Biophysical Properties of the Synucleins and Their Propensities to Fibrillate

INHIBITION OF \( \alpha \)-SYNUCLEIN ASSEMBLY BY \( \beta \)- AND \( \gamma \)-SYNUCLEINS*

The pathological hallmark of Parkinson’s disease is the presence of intracellular inclusions, Lewy bodies, and Lewy neurites, in the dopaminergic neurons of the substantia nigra and several other brain regions. Filamentous \( \alpha \)-synuclein is the major component of these deposits and its aggregation is believed to play an important role in Parkinson’s disease and several other neurodegenerative diseases. Two homologous proteins, \( \beta \)- and \( \gamma \)-synucleins, are also abundant in the brain. The synucleins are natively unfolded proteins. \( \beta \)-Synuclein, which lacks 11 central hydrophobic residues compared with its homologs, exhibited the properties of a random coil, whereas \( \alpha \)- and \( \gamma \)-synucleins were slightly more compact and structured. \( \gamma \)-Synuclein, unlike its homologs, formed a soluble oligomer at relatively low concentrations, which appears to be an off-fibrillation pathway species. Here we show that, although they have similar biophysical properties to \( \alpha \)-synuclein, \( \beta \)- and \( \gamma \)-synucleins inhibit \( \alpha \)-synuclein fibril formation. Complete inhibition of \( \alpha \)-synuclein fibrillation was observed at 4:1 molar excess of \( \beta \)- and \( \gamma \)-synucleins. No significant incorporation of \( \beta \)-synuclein into the fibrils was detected. The lack of fibrils formed by \( \beta \)-synuclein is most readily explained by the absence of a stretch of hydrophobic residues from the middle region of the protein. A model for the inhibition is proposed.

Synucleins belong to a family of closely related presynaptic proteins that are encoded by three distinct genes, described only in vertebrates (reviewed in Ref. 1). They are soluble, relatively small, intracellular and especially abundant in neural tissues. Synucleins are characterized by the presence of acidic stretches within the COOH-terminal region and a repetitive, degenerative amino acid motif KTKEGV throughout the first 87 residues, causing a variation in hydrophobicity with a strictly conserved periodicity of 11 amino acids (2). Such a periodicity is characteristic of the amphipathic helices of apolipoproteins (2). The name “synuclein” was first given to a protein that was isolated from the electric organ of Torpedo californica, where it was found in both nerve terminals and the nuclear envelope (3). The family of synuclein proteins includes: \( \alpha \)-synuclein, which was also called non-amyloid component precursor protein, or synelfin (2–5); \( \beta \)-synuclein, also referred to as phosphonpeptide protein 14 (5–7) and \( \gamma \)-synuclein, also known as breast cancer-specific gene 1 or persyn (8–10).

Human \( \alpha \)-synuclein is an abundant 140-residue brain protein. It has been estimated to account for as much as 1% of the total protein in soluble cytosolic brain fractions (11). \( \alpha \)-Synuclein is located in close vicinity to, and is loosely associated with, synaptic vesicles and thus may play a role in the release of neurotransmitters (5, 11). It belongs to the rapidly growing family of natively unfolded proteins, which have little or no ordered structure under physiological conditions (12, 13). Natively unfolded proteins are characterized by a unique combination of low overall hydrophobicity and large net charge (14).

Human \( \beta \)-synuclein is a 134-amino acid neuronal protein showing 78% identity to \( \alpha \)-synuclein. \( \alpha \)- And \( \beta \)-synucleins share a conserved COOH terminus, with three identically placed tyrosine residues. However, \( \beta \)-synuclein lacks 11 residues within the middle region (residues 73–83) (1). Like \( \alpha \)-synuclein, it is expressed predominantly in the brain (5, 15). The third member of the human synuclein family is the 127-amino acid \( \gamma \)-synuclein, which shares 60% similarity with \( \alpha \)-synuclein at the amino acid sequence level (1). It lacks the tyrosine-rich COOH-terminal signature of \( \alpha \)- and \( \beta \)-synucleins. \( \gamma \)-Synuclein is abundant in spinal cord and sensory ganglia (10). Interestingly, it is more widely distributed within the neuronal cytoplasm than \( \alpha \)- and \( \beta \)-synucleins, being present throughout cell bodies and axons (10). The presence of synucleins has also been established in other cell types: thus, \( \alpha \)-synuclein was found in platelets (16), \( \beta \)-synuclein in Sertoli cells of the testis (17), and \( \gamma \)-synuclein in the epidermis (18) and in metastatic breast cancer tissue (8).

Several observations have implicated \( \alpha \)-synuclein in the pathogenesis of Parkinson’s disease (PD)\(^1\) and several other neurodegenerative disorders. Clinically, PD is a movement disorder characterized by tremor, rigidity, and bradykinesia.

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† These authors contributed equally to the results of this paper.
‡ To whom correspondence should be addressed. Tel.: 831-459-2744; Fax: 831-459-2744; E-mail: enzyme@cats.ucsc.edu.

\(^1\) The abbreviations used are: PD, Parkinson’s disease; LB, Lewy bodies; LN, Lewy neurites; ThT, thioflavin T; FTIR, Fourier transform infrared; SAXS, small angle X-ray scattering; \( \theta _R \), Stokes radius.
These symptoms are attributed to the progressive loss of dopaminergic neurons from the substantia nigra. Surviving neurons contain cytosolic filamentous inclusions known as Lewy bodies (LBs) and Lewy neurites (LNs) (19, 20). In addition to the substantia nigra, LBs and LNs are also found in other brain regions, such as the dorsal motor nucleus of the vagus, the nucleus basalis of Meynert, and the locus coeruleus (20).

α-Synuclein has been shown to be a major fibrillar component of LBs and LNs (21, 22). Moreover, two different missense mutations in the α-synuclein gene, resulting in the A53T and A30P substitutions, have been identified in a small number of kindreds with autosomal dominantly inherited, early onset PD (23, 24). In addition, the expression of wild type (WT) α-synuclein in transgenic mice (25) or of WT, A30P, and A53T α-synuclein in transgenic flies (26) leads to motor deficits and neuronal inclusions reminiscent of PD. Abundant α-synuclein-positive LBs and LNs in the cerebral cortex are also neuropathological hallmarks of dementia with Lewy bodies, a common late-life dementia that is clinically similar to Alzheimer’s disease (21, 22, 27). Furthermore, LBs and aggregated α-synuclein have been detected in diffuse Lewy body disease (28, 29), some cases of Alzheimer’s disease (30) and Down’s syndrome (31), as well as some cases of Hallervorden-Spatz disease (32). Finally, α-synuclein is the major component of the filamentous neuronal and glial inclusions of multiple system atrophy (33).

It has recently been reported that in addition to α-synuclein-containing LBs and LNs, the development of PD and dementia with Lewy bodies is accompanied by the appearance of non-filamentous α-, β-, and γ-synuclein-positive accumulations in axon terminals of the hippocampus (34). This implicates β- and γ-synucleins, in addition to α-synuclein, in the progression of PD and dementia with Lewy bodies.

The aggregation of recombinant α-synuclein and its A30P and A53T mutants has been studied in vitro. It has been established that all three proteins, as well as carboxyl-terminal truncated forms thereof, readily assemble into filaments, with morphologies and staining characteristics similar to those of filaments extracted from disease-affected brain (13, 35–45). Fibrillation is believed to occur via a nucleation-dependent mechanism (40, 46) with the critical primary stage being the structural transformation of the protein from the unfolded conformation to a partially folded intermediate (13). Under these experimental conditions, β- and γ-synucleins failed to assemble into filaments (41, 43, 45).

Comparatively little is known about the structural properties of β- and γ-synucleins. The question of reciprocal effects of individual family members on the aggregation of other synucleins has not been considered as yet. Here, we compare the structural properties of α-synuclein and its propensity to form fibrils with those of β- and γ-synucleins. Data on the in vitro fibrillation of α-synuclein in the presence of β- or γ-synuclein are also presented.

**EXPERIMENTAL PROCEDURES**

**Materials**—Thioflavin T (ThT) and porcine intestinal heparin (Grade I-A, molecular weight 18,000) were obtained from Sigma. All other chemicals were of analytical grade from Fisher Chemicals. Human recombinant α-, β-, and γ-synucleins were expressed and purified as described (5, 22, 47).

**Fibril Formation**—Assay solutions contained proteins at a concentration of 70–280 μM (1–4.0 mg/ml) in 20 mM sodium phosphate, 0.1 mM NaCl, pH 7.5, buffer, containing 10 μM ThT. A volume of 100 μl of the mixture was pipetted into a well of a 96-well plate (white plastic, clear bottom) and a 1/8th-inch diameter Teflon sphere (McMaster-Carr, Los Angeles, CA) was added. Each sample was run in triplicate or quadruplicate. The plate was loaded into a fluorescence plate reader (Fluoroskan Ascent) and incubated at 37 °C with shaking at 600–900 rpm with a shaking diameter of 1 mm. The fluorescence was measured at 30-min intervals with excitation at 444 nm and emission at 485 nm, with a sampling time of 40 ms. Data from replicate wells were averaged before plotting fluorescence versus time.

**Analysis of Fibrillation Kinetics**—The kinetics of α-synuclein fibrillation are sigmoidal, defined by an initial lag phase, a subsequent growth phase in which ThT fluorescence increased, and a final equilibrium phase, where ThT fluorescence reached a plateau indicating the end of fibril formation. ThT fluorescence measurements were plotted as a function of time and fitted to a sigmoidal curve using the empirical approach described in Ref. 48.

**Circular Dichroism Measurements**—CD spectra were obtained with an AVIV 60DS spectrophotometer (Lakewood, NJ) using protein concentrations of ~35 μM. Spectra were recorded in a 0.01-cm path length cell from 250 to 190 nm with a step size of 0.5 nm, a bandwidth of 1.5 nm, and an averaging time of 10 s. For all spectra, an average of 5 scans was obtained. CD spectra of the appropriate buffers were recorded and subtracted from the protein spectra.

**FTIR Spectra**—Attenuated total reflection data were collected on a Nicolet 800SX FTIR spectrometer equipped with an MCT (mercurycadmium-telluride) detector. The IRE (72 × 10 × 6 mm, 45° germanium trapezoid) was held in a modified SPECAC out-of-compartment attenuated total reflection apparatus. The hydrated thin films were prepared as described previously (49, 50). Previous investigations have shown that comparable secondary structure analyses are obtained for native proteins from thin film attenuated total reflectance–FTIR as from transmission mode FTIR and x-ray crystallography (50, 51). Typically 1024 interferograms were co-added at 4 cm⁻¹ resolution. Data analysis was performed with GRAMS32 (Galactic Industries). Secondary structure content was determined from curve fitting to spectra deconvoluted using second derivatives and Fourier self-deconvolution to identify component band positions. Hydrated thin film samples were prepared by drying 50 μl of 70 μM α-synuclein solution on a ZnSe crystal with dry N₂. The IR spectra were collected, followed by Fourier transformation using the spectrum of the clean crystal as a background. Water (liquid and vapor) components were subtracted from the protein spectrum.

**Fluorescence Measurements**—Fluorescence measurements were performed in semimicro quartz cuvettes (Hellma) with a 1-cm excitation path length using a FluoroMax-2 spectrofluorometer (Instruments S.A., Inc., Jobin Yvon-Spex). The light source was a 150-W xenon lamp.

**Electron Microscopy**—Transmission electron micrographs were collected using a JEOL JEM-100B microscope operating with an accelerating voltage of 80 kV. Typical nominal magnifications ranged from ×20,000 to 50,000. Samples were deposited on Formvar-coated 300-mesh copper grids and negatively stained with 1% aqueous uranyl acetate.

**Small Angle X-ray Scattering Experiments**—Small angle x-ray scattering (SAXS) measurements were made using Beam Line 4-2 at Stanford Synchrotron Radiation Laboratory (52). X-ray energy was selected at 8980 eV (Cu edge) by a pair of Mo,B,C multilayer monochromator crystals (53). Scattering patterns were recorded by a linear position-sensitive proportional counter, which was filled with an 80% Xe, 20% CO₂ gas mixture. Scattering patterns were normalized by incident x-ray fluctuations, which were measured with a short length ion chamber before the sample. The sample-to-detector distance was calibrated to be 230 cm, using a cholesteryl myristate sample. To avoid radiation damage of the sample in SAXS measurements, the protein solution was continuously passed through a 1.3-mm path length observation flow cell with 25-μm mica windows. Background measurements were performed before and after each protein measurement and then averaged before being used for background subtraction. All SAXS measurements were performed at 23 ± 1 °C.

The radius of gyration (Rg) was calculated according to the Guinier approximation (54),

\[ \ln I(Q) = \ln I(0) - \frac{R_g^2 Q^2}{2} \]  
(Eq. 1)

where I(0) is the forward scattering amplitude, Q is the scattering vector given by \( Q = 4 \pi \sin \theta / \lambda \), where 2θ is the scattering angle, and \( \lambda \) is the wavelength of x-ray.

**Gel Filtration Experiments**—The hydrodynamic dimensions (Stokes radius, \( R_g \)) of synucleins were measured by size-exclusion chromatography. Size-exclusion measurements were performed on a Superose-12 column using a Amersham Bioscience FPLC chromatographic system. A set of globular proteins (Gel Filtration Chromatography Standards from Bio-Rad Laboratories) with known \( R_g \) values was used to create a calibration curve, 1000V₉₂ versus \( R_g \) (55–57).

Hydrodynamic dimensions of native and completely unfolded globu-
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RESULTS

\textbf{\alpha, \beta, and \gamma-Synucleins Are Natively Unfolded to Different Degrees}

Secondary Structure from Far-UV CD Spectra—Fig. 1A represents the far-UV CD spectra of human recombinant \(\alpha\)-, \(\beta\)-, and \(\gamma\)-synucleins measured at pH 7.5 and 3.0 at 20°C. At neutral pH, all three proteins show far-UV CD spectra typical of an essentially unfolded polypeptide chain. This includes the characteristic minimum in the vicinity of 196 nm and the absence of bands in the 210–230-nm region. Interestingly, \(\alpha\)- and \(\gamma\)-synucleins possess almost indistinguishable spectra, whereas the far UV-CD spectrum of \(\beta\)-synuclein shows a slightly increased degree of disorder, manifested by a small increase in negative ellipticity in the vicinity of 196 nm and somewhat lower intensity in the vicinity of 222 nm (see Table I). This suggestion was further confirmed by hydrodynamic studies (see below). As the pH is decreased changes were observed in the spectral shape for all three proteins. Fig. 1 shows that the decrease in the minimum at 196 nm is accompanied by an increase in negative intensity around 222 nm, reflecting pH-induced formation of secondary structure. All three proteins possess almost identical far-UV CD spectra at acidic pH (see also Table I). Importantly, the pH-induced changes in the far-UV CD spectra of the synucleins were completely reversible. Previously we have shown that the pH-induced increase in the structure of WT \(\alpha\)-synuclein represents an intramolecular process involving the formation of a partially folded intermediate, and not self-association (13). Thus, this also appears to be the situation for the \(\beta\)- and \(\gamma\)-synucleins.

Hydrodynamic Properties of the Synucleins from Size-exclusion Chromatography—Hydrodynamic techniques (such as size-exclusion/gel-filtration, viscometry, SAXS, SANS, sedimentation, dynamic and static light scattering) are useful in ascertaining the degree of compactness of a protein, and can distinguish between partially and fully unfolded states, since an increase in the hydrodynamic volume is associated with unfolding. Transformation into the molten globule state results in a \(-15\%\)–\(-20\%) increase in the hydrodynamic radius of globular proteins (57, 59, 60). The increase in hydrodynamic volume of less folded intermediates is even larger (60–62). The native and unfolded conformations of globular proteins possess very different molecular mass dependence of their hydrodynamic radii, \(R_g\) (57, 58). Thus, equilibrium conformations of globular proteins (native, molten globule, unfolded states etc.) can easily be discriminated by the degree of compactness of the polypeptide chain (57–62).

The hydrodynamic properties of the synucleins were studied under various experimental conditions by size exclusion chromatography and SAXS (see Table I). Chromatographic analysis confirmed the results of far-UV CD studies (see Fig. 1B) and showed that under conditions of neutral pH \(\beta\)-synuclein was slightly more extended than \(\alpha\)- and \(\gamma\)-synucleins, whereas in acidic solutions all three proteins possessed the same degree of compaction.

In fact, the data presented in Table I show that at neutral pH the hydrodynamic dimensions of \(\beta\)-synuclein are typical of a completely unfolded polypeptide chain, while \(\alpha\)- and \(\gamma\)-synucleins are more compact than expected for a random coil. This follows from comparison of values of the measured Stokes radius, \(R_o\), with those calculated for a completely unfolded polypeptide chain of the appropriate molecular mass (57). In the case of \(\beta\)-synuclein the experimentally determined value perfectly matched the calculated one, but the Stokes radii measured for \(\alpha\)- and \(\gamma\)-synucleins were notably lower than the corresponding calculated values (see Table I). Furthermore, in the case of \(\alpha\)-synuclein, this conclusion was confirmed by measurement of \(R_g\) in the presence of 8 M urea. Table I shows that under such conditions this protein behaves as a random coil.

Fig. 1B compares hydrodynamic volumes measured for synucleins under different experimental conditions with the results of far-UV CD analysis. Comparison of the degree of compactness (measured as the ratio \(V_h/V_n\), where \(V_h\) and \(V_n\) correspond to hydrodynamic volume calculated for given molecular mass and measured hydrodynamic volume, respectively) with the amount of secondary structure (determined as \(|\theta|_{222}/|\theta|_{222,\text{coil}}\)) showed an excellent correlation. These data demonstrate that under physiological conditions \(\beta\)-synuclein is more unfolded than the \(\alpha\)- and \(\gamma\)-synucleins.

Hydrodynamic Properties of the Synucleins from SAXS—SAXS is a very useful method for the investigation of conformation, shape, and dimensions of biopolymers in solution. Analysis of the scattering curves using the Guinier approxima-
The radius of gyration, \( R_g \), Scattering data in the form of Kratky plots provides information about the globularity (packing density) and conformation of a protein (54, 71): for a native globular protein this plot has a characteristic maximum, whereas unfolded and partially folded polypeptides have significantly different-shaped Kratky plots. On the other hand, \( I(0) \), the forward scattering amplitude, is proportional to \( n \rho_c^2 V_s^2 \), where \( n \) is the number of scatterers (protein molecules) in solution; \( \rho_c \) is the electron density difference between the scatterer and the solvent; and \( V \) is the volume of the scatterer. Thus, \( I(0) \) is proportional to the square of the molecular weight of the molecule (54). This means that \( I(0) \) for a pure \( N \)-mer will be \( N \)-fold that for the same number of monomers since each \( N \)-mer will scatter \( N^2 \) times as strongly as the monomer, but in this case the number of scattered particles (\( N \)-mers) will be \( N \) times less than that in the pure monomer sample.

Guinier analysis of the scattering data shows that at neutral \( pH \) the synucleins are characterized by rather different \( R_g \) values (Fig. 2A, Table I). The linear Guinier plots indicate that the solutions of these three proteins are homogeneous under the conditions studied. The radius of gyration of a completely unfolded (random coil) polypeptide, \( R_g^{\text{unc}} \), may be estimated from the corresponding Stokes radius, \( R_s^\text{S} \), using the relation \( R_g^{\text{unc}} / R_s^\text{S} = 1.51 \) (63). The observed \( R_g \) value for \( \alpha \)-synuclein at neutral \( pH \) (40 ± 1 Å) is smaller than that estimated for a random coil conformation for a protein of this size (52 Å), indicating that the natively unfolded conformation of this protein is more compact than that of a random coil. A decrease in \( pH \) is accompanied by further decrease in the hydrodynamic volume (cf. Table I and data presented in Ref. 13). On the other hand, the observed \( R_g \) value for \( \beta \)-synuclein (49 ± 1 Å) matches that expected for a completely unfolded polypeptide chain of this length (51 Å), which indicates the random coil conformation for \( \beta \)-synuclein at neutral \( pH \). Interestingly, \( \gamma \)-synuclein had a much larger \( R_g \) (61 ± 1 Å) under the conditions studied. This may be due to very significant asymmetry of this protein or because of its self-association. The latter explanation is more plausible, as the SAXS measurements were performed at relatively high protein concentrations (280 \( \mu \)g), and the size exclusion experiments, which were performed at low protein concentrations (7 \( \mu \)g), showed that \( \gamma \)-synuclein has hydrodynamic dimensions smaller than those of a random coil (see Table I). This suggests that \( \gamma \)-synuclein has a higher propensity to aggregate (forming soluble oligomers) than the other two synucleins. Analysis of the SAXS forward-scattering intensity values, \( I(0) \), confirmed this conclusion. In fact, Table I shows that for \( \gamma \)-synuclein the \( I(0) \) value is more than twice as large as that determined for \( \alpha \) and \( \beta \)-synucleins. Thus, \( \gamma \)-synuclein, is monomeric at low concentrations, but associates to form oligomers (probably dimers) when the protein concentration increases.

Analysis of the x-ray scattering in the form of a Kratky plot (Fig. 2A) shows that the \( \alpha \) and \( \beta \)-synucleins do not have a well developed globular structure (Fig. 2B). However, the profile of the Kratky plot for \( \gamma \)-synuclein shows very distinctive features. In particular, a characteristic maximum at low angles was observed, which may be indicative of some globular structure in the oligomer. Analogous behavior has been previously observed during the association of partially folded intermediates of staphylococcal nuclease (64, 65).

**TABLE I**

*Major structural characteristics of the human recombinant \( \alpha \)-, \( \beta \)-, and \( \gamma \)-synucleins*

| Protein, conditions | M | \( \text{RSN} \) (E) | \( \text{RSU} \) (E) | \( R_g \) (E) | \( R_s \) (E) | \( I(0) \) | \( M/M_c^a \) | \( M/M_c^b \) | [\( \theta \) in] deg cm\(^2\) dmol\(^{-1}\) |
|---------------------|---|----------------|----------------|-----------|-----------|---------|------------|------------|------------------|
| \( \alpha \)-synuclein | | | | | | | | | |
| pH 7.5, 100 mM NaCl | 14,460 ± 1 | 19.1 | 34.3 | 31.8 ± 0.4 | 41 ± 1 | 0.03152 ± 0.00008 | 1.000 | 1.000 | -2220 ± 50 |
| pH 3.0, 100 mM NaCl | 27.9 ± 0.4 | 30 ± 1 | 34.5 ± 0.4 | 38.5 ± 0.4 | 41 ± 1 | 0.03152 ± 0.00008 | 1.000 | 1.000 | -2860 ± 60 |
| 8 M urea | 1500 ± 90 | 1500 ± 90 | 1500 ± 90 | 1500 ± 90 | 1500 ± 90 | 1500 ± 90 | 1500 ± 90 | 1500 ± 90 | 1500 ± 90 |
| \( \beta \)-synuclein | | | | | | | | | |
| pH 7.5, 100 mM NaCl | 14,276.9 | 19.0 | 34.1 | 33.9 ± 0.4 | 49 ± 1 | 0.03072 ± 0.00007 | 0.975 | 0.987 | -1890 ± 50 |
| pH 3.0, 100 mM NaCl | 27.5 ± 0.4 | 27.5 ± 0.4 | 27.5 ± 0.4 | 27.5 ± 0.4 | 27.5 ± 0.4 | 27.5 ± 0.4 | 27.5 ± 0.4 | 27.5 ± 0.4 | 27.5 ± 0.4 |
| 8 M urea | 1500 ± 90 | 1500 ± 90 | 1500 ± 90 | 1500 ± 90 | 1500 ± 90 | 1500 ± 90 | 1500 ± 90 | 1500 ± 90 | 1500 ± 90 |
| \( \gamma \)-synuclein | | | | | | | | | |
| pH 7.5, 100 mM NaCl | 13,300.8 | 18.5 | 32.8 | 30.4 ± 0.4 | 61 ± 1 | 0.07874 ± 0.00015 | 2.498 | 0.920 | -2180 ± 50 |
| pH 3.0, 100 mM NaCl | 26.5 ± 0.4 | 26.5 ± 0.4 | 26.5 ± 0.4 | 26.5 ± 0.4 | 26.5 ± 0.4 | 26.5 ± 0.4 | 26.5 ± 0.4 | 26.5 ± 0.4 | 26.5 ± 0.4 |
| 8 M urea | 1500 ± 90 | 1500 ± 90 | 1500 ± 90 | 1500 ± 90 | 1500 ± 90 | 1500 ± 90 | 1500 ± 90 | 1500 ± 90 | 1500 ± 90 |

*Estimated from the Guinier region of SAXS data. These measurements have been performed in solution containing 4.0 mg/ml of a given protein.*

*Calculated from theoretical molecular masses (see second column of this table).*
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Comparison of the Fibrillation Kinetics of the Members of Synuclein Family

Thioflavin T is a fluorescent dye, which interacts with fibrils relatively specifically. This process is accompanied by a characteristic increase in fluorescence intensity in the vicinity of 480 nm (66, 67). Among members of the synuclein family only α-synuclein formed fibrils at relatively low (50 μM) protein concentrations. However, γ-synuclein did form fibrils when the protein concentration was increased 5-fold (250 μM). In contrast, β-synuclein did not form fibrils, even at 500 μM. Using ThT fluorescence, Fig. 4 compares the fibrillation patterns of α-, β-, and γ-synucleins under different experimental conditions. These measurements were performed at different protein concentrations (70, 500, and 250 μM for α-, β-, and γ-synucleins, respectively) to have comparable time scales for the different synucleins. The rates of fibrillation for α- and γ-synuclein were concentration dependent, the rate of fibrillation increasing with increasing concentration, as previously reported (68). In agreement with earlier studies (43), at neutral pH, α-synuclein formed fibrils faster than γ-synuclein, whereas β-synuclein did not form fibrils within a time scale of several weeks. The fact that γ-synuclein self-associates to form oligomers with globular structure and enhanced β-sheet content, yet fibrillates more slowly than α-synuclein, suggests that these oligomers may be off the fibrillation pathway. This also may explain the lower ThT signal observed for γ-synuclein in comparison with α-synuclein (cf. Fig. 4, A and C).

Previously, we have shown that the fibrillation rate of α-synuclein is enhanced by a decrease in pH (13) or by the addition of heparin (or other sulfated glycosaminoglycans) to the protein solution at neutral pH (69). Fig. 4A shows that a decrease in pH accelerates fibrillation more strongly than the addition of heparin. The data presented in Fig. 4C are consistent with the conclusion that these factors affect α- and γ-synuclein fibrillations in a similar way. Interestingly, β-synuclein remained soluble for several weeks under all conditions studied (high protein concentration, low pH, or the presence of heparin).

Inhibition of α-Synuclein Fibrillation in the Presence of β- and γ-Synucleins

Fig. 5 shows the effects of β- and γ-synucleins on the fibrillation kinetics of α-synuclein. The addition of either β-synuclein or γ-synuclein in a 1:1 molar ratio to 70 μM α-synuclein solution substantially reduced the fibrillation rate and increased the duration of the lag-time (see squares in Fig. 5). A further increase in the relative concentration of β- or γ-synuclein (2:1 molar ratio) led to additional inhibition of α-synuclein fibrillation (see triangles in Fig. 5). Finally, with a 4:1 molar excess of β- over α-synuclein, very few fibrils formed over a period of weeks. Fig. 6 illustrates that the capacity to inhibit α-synuclein fibrillation is rather specific for β- and γ-synucleins; thus, the addition of hen egg white lysozyme to α-synuclein solution in a 2:1 molar ratio led to enhancement, rather than inhibition, of synuclein fibrillation.

To shed more light on the mechanism of inhibition of α-synuclein fibrillation by β-synuclein, the possible incorporation of β-synuclein into the α-synuclein fibrils was analyzed by mass spectrometry. α-Synuclein fibrils were grown for 1 week in a 2-fold molar excess of β-synuclein, isolated by centrifugation, washed twice with distilled water, dissolved in alkaline solution, and subjected to MS analysis. The results showed a single component of molecular mass 14,460 Da, which corresponds to the calculated mass of recombinant human α-synuclein.

**Fig. 3.** Secondary structure analysis of α-synuclein (A), β-synuclein (B), and γ-synuclein (C) by FTIR. FTIR spectra of the amide I region were measured at pH 7.5 (solid line). Curve fit spectra are present by dashed lines. The major β-structure bands are in the 1620 to 1640 cm⁻¹ region.

Secondary Structure Analysis by FTIR—FTIR is much more sensitive to β-structure than CD, although higher concentrations of sample are required in the FTIR experiments. Fig. 3 represents the FTIR (amide I region) spectra measured for the three synucleins at pH 7.5. The FTIR spectra of α- and β-synucleins are typical of a substantially unfolded polypeptide chain, whereas the spectrum of γ-synuclein shows significant differences, indicative of increased ordered structure. The most evident change is the appearance of a new band in the vicinity of 1626 cm⁻¹, which corresponds to β-sheet. This means that in contrast to the α- and β-proteins, γ-synuclein contains significant amounts of β-structure under these conditions. This is attributed to association of this protein under the higher protein concentrations used in the FTIR experiments, compared with circular dichroism.

Deconvolution (FSD and second derivative) of the FTIR spectra, followed by curve fitting, permitted quantitative analysis of the secondary structure content in α-, β-, and γ-synucleins. These results are summarized in Table II. The β-structure content of the synucleins increases in the following order: β < α < γ. The much higher contribution of β-structure in γ-synuclein is attributed to its self-association under the concentrations used.

This observation correlates with the results of far-UV CD and SAXS analysis. In fact, regardless of the concentration, β-synuclein had the properties expected of a random coil conformation, whereas α-synuclein showed the properties of a slightly collapsed unfolded conformation. γ-Synuclein, although unfolded in its monomeric form, associated at protein concentrations ≥70 μM, leading to the appearance of β-structure and some globular character.
The morphologies of fibrils of \( \alpha \)-synuclein were essentially indistinguishable from those of \( \beta \)-synuclein by electron microscopy (Fig. 7). In the presence of heparin, \( \alpha \)-synuclein formed long fibrils. However, in the absence of heparin, substantial amounts of amorphous aggregates were also observed. For \( \beta \)-synuclein only amorphous precipitates were observed. No morphological differences were detected between \( \alpha \)-synuclein fibrils grown in the absence or presence of \( \beta \)- or \( \gamma \)-synuclein. More amorphous aggregates were observed when two synucleins were incubated together than when \( \alpha \)-synuclein was incubated by itself (Fig. 7).

**DISCUSSION**

The pathological hallmark of PD is the presence of intracellular inclusions, Lewy bodies, and Lewy neurites, in the dopaminergic neurons of substantia nigra and several other brain regions. Filamentous \( \alpha \)-synuclein has been shown to be the major component of these deposits (21, 22). This observation, in conjunction with the identification of mutations in the \( \alpha \)-synuclein gene in early onset cases of familial PD (23, 24), strongly suggest that the aggregation of \( \alpha \)-synuclein plays a

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**TABLE II**

**Secondary structure analysis of human \( \alpha \)-, \( \beta \)-, and \( \gamma \)-synucleins determined by FTIR**

| Structural assignment | \( \alpha \)-Synuclein | % | \( \beta \)-Synuclein | % | \( \gamma \)-Synuclein | % |
|----------------------|------------------------|---|----------------------|---|----------------------|---|
| Wavenumber \( \text{cm}^{-1} \) | Turn 1692 1.4 | Turn 1691 1.8 | Turn 1688 3.9 |
| Loops 1664 16.4 | Loops 1678 12.5 | Loops 1663 22.6 |
| Loops/disordered 1651 27.1 | Loops/disordered 1649 29.1 | Loops/disordered 1652 23.1 |
| Disordered/extended* 1637 28.7 | Disordered/extended* 1636 26.0 | Disordered/extended* 1640 19.2 |
| \( \beta \)-Sheet 1627 13.8 | \( \beta \)-Sheet 1625 8.0 | \( \beta \)-Sheet 1623 31.9 |

* The assignment of this band is uncertain, it may also reflect disordered secondary structure, or a combination of extended and disordered.

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**Fig. 4.** Kinetics of fibrillation of \( \alpha \)-synuclein (A), \( \beta \)-synuclein (B), and \( \gamma \)-synuclein (C) under different experimental conditions were monitored by the characteristic enhancement of thioflavin T fluorescence intensity. Measurements were performed at 70, 500, and 250 \( \mu \)M for \( \alpha \)-, \( \beta \)-, and \( \gamma \)-synuclein, respectively, to have comparable time scales for the different proteins. Propensities to fibrillate were studied at pH 7.5 (black circles), pH 3.0 (open circles), and pH 7.5 in the presence of 75 \( \mu \)g/ml heparin (black triangles). Measurements were performed at 37 °C as described under “Experimental Procedures.”

**Fig. 5.** Inhibition of \( \alpha \)-synuclein fibrillation in the presence of \( \beta \)-synuclein (A) or \( \gamma \)-synuclein (B). The fibrillation kinetics were studied for \( \alpha \)-synuclein (70 \( \mu \)M) in the absence (circles) or presence of either \( \beta \)-synuclein or \( \gamma \)-synuclein in 1:1 (squares), 1:2 (triangles) and 1:4 molar ratios (diamonds).

**Fig. 6.** Fibrillation kinetics of \( \alpha \)-synuclein (35 \( \mu \)M) in the absence (black circles) or presence of hen egg white lysozyme in a 2:1 molar ratio (open circles).

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**Morphology of Synuclein Fibrils**

The morphologies of fibrils of \( \gamma \)-synuclein were essentially indistinguishable from those of \( \alpha \)-synuclein by electron microscopy (Fig. 7). In the presence of heparin, \( \gamma \)-synuclein formed long fibrils. However, in the absence of heparin, substantial amounts of amorphous aggregates were also observed. For \( \beta \)-synuclein only amorphous precipitates were observed. No morphological differences were detected between \( \alpha \)-synuclein fibrils grown in the absence or presence of \( \beta \)- or \( \gamma \)-synuclein. More amorphous aggregates were observed when two synucleins were incubated together than when \( \alpha \)-synuclein was incubated by itself (Fig. 7).
critical role in PD and related neurodegenerative diseases. Although the etiology of idiopathic PD is unknown, it is most likely to be multifactorial. One possibility is that the feature common to the various different processes that can lead to PD is the aggregation of α-synuclein. It could be triggered by a range of factors, both environmental and endogenous. Our results suggest that the cellular levels of β- and γ-synucleins could constitute such an endogenous factor.

Synucleins are abundant brain proteins of unknown function, with relatively well conserved NH₂-terminal sequences. Major differences between them are that β-synuclein lacks 11 residues (between 73 and 83) from the hydrophobic repeat region of α-synuclein and that γ-synuclein is significantly shorter than the other two, but that it retains the central hydrophobic sequence (Fig. 8) (1).

Investigation of the hydrodynamic size and secondary structure characteristics of the synucleins reveals that β-synuclein is both somewhat more unfolded and less structured than the other synucleins. In fact, its properties correspond to those expected for a random coil, whereas both α- and γ-synucleins are slightly more compact and structured. The latter is especially apparent in the Kratky plots and the hydrodynamic radii. These properties to those of other synucleins. In fact, its properties correspond to those of other synucleins. The latter is especially apparent in the Kratky plots and the hydrodynamic radii. These properties correspond to those of other synucleins.

It has previously been shown that α-synuclein forms a partially folded intermediate that is maximally stabilized at pH 3 (13). Presumably, this species is in equilibrium with the naturally unfolded conformation at pH 7.5, but present at only low levels. This partially folded conformation has also been shown to be a critical intermediate in the fibrillation pathway (13). The biophysical properties of the partially folded intermediate of α-synuclein observed at pH 3 are similar to those of β- and γ-synucleins, indicating that they also adopt similar intermediate conformations. We have previously shown that the build-up of this intermediate at pH 3 is due to decreased charge-charge repulsion, resulting from the neutralization of carboxylates (13).

The fibrillation of α- and γ-synucleins exhibits the classical behavior of a nucleated polymerization process, in which there is an initial lag phase (during which the nuclei are formed), followed by the exponential growth of the fibrils. Monitoring fibril formation using ThT demonstrated that, regardless of the pH, α-synuclein formed fibrils most rapidly, followed by γ-synuclein, about 20-fold more slowly, whereas β-synuclein did not form fibrils under the conditions used for a period of weeks. The latter observation is most readily explained by the absence of a stretch of 11 amino acids from the repeat region of β-synuclein (38, 43). Since this hydrophobic region is implicated in α-synuclein aggregation, it is not surprising that the propensity for β-synuclein to aggregate is substantially less than that of α-synuclein (38, 43, 44). Thus, members of the synuclein family show different degrees of unfolding and distinct propensities to form soluble oligomers and fibrils, with α-synuclein being the most prone to fibrillate. We also attribute the more unfolded state of β-synuclein to the missing hydrophobic residues of the repeat region of the molecule. It follows that a major factor in the fibrillation of α-synuclein involves interactions between the hydrophobic central region of the molecule. Since the partially folded intermediate of β-synuclein observed at low pH has apparently identical conformational properties to those of α-synuclein, this suggests that the decreased hydrophobicity of the former leads to a decreased propensity to associate and hence to fibrillate.

Since β-synuclein is present in comparable amounts in human brain to α-synuclein (5), it was of interest to determine the effect of the presence of β-synuclein in stoichiometric amounts on the fibrillation of α-synuclein. Whereas substoichiometric levels of β-synuclein had negligible effects on the kinetics of α-synuclein fibrillation, equivalent or excess concentrations of β-synuclein led to significant inhibition of α-synuclein fibril formation. Similarly, the addition of excess stoichiometries of β-synuclein led to inhibition of α-synuclein fibrillation. These observations indicate that β- and γ-synucleins may act as regulators of α-synuclein fibrillation in vivo, in effect acting as chaperones to minimize the aggregation of α-synuclein. In particular, excess stoichiometries of β-synuclein would be expected

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**Fig. 7.** Electron micrographs of synuclein aggregates. a, α-synuclein fibrils grown at pH 7.5. b, β-synuclein amorphous aggregates grown at pH 7.5. c, γ-synuclein fibrils grown in the presence of heparin at pH 7.5. d, fibrils of α-synuclein grown in the presence of a 2-fold excess of β-synuclein. e, α-synuclein fibrils grown in the presence of a 2-fold excess of γ-synuclein. g, γ-synuclein fibrils grown at pH 3.

**Fig. 8.** Optimally aligned sequences of α-, β-, and γ-synucleins. The total number of residues in each protein is 140, 134, and 127, respectively. Gaps in the β and γ sequences relative to α-synuclein are indicated with dashes. Residues in the β- and γ-synucleins different from those in the α-sequence are highlighted.
to limit the amount of fibrillation. Consequently, one possible factor in the etiology of PD may be a decrease in the levels of β- or γ-synuclein.

We believe that the most likely mechanism for the inhibitory effect of β-synuclein involves the incorporation of β- or γ-synuclein into oligomeric intermediates, leading to their stabilization. Most likely the β- or γ-synuclein interacts with a transient oligomeric intermediate, normally on the fibrillation pathway of α-synuclein, and results in stabilizing the intermediate and blocking its conversion into fibrils. This hypothesis is supported by the observations that the final ThT signals decrease in the presence of increasing amounts of β- or γ-synuclein, indicating fewer fibrils formed in the presence of the β- or γ-synucleins, as well as the EM images which show that the presence of β- or γ-synuclein leads to increasing amounts of amorphous deposits, and no obvious decrease in length of the α-synuclein fibrils. Similarly, the mass spectrometry results are consistent with this model. According to this hypothesis we conclude that the presence of β- or γ-synuclein leads to stabilization of an oligomeric intermediate, which leads to amorphous rather than fibrillar aggregates. The lack of detectable differences in the fibrils grown either from α-synuclein or from α-synuclein in the presence of β- or γ-synuclein, is also consistent with the inhibitory synucleins having a minimal impact on fibril structure.

An alternative model involves the incorporation of a molecule of β-synuclein (or γ-synuclein) into the growing end of an α-synuclein fibril, leading to “capping off” of the fibril. The general similarity in structure between α-synuclein and β- and γ-synuclein is expected to be consistent with both models. However, if the central hydrophobic region in α-synuclein is critical for fibrillation, especially for fibril elongation, then the fact that both β- and γ-synuclein inhibit fibrillation suggests that fibril capping is not the mechanism of inhibition.

The fact that both β- and γ-synucleins inhibit fibrillation of α-synuclein also indicates that it is not just the central hydrophobic region of α-synuclein which is involved in synuclein self-association, since this region, which is absent in β-synuclein, is present in γ-synuclein. Thus there must be other regions of the protein that are also critical for association, and probably fibril formation.

After submission of this manuscript, a study was published reporting that β-synuclein inhibited the aggregation of α-synuclein in transgenic mouse brain and in transfected non-neuronal cells (70). A similar effect was reported in vitro, when recombinant human α-synuclein was incubated at 65 °C and its aggregation monitored by SDS-PAGE and immunoblotting. Thus, our in vitro results, demonstrating inhibition of α-synuclein fibrillation by small molar excesses of β- and γ-synucleins is clearly relevant to the in vivo situation. There are several important consequences from these findings. First, they suggest that β- and γ-synucleins may regulate α-synuclein fibrillation in vivo, and thus factors that lead to a drop in their levels could potentially predispose to Parkinson’s disease. Second, they suggest that elevated levels of β- and γ-synucleins could be protective (see caveat below). Finally, the combined results suggest that the in vitro system will be valuable for testing mechanistic and other aspects of the interactions among the synucleins. With respect to the potential of β- and γ-synucleins to inhibit α-synuclein fibrillation in vivo, it is important to note that the neurotoxic species is unknown, and could be an oligomeric intermediate such as that stabilized by the presence of β- and γ-synuclein. If this were the case, then elevated levels of β- and γ-synuclein would be undesirable.
Synucleins and Their Propensities to Fibrillate

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