} exhibit specific binding with dissociation constants in the vicinity of 2 mM.

The morphology of the fibrillar material formed by β-synuclein in the presence of these metals was analyzed by transmission electron microscopy (data not shown) and atomic force microscopy (Figure 6). Neither EM nor AFM of β-synuclein alone showed any fibrils or large aggregates. Instead, the incubation of β-synuclein alone leads to formation of spherical oligomers (Figure 6A). There appear to be two major populations differing in size by a factor of 2, with a few additional ones ~4 times as large. Fibrils of β-synuclein incubated in the presence of zinc appear to be very similar to fibrils of α-synuclein (*49*). Samples from incubations with Zn^{2+} and Pb^{2+} showed evidence of a mixture of oligomers and amyloid fibrils, whereas samples with alu-

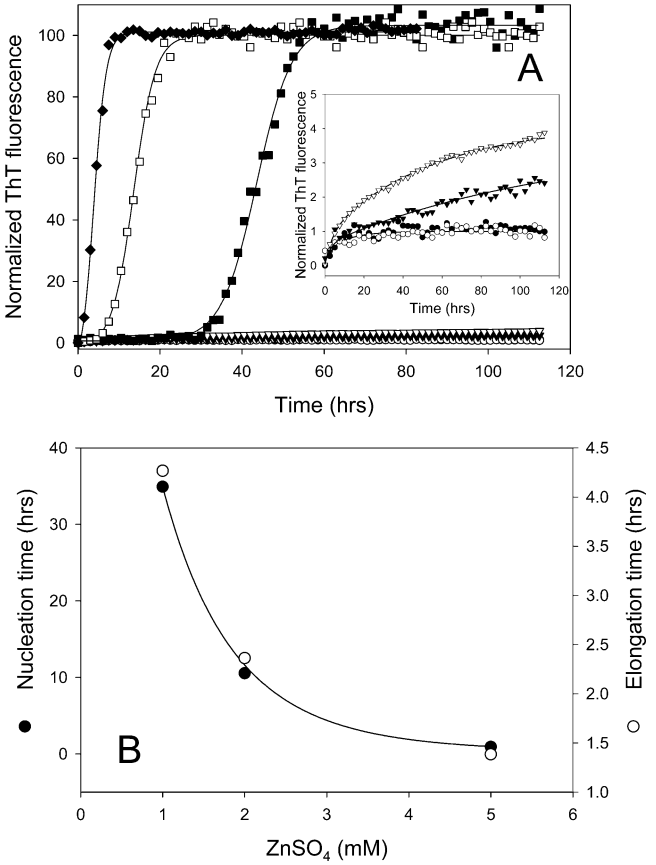

FIGURE 4: Effect of the concentration of Zn^{2+} in inducing fibrillation of β-synuclein. (A) Kinetics of β-synuclein fibrillation measured in the presence of different concentrations of Zn^{2+} monitored by ThT: control, no cation (●), 10 μM (○), 50 μM (▼), 500 μM (▽), 1 mM (■), 2 mM (□), and 5 mM (◆). The inset has an expanded ThT intensity scale. (B) Dependence of the nucleation (●) and elongation (○) times on the $ZnSO_4$ concentration.

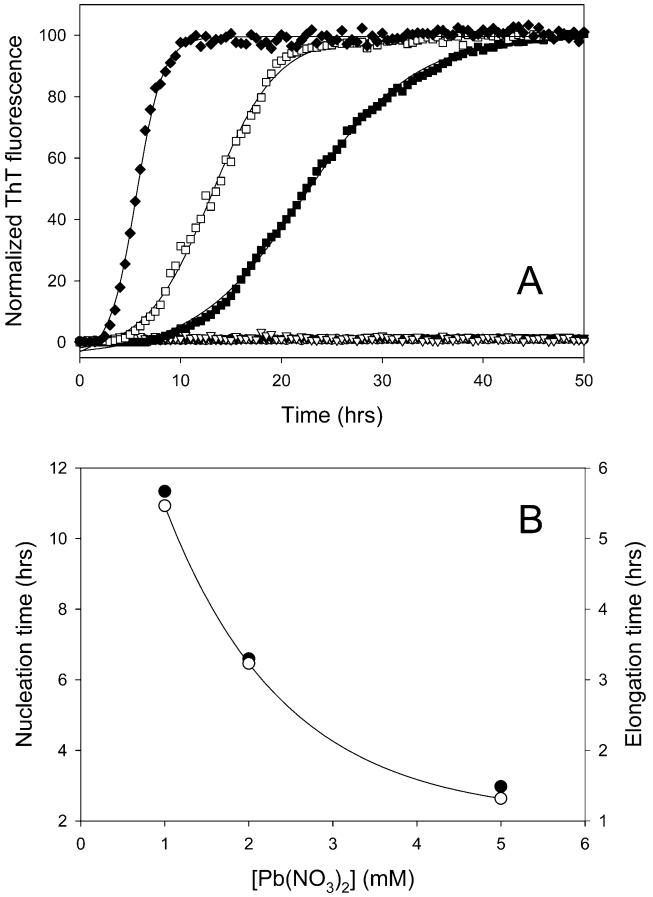

FIGURE 5: Effect of Pb^{2+} concentration in inducing fibrillation of β-synuclein. (A) Kinetics of β-synuclein fibrillation monitored by ThT fluorescence in the presence of different concentrations of Pb^{2+}: control, no cation (●), 10 μM (○), 50 μM (▼), 500 μM (▽), 1 mM (■), 2 mM (□), and 5 mM (◆). (B) Dependence of the nucleation (●) and elongation (○) times on $Pb(NO_3)_2$ concentration. The protein concentration was 70 μM. Measurements were carried out at 37 °C with agitation.

minum showed only nonfibrillar aggregates. Incubation in the presence of lead gave large amorphous deposits, consisting of aggregates of smaller (but still relatively large) amorphous globules. Incubation of α- and β-synuclein in a 1:1 mixture showed only spherical oligomers, as with β-synuclein alone, predominantly of two sizes, apparently slightly smaller than those of β-synuclein alone. However, in the presence of Pb^{2+}, the mixture gave nice fibrils (Figure 6 E), as well as clusters of fibrils, possibly combined with an amorphous core (Figure 6F) (the fibrils are most readily seen around the periphery, because of the loss of resolution due to the height of the cluster).

Effect of Metal Binding on β-Synuclein Conformation. To determine if the metals induced a conformational change in β-synuclein, we examined the effect of different metals on the secondary structure of β-synuclein. The far-UV CD spectrum of β-synuclein, shown in Figure 7, has the typical profile of a "natively" unfolded protein exhibiting slight β-structure. The addition of cations Zn^{2+}, Pb^{2+}, and Cu^{2+} led to a slight increase in the level of ordered secondary structure, while the addition of Al^{3+} had a much weaker effect. In previous studies of interaction of metal with natively unfolded α-synuclein, many metals showed similar structural changes, reflecting the cation-induced formation of a stable partially folded conformation (*37*, *46*). The stabilization of such a partially folded intermediate correlates with the accelerated fibrillation. Further structural analysis was performed using FTIR (amide I region) of β-synuclein, as shown in Figure 8. The FTIR spectrum of β-synuclein alone is typical of a substantially unfolded polypeptide chain, whereas interaction with metals leads to spectral changes, consistent with an increase in the level of ordered structure. The most evident change is the decrease in the magnitude of the band in the vicinity of 1655 cm^{-1}, which corresponds to the disordered conformation, accompanied by the appearance of a new band in the vicinity of 1620 cm^{-1}, which corresponds to β-sheet. These observations are further illustrated by Figure 8B, which depicts difference FTIR spectra and shows that the depletion in the magnitude of the signal in the vicinity of 1655 cm^{-1} occurs concomitantly with the increase in magnitude of the the signal around 1620 cm^{-1}. Thus, the presence of metals leads to increased amounts of β-structure in β-synuclein. We have previously observed increased β-sheet content in α-synuclein which has been attributed to formation of soluble oligomers (*16*, *24*, *50*−*52*). These data, along with those from CD, suggest that β-synuclein forms a partially folded intermediate in the presence of metals. This intermediate might be similar, but not necessarily identical, to the one formed by α-synuclein under conditions favoring fibrillation and oligomerization.

Accelerated Fibrillation of α-Synuclein/β-Synuclein Mixtures in the Presence of Zn^{2+} and Pb^{2+}. Since β-synuclein

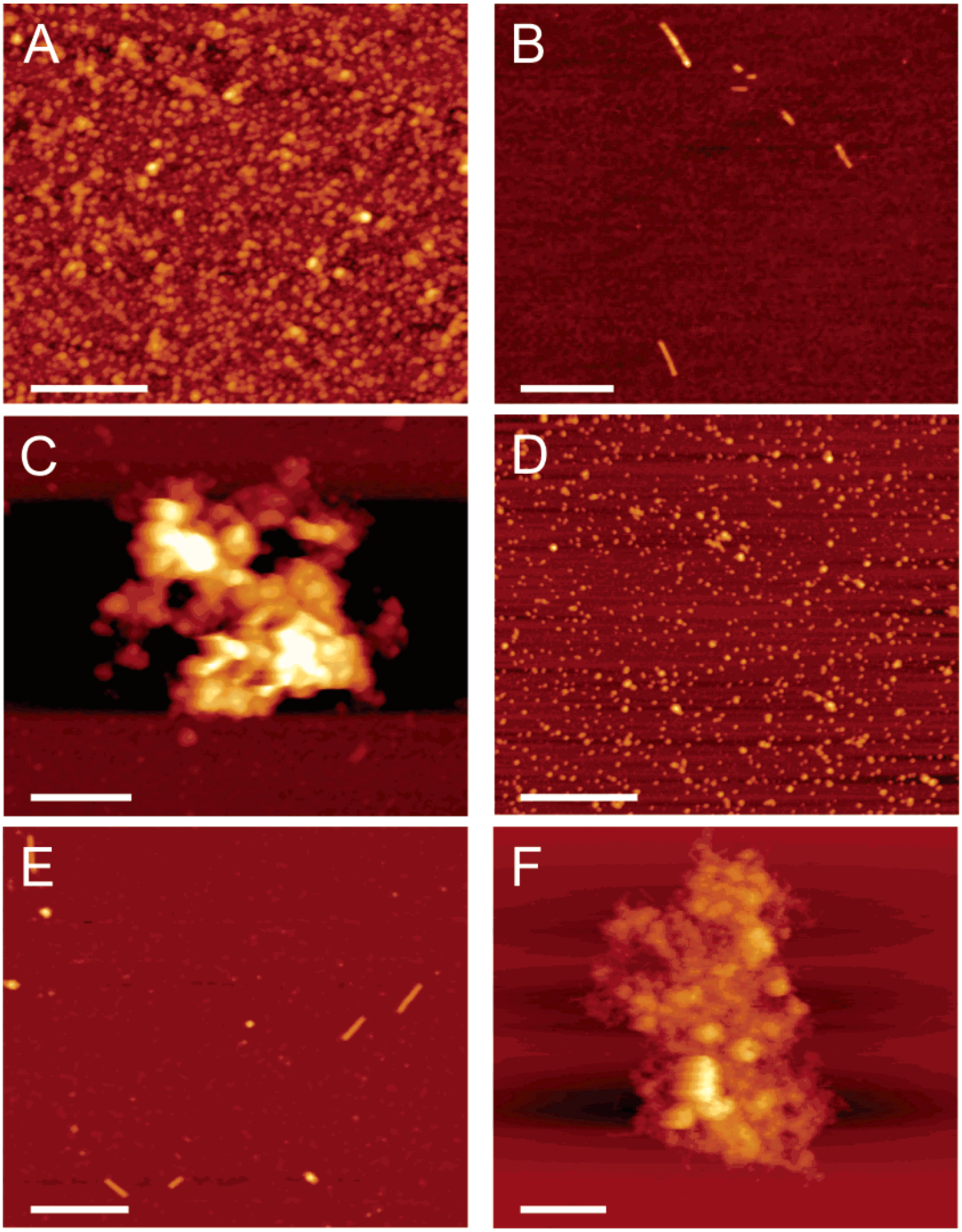

FIGURE 6: Aggregated forms of β-synuclein imaged by AFM: (A) β-synuclein alone showing spherical oligomers, (B) β-synuclein incubated in the presence of Zn^{2+} showing fibrils, (C) β-synuclein incubated in the presence of Pb^{2+} showing amorphous aggregates, (D) a 1:1 mixture of β- and α-synucleins, no metals, showing spherical oligomers, (E) a 1:1 mixture of β- and α-synucleins incubated in the presence of Pb^{2+} showing fibrils, and (F) a 1:1 mixture of β- and α-synucleins incubated in the presence of Pb^{2+} showing a large cluster of fibrils. The scale bar for all the panels except F is 0.5 μm; in panel F, the scale bar is 1 μm.

has been shown to inhibit the fibrillation of α-synuclein (*24*−*26*), it was of interest to determine if this inhibitory effect of β-synuclein can be overcome by metals. For purposes of comparison, Figure 9A shows the kinetics of fibrillation of α- and β-synuclein in the presence of 500 μM Zn^{2+} and Pb^{2+}. As shown in Figure 9B, the effect of Zn^{2+} and Pb^{2+} on the kinetics of a 1:1 stoichiometric mixture of β- and α-synuclein was to dramatically overcome the inhibitory effect of β-synuclein. For example, Zn^{2+} and Pb^{2+} decreased the lag times of a 1:1 mixture from ~115 h to 8.3 ± 0.5 and 19.9 ± 0.7 h, respectively. Similarly, these metals accelerated the elongation phase of fibril growth in a 1:1 mixture of β- and α-synucleins. In fact, Pb^{2+} and Zn^{2+} decreased the elongation time ($1/k_{obs}$) from 33.2 ± 0.9 h to 4.2 ± 0.5 and 3.4 ± 0.4 h, respectively. In addition, Figure 9B shows that the addition of Zn^{2+} induces very effective fibrillation in a 4:1 mixture of β- and α-synucleins (see the gray circles), i.e., under conditions when, in the absence of metal, the proteins do not fibrillate (*24*).

The kinetics shown in Figure 9 for the metal-catalyzed fibrillation of α-synuclein are slower than those shown in Figure 1 for comparable experiments, because different

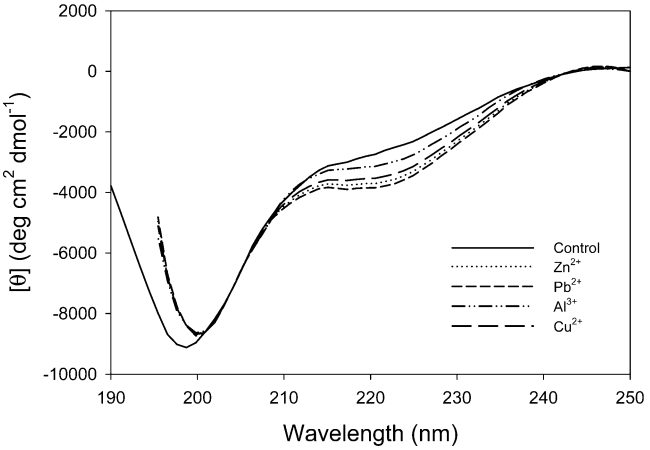

FIGURE 7: Metal-induced conformational changes in β-synuclein. Far-UV CD spectral changes in β-synuclein: β-synuclein alone (—) and β-synuclein in the presence of 500 μM $ZnSO_4$ (···), $Pb(NO_3)_2$ (- - -), $AlCl_3$ (—··—), and $CuCl_2$ (— — —). Measurements were performed at pH 7.5 and 23 °C. The protein concentration was 0.5 mg/mL.

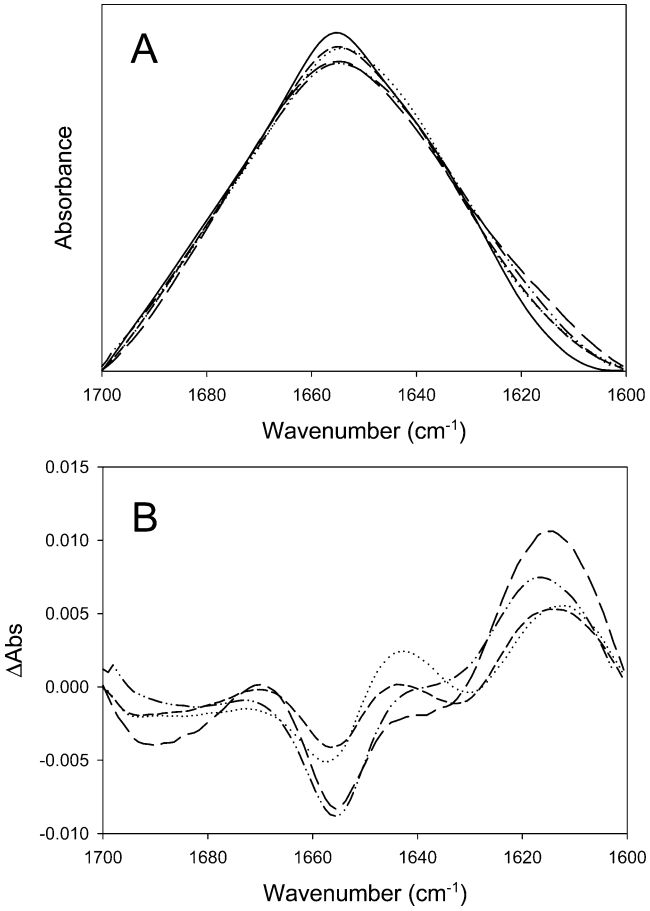

FIGURE 8: FTIR spectra of the amide I region of β-synuclein. β-Synuclein alone (—) or in the presence of 500 μM $ZnSO_4$ (···), $Pb(NO_3)_2$ (- - -), $AlCl_3$ (—··—), and $CuCl_2$ (— — —). (A) Amide I region spectra measured at pH 7.5 and 23 °C. The protein concentration was 0.5 mg/mL. (B) Difference FTIR spectra of the metal-induced changes in β-synuclein secondary structure: the spectrum of β-synuclein alone has been subtracted from the spectra in the presence of the metals. The difference spectra indicate the excess (positive values) or deficit of a particular structure (negative values) in the presence of the metals compared to their absence, and show loss of disordered structure (around 1655 cm^{-1}) and gain of β-structure (around 1620 cm^{-1}).

instruments were used. As is well-known, agitation is a major determinant in the rate of polypeptide fibrillation, and even though the rates of agitation may be set identically between instruments, there will inevitably be small differences, which can have a significant impact on the rate of fibrillation. To minimize this effect, all experiments in a given series (i.e., in a given figure) were done on the same 96-well plate at the same time; each sample was replicated four to five times, and the results were averaged. The variance in this case is small, 10−15%.

AFM analysis further verifies the formation of bona fide fibrils from this mixture incubated in the presence of either metal (Figure 6). The morphology of the β-synuclein fibrils is similar to that of α-synuclein. By comparison with Figure 9A, the results of the fibrillation of a 1:1 β-synuclein/α-synuclein mixture show that under these experimental conditions the mixture fibrillated almost as rapidly as the individual proteins in the presence of the metals. Fibril formation was also confirmed by EM (see the Supporting Information).

Other Factors Affecting Aggregation and Fibrillation of β-Synuclein in Vitro

Effect of Molecular Crowding and Metals. The intracellular environment is crowded with macromolecules whose concentration can reach 400 g/L. These intracellular solutes occupy as much as 20−30% of the total cellular volume, creating a crowded medium, with considerably restricted amounts of free water, but where, in general, no individual macromolecular species is present at a high concentration (*53*, *54*). This means that the inside of a cell is a very crowded environment, which is poorly modeled by dilute solutions. Since the volume occupied by these macromolecular solutes is unavailable to other molecules, this gives rise to excluded volume effects (*55*, *56*), which may have a large influence on the behavior of biological macromolecules and protein−protein interactions. Since macromolecular crowding accelerates the fibrillation of apolipoprotein C-II (*57*) and α-synuclein (*58*−*60*), we investigated the effect of macromolecular crowding agents on the fibrillation of β-synuclein. Figure 10 shows that the addition of 200 mg/mL PEG 10000, a polymer frequently used to model the effects of macromolecular crowding, does not result in the fibrillation of β-synuclein (at least within 120 h). However, the addition of PEG dramatically accelerated the fibrillation of β-synuclein in the presence of Zn^{2+} (see Figure 10). The data suggest that the crowding agent primarily affects the interaction of β-synuclein with the metal, stabilizing the resulting complex and, thus, the partially folded amyloidogenic intermediate, leading to the acceleration of β-synuclein fibrillation.

Effect of Glycosaminoglycans and Metals. There is abundant evidence that glycosaminoglycans are involved in the formation of the amyloid fibrils in a variety of human diseases (reviewed in refs *61* and *62*). Proteoglycans containing heparan sulfate, chondroitin sulfate, keratan sulfate, and/or dermatan sulfate have been found in all amyloid deposits (*63*). Certain GAGs (heparin and heparan sulfate) and other highly sulfated polymers (dextran sulfate) have been observed to significantly stimulate the rate of formation of α-synuclein fibrils in vitro (*64*). Figure 11 shows that, in contrast to that of α-synuclein, the aggregation behavior of

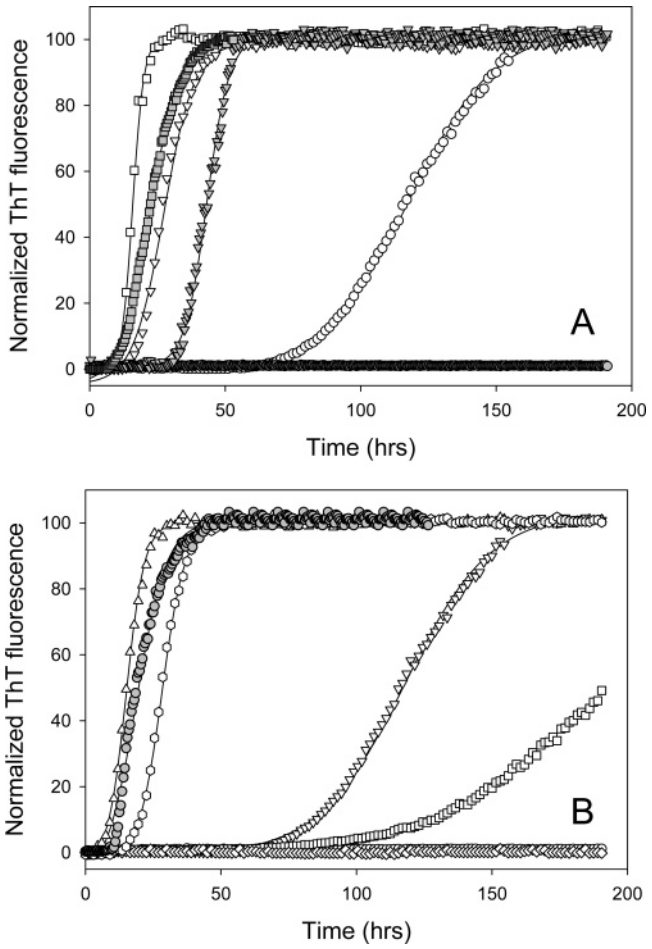

FIGURE 9: Effect of Zn^{2+} and Pb^{2+} on the fibrillation of α- and β-synucleins and their mixtures. (A) The fibrillation of the individual proteins was analyzed in the absence (circles) or presence of 5 mM Zn^{2+} (squares) or Pb^{2+} (inverted triangles). Data for α-synuclein and β-synuclein are depicted with white and gray symbols, respectively. (B) Fibrillation of α-synuclein (▽) and β-synuclein (○), and their 1:1 (□) or 4:1 (◇) mixtures incubated alone or in the presence of 2 mM Zn^{2+} (△) or Pb^{2+} (○). Gray circles show data for fibrillation of a 4:1 mixture of β- and α-synucleins in the presence of Zn^{2+}.

β-synuclein is not affected by heparin. However, the rate of fibrillation of β-synuclein with both heparin and metals, e.g., Cu^{2+} (cf. Figure 2), present was greatly accelerated.

Effect of Pesticides. Both epidemiological and clinical observations indicate that pesticides and herbicides are potential risk factors for PD (*27*, *30*, *65*). In vitro, certain pesticides significantly stimulate the rate of fibril formation when incubated with α-synuclein at pH 7.5 and 37 °C (*2*, *66*). The efficiency of a given pesticide to accelerate fibrillation was shown to be directly correlated with its ability to induce partial folding in α-synuclein (*2*, *24*, *66*, *67*). To determine if pesticides might trigger the aggregation of β-synuclein, we used ThT and SEC HPLC to investigate the effects of some pesticides on the association state of β-synuclein. The ThT assays (Figure 12A) show that pesticides differ considerably in their effectiveness in stimulating the aggregation and fibrillation of β-synuclein. The widely used pesticide rotenone induced rapid and efficient fibrillation of β-synuclein, whereas dieldrin and paraquat promoted rapid oligomerization of the protein, leading to amorphous aggregates. Figure 12B compares the

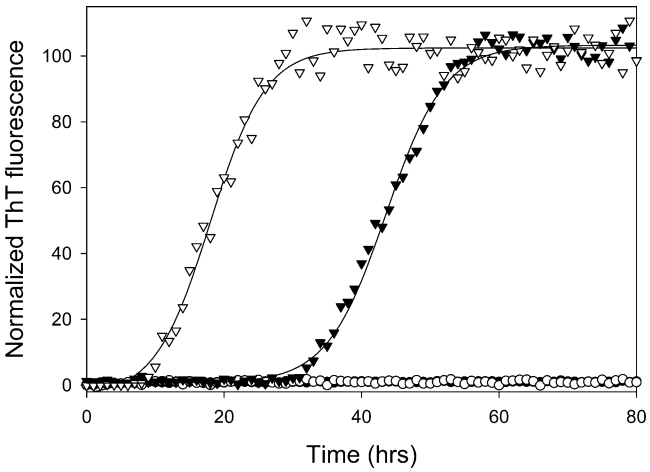

FIGURE 10: Effect of macromolecular crowding and metals on the fibrillation of β-synuclein. The symbols represent ThT fluorescence intensities determined experimentally, and the lines are fitted according to ref *39*: control, no cation or crowding agent (●), 200 mg/mL PEG 10000 (○), 1 mM Zn^{2+} (▼), 500 μM Zn^{2+} and 200 mg/mL PEG 10000 (▽). Data for β-synuclein alone or in the presence of PEG are superimposed. The protein concentration was 70 μM. Incubation was carried out at 37 °C with agitation.

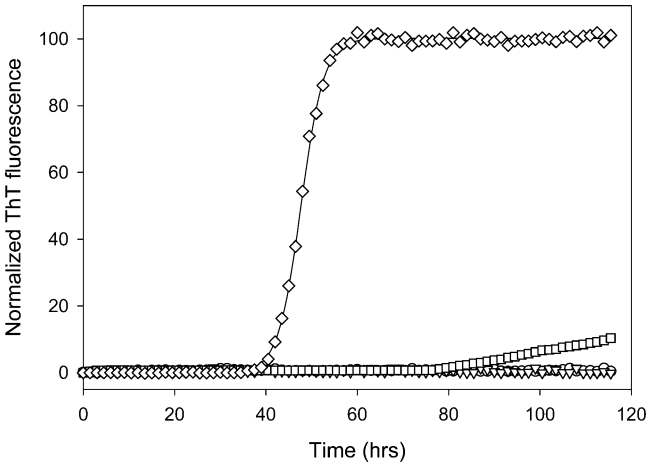

FIGURE 11: Effect of heparin (500 μg/mL) and Cu^{2+} (500 μM) on fibrillation of β-synuclein: control, β-synuclein in the absence of additives (○) and in the presence of heparin (▽), Cu^{2+} (□), or a mixture of heparin and Cu^{2+} (◇). Data for β-synuclein alone and for the protein in the presence of heparin are superimposed. The protein concentration was at 70 μM. Incubation was carried out at 37 °C with agitation.

SEC profile of nonincubated β-synuclein with those of the soluble fraction after incubation of β-synuclein for 60 h either alone or in the presence of several pesticides. Nonincubated β-synuclein elutes as a monomer, and the incubated protein alone elutes as a monomer or dimer. Incubation with paraquat, rotenone, or dieldrin led to loss of monomer and formation of oligomers of various sizes in the soluble fraction. Interestingly, the amount of soluble material was significantly decreased when β-synuclein was incubated with rotenone, indicating that incubation of β-synuclein with this pesticide induces effective aggregation and/or fibrillation.

Effect of Organic Solvents. The structural properties and aggregation and fibrillation propensities of α-synuclein in mixtures of water with alcohols with different lengths of aliphatic chain (methanol, ethanol, and propanol) and fluoro alcohols (trifluoroethanol, TFE, and hexafluoro-2-propanol, HFiP) have been analyzed (*50*). The structure-forming and

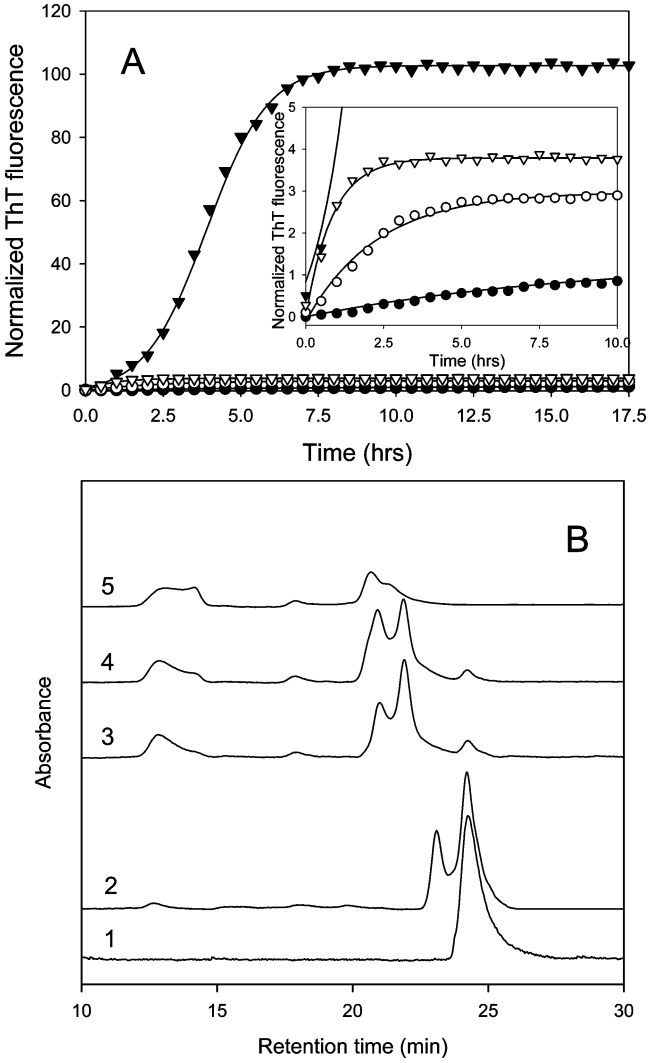

FIGURE 12: Effect of pesticides on the aggregation of β-synuclein. (A) Kinetics of aggregation as detected by changes in ThT fluorescence intensity: control, no pesticide (●), 200 μM paraquat (○), 200 μM dieldrin (▽), and 200 μM rotenone (▼). The inset depicts expanded data. (B) SEC HPLC analysis of the association state of β-synuclein before (trace 1) and after incubation alone (2) or in the presence of 200 μM paraquat (3), dieldrin (4), or rotenone (5). Samples were incubated at 37 °C for 60 h with agitation. Prior to measurements, solutions were filtered with a 0.1 μm filter. The SEC flow rate was 0.4 mL/min, monitored by the absorbance at 280 nm at 25 °C.

aggregation-promoting effects of solvents studied were characterized by a common first stage leading to the formation of a partially folded intermediate. Furthermore, under all the conditions in which the intermediate was populated, α-synuclein fibrillated significantly faster than in the absence of the solvent. High concentrations of simple alcohols and moderate concentrations of TFE induced β-structure-enriched species in α-synuclein, whereas high concentrations of fluoro alcohols gave rise to an α-helical conformation; with time, both α-helical and β-structural species formed insoluble aggregates (*50*). Figure 13A compares the effects of low concentrations of 2.5% HFiP and 10% TFE on the aggregation of β-synuclein, and shows that both solvents dramatically accelerated self-association of this protein. EM analysis revealed that the major form of the protein after the prolonged incubation in the presence of organic solvents is amorphous aggregates (data not shown). Interestingly, prior

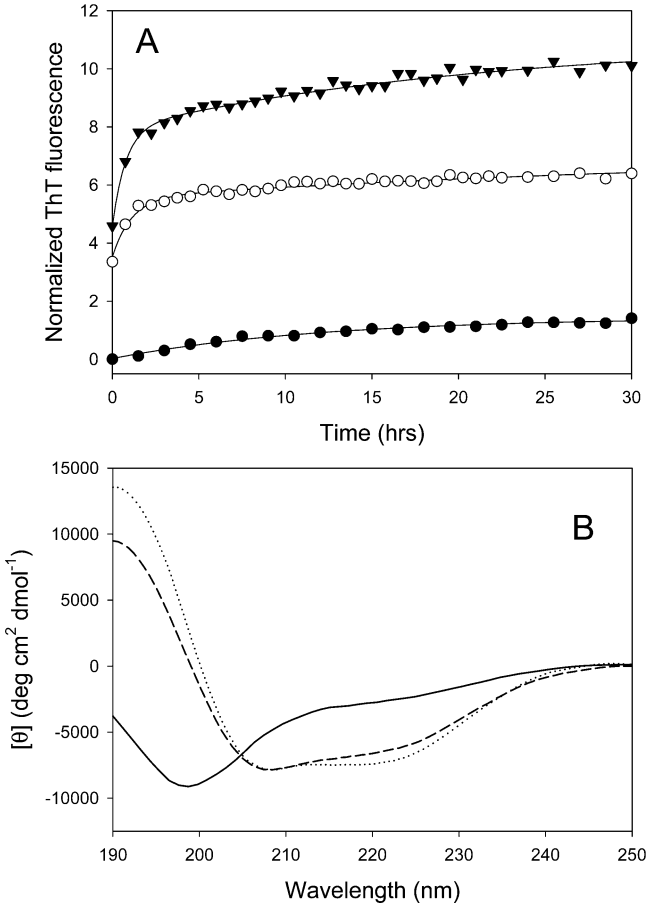

FIGURE 13: Effect of organic solvents on the aggregation (A) and secondary structure (B) of β-synuclein. (A) Kinetics of aggregation as monitored by ThT fluorescence intensity: control, no organic solvents (●), 2.5% HFiP (○), and 10% TFE (▼). The protein concentration was 70 μM. Samples were incubated at 37 °C with agitation. (B) Effect of organic solvents on the secondary structure of β-synuclein monitored by changes in far-UV CD spectra. Spectra were measured for β-synuclein alone (—) or in the presence of 2.5% HFiP (- - -) or 10% TFE (•••). Measurements were performed at pH 7.5 and 23 °C using a cell with a path length of 0.1 mm. The protein concentration was 0.5 mg/mL.

to aggregation, β-synuclein showed substantial α-helical conformation under these conditions (Figure 13B).

DISCUSSION

As noted, both α- and β-synuclein are abundant brain proteins (*21*). α-Synuclein has been shown to be implicated in the pathogenesis of several neurodegenerative diseases (*4*, *19*, *68*−*71*). Although β-synuclein has not been implicated in PD, abnormal accumulations of β-synuclein have been reported in axonal lesions in the brains of patients with PD, dementia with LB, and Hallervorden-Spatz syndrome (*23*, *72*), and in several neuronal cell types in the multiple-system atrophy brain (*73*) and in Pick bodies in the dentate gyrus in Pick's disease (*74*). Notably, β-synuclein is not found in Lewy bodies, although if these are protective, it does not automatically rule out a role for β-synuclein in PD.

It has been shown that β-synuclein is even more unfolded than α-synuclein, and its lack of fibrillation has been attributed to the 11 missing hydrophobic residues in the NAC region of the molecule (*24*). Furthermore, recent studies have shown that β-synuclein inhibits α-synuclein aggregation in vitro and in model in vivo experiments with transgenic mice

brain and transfected non-neuronal cells (*24*−*26*). These observations suggest that β-synuclein might regulate α-synuclein fibrillation in vivo, acting as a chaperone to minimize the aggregation of α-synuclein. Consequently, decreased levels of β-synuclein could act as one possible factor in the etiology of PD (*24*, *25*).

Although the exact cause of neurodegeneration in PD is unknown, a growing body of evidence suggests multiple causative pathways, with a dramatic role of several environmental factors (*1*, *2*, *27*, *36*, *75*, *76*). In accord with this hypothesis, it has been shown that metals (*2*, *37*) and pesticides and herbicides (*2*, *24*, *66*, *67*) can greatly accelerate the fibrillation of α-synuclein in vitro.

There has been substantial interest in the molecular mechanism of α-synuclein fibrillation. Transformation of this natively unfolded protein into a partially folded conformation represents an important primary step in fibrillation. Conditions that stabilize this amyloidogenic intermediate lead to acceleration of fibrillation in vitro and in vivo (*2*, *37*, *66*, *67*). A variety of different mechanisms have been suggested to account for the accelerating effects of different factors that stimulate α-synuclein fibrillation, including masking of the Coulombic charge−charge repulsion for metals (*2*, *37*), preferential binding of ligand to the partially folded intermediate for pesticides, specific solvent-induced stabilization of the amyloidogenic intermediate by solvents (*50*, *66*), and minimization of the electrostatic repulsion between negatively charged α-synuclein molecules and polycations (*38*, *77*). Factors that inhibit fibrillation of α-synuclein frequently do so by stabilizing off-pathway soluble oligomers. Interestingly, there are several similarities in the structural properties and aggregation behavior of β-synuclein and methionine-oxidized α-synuclein. Both β-synuclein and oxidized α-synuclein are more unfolded than α-synuclein (*24*, *46*). Neither β-synuclein nor methionine-oxidized human α-synuclein forms fibrils in vitro (*24*, *46*).

The results reported here show that conditions promoting accelerated fibril formation in α-synuclein also induce fibrillation and aggregation of "nonamyloidogenic" β-synuclein. This includes some metals (Zn^{2+}, Pb^{2+}, and Cu^{2+}) alone or in combination with crowding agents and GAGs, as well as pesticides (rotenone, paraquat, and dieldrin) and low concentrations of organic solvents. That β-synuclein can form fibrils under some conditions is, perhaps, not unexpected as there is a growing belief that the ability to fibrillate is a generic property of a polypeptide chain, and all proteins are potentially able to form amyloid fibrils under appropriate conditions (*78*−*80*). An interesting aspect of our findings is that these fibrillation-promoting conditions include the environmental pollutants (metals and pesticides) assumed to be involved into the pathogenesis of PD, and that they induce fibrillation and aggregation of β-synuclein, which has been assumed to be a benign counterpart of amyloidogenic α-synuclein. It is noteworthy that factors such as macromolecular crowding and GAGs do not promote fibrillation of β-synuclein on its own: it is only in combination with metals that these factors lead to very efficient fibrillation. Furthermore, in the presence of some metals, the "normal" inhibitory effect of β-synuclein on α-synuclein fibrillation is overcome, and mixtures of α- and β-synucleins exhibit rapid fibrillation, almost as fast, as that of α-synuclein incubated with these metals in the absence of β-synuclein.

It is of interest to enquire whether these in vitro observations may be relevant to the in vivo situation. A difficult question to answer is the available metal ion concentration in the appropriate regions of the brain. Total zinc levels in brain are on the order of 0.3 mM (*81*) and, for iron, as much as 1 mM (*82*). However, the available levels will be much lower, and thus considerably less than those used in the study presented here. Consequently, it is unlikely that β-synuclein fibrillation will occur in vivo. However, it is possible that the presence of combined high concentrations of metals and pesticides may weaken the putative inhibitory effect of β-synuclein on α-synuclein fibrillation.

There are two most likely mechanisms for the lack of fibrillation of β-synuclein: either lack of association to form the critical nucleus, due to the absence of the relatively hydrophobic residues in the NAC region, or the formation of stable oligomeric intermediates, which are sufficiently stable to resist fibrillation. The AFM data (Figure 6A) show that β-synuclein forms oligomers, indicating that the second explanation is correct. This, of course, then raises the question of why such stable oligomers should be formed. Probably there is a kinetic competition between the formation of different types of associated states of β-synuclein, in particular, those observed, and those that can lead to fibrils, and the former are formed much more rapidly. Presumably, in the case of α-synuclein, such a corresponding kinetic competition occurs, and the associated state (nucleus) leading to fibrils is formed more rapidly.

The simplest explanation for the effect of metals on β-synuclein fibrillation is that their presence overcomes electrostatic repulsions allowing formation of a partially folded intermediate that can associate and form fibrils. However, since only a limited number of metals were able to induce β-synuclein to form fibrils, there must be something specific to the properties of those metals. One possibility is that the nature of their coordination properties allows for intramolecular bridging between carboxylate side chains, bringing certain regions of the polypeptide chain into close contact, favoring collapse to the partially folded intermediate and subsequent association. Another factor is the kinetic competition between pathways leading to soluble oligomers, amorphous deposits, and fibrils. As seen in Figure 6, for example, it appears that with Pb^{2+} the fastest pathway is to amorphous deposits, whereas in contrast, with Zn^{2+} it is to fibrils.

In the case of the fibril-promoting effect of pesticides, the most likely explanation is that they preferentially bind to the amyloidogenic partially folded intermediate, due to their hydrophobicity. Again, the specific nature of the interaction between the pesticide and the conformation of the partially folded intermediate cam account for specificity.

ACKNOWLEDGMENT

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SUPPORTING INFORMATION AVAILABLE

Electron microscope images of β-synuclein deposits. This material is available free of charge via the Internet at http://pubs.acs.org.

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