The Association of α-Synuclein with Membranes Affects Bilayer Structure, Stability, and Fibril Formation*

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The aggregation of α-synuclein is believed to be a critical factor in the etiology of Parkinson’s disease. α-Synuclein is an abundant neuronal protein of unknown function, which is enriched in the presynaptic terminals of neurons. Although α-synuclein is found predominantly in the cytosolic fractions, membrane-bound α-synuclein has been suggested to play an important role in fibril formation. The effects of α-synuclein on lipid bilayers of different compositions were determined using fluorescent environment-specific probes located at various depths. α-Synuclein-membrane interactions were found to affect both protein and membrane properties. Our results indicate that in addition to electrostatic interactions, hydrophobic interactions are important in the association of the protein with the bilayer, and lead to disruption of the membrane. The latter was observed by atomic force microscopy and fluorescent dye leakage from vesicles. The kinetics of α-synuclein fibril formation were significantly affected by the protein association and subsequent membrane disruption, and reflected the conformation of α-synuclein. The ability of α-synuclein to disrupt membranes correlated with the binding affinity of α-synuclein for the particular membrane composition, and to the induced helical conformation of α-synuclein. Protofibrillar or fibrillar α-synuclein caused a much more rapid destruction of the membrane than soluble monomeric α-synuclein, indicating that protofibrils (oligomers) or fibrils are likely to be significantly neurotoxic.

Parkinson’s disease (PD)† is the second most common neurodegenerative disease in the United States, currently affecting as many as 1.5 million individuals. The two pathological hallmarks of PD are the loss of dopaminergic neurons in the substantia nigra region of the brain, and the presence of intracellular inclusions, Lewy bodies, and Lewy neurites. The cause of Parkinson’s disease is unknown. Recent observations indicate that PD is a protein deposition disease involving the aggregation of α-synuclein. The evidence includes the discovery that α-synuclein is the major fibrillar protein in Lewy bodies (1), and the finding of mutant α-synucleins (A53T (2) and A30P (3)) in rare cases of familial early-onset Parkinson’s disease.

Substantial evidence supports the hypothesis that “pathological” or abnormal aggregation (4) arises from a key partially folded intermediate precursor. Such intermediates have sizable non-polar patches (i.e. contiguous hydrophobic side-chains) on their surface, which lead to hydrophobic interactions between molecules, resulting in specific intermolecular interactions and aggregation. These hydrophobic patches are absent in the fully unfolded state. Factors that increase the concentration of such intermediates will favor aggregation. A partially folded conformation has been shown to be a critical amyloidogenic intermediate in the formation of α-synuclein fibrils (5). In addition, soluble oligomers, in the form of annular protofibrils, have been shown to lead to vesicle permeability (6, 7).

α-Synuclein is a relatively abundant brain protein of 140 amino acids and of unknown function, and is intrinsically unstructured, i.e. natively unfolded (8, 9). The amino acid sequence of human α-synuclein shows seven repeats of 11 residues in the N-terminal half of the molecule, with a consensus sequence of KTKEGV. This repeat is reminiscent of the lipid-binding domains of some apolipoproteins, and the first five repeats are predicted to form an amphipathic helix (10). α-Synuclein has been shown to be present in high concentrations at presynaptic terminals (11), and to inhibit phospholipase D2 (12).

The existing literature on the interactions of α-synuclein with membranes is very contradictory. It has been reported that small oligomeric forms of α-synuclein preferentially associated with lipid droplets and cell membranes (13), and that α-synuclein binds preferentially to small unilamellar vesicles (SUVs) containing acidic phospholipids (PLs), such as PA and PS (PS is abundant (12–22% of total phospholipid) in nerve tissue, whereas PA comprises only 1–3%) but not to vesicles with a net neutral charge (10). In contrast, strong binding of α-synuclein (and its mutants and β-synuclein) to large unilamellar vesicles (LUVs) with either anionic or zwitterionic head groups has also been reported (14). Differential affinity of α-synuclein for specific phospholipids may play an important role in determining the location of the protein and the rate of fibril formation. The A30P mutation has been reported to either abolish lipid binding (15) or not (16). Wild-type and mutant α-synucleins are reported to associate with neural membranes, via strong interactions of both N- and C-terminal regions (17). A lipid binding motif and strong binding of α-synuclein to oleic acid have been reported (18). Previous reports have indicated that α-synuclein interaction with phospholipids may transform it into a helical conformation (13). Membranes have been reported to accelerate the fibrillation of α-synuclein (19) and to inhibit its fibrillation (14). A recent report suggests that α-synuclein aggregation may occur on

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† The abbreviations used are: PD, Parkinson’s disease; PL, phospholipid; LUV, large unilamellar vesicle; SUV, small unilamellar vesicle; NBD, 7-nitrobenzofurazan-labeled; PA, 1,2-dipalmitoyl-sn-glycero-3-phosphate; PG, 1,2-dipalmitoyl-sn-glycero-3-phospho-RAC-(1-glycerol); PS, 1,2-dipalmitoyl-sn-glycero-3-phospho-t-serine; PE, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine; AFM, atomic force microscopy.
membrane surfaces (13), and that membranes preferentially induce α-synuclein oligomers. Oligomers of α-synuclein have been reported to be induced in the presence of vesicles containing polyunsaturated fatty acids (20), with similar effects observed for β- and γ-synuclein and the familial mutants of α-synuclein. Recently, we have shown that lipid-bound, helical α-synuclein does not form fibrils (21). The mechanism of cell death in PD is unknown, but it has been suggested that membrane permeability, induced by some form of aggregated α-synuclein could be a factor (7, 22, 23). It is likely that interactions between hydrophobic residues of the amphipathic α-helices of α-synuclein play an important role in the association of the protein to the membrane. The significance of the presence of oligomers of α-synuclein is that they are suspected of being cytotoxic (6, 23, 24).

The present study was triggered by results of preliminary investigations that suggested that the outcome of the interaction of α-synuclein with membranes is very dependent on factors such as the relative concentrations of α-synuclein and lipids, nature of the vesicles, nature of the head groups etc. These factors could explain many of the apparently contradictory reports regarding the interaction of α-synuclein with membranes. We show that α-synuclein-membrane interactions affect both protein and membrane properties, and lead to disruption of the membrane, as well as affecting the kinetics of α-synuclein fibril formation. The presence of protofibrillar (oligomeric) or fibrillar α-synuclein caused a much more rapid destruction of the membrane than soluble monomeric α-synuclein.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of Protein—**Recombinant α-synuclein was expressed (in Escherichia coli) and purified as described previously (5), and stored at −80 °C. A stock solution of purified α-synuclein with a concentration <100 μM was treated with NaOH, then centrifuged for 15 min at 14,000 rpm to remove insoluble aggregated species, and used within 1 day.

**Materials—**1,2-Dipalmitoyl-sn-glycero-3-phospho-(PA), 1,2-dipalmitoyl-sn-glycero-3-phospho-RAC (PG), 1,2-dipalmitoyl-sn-glycero-3-phospho-i-serine (PS), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (PC), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (PE), and NBD (7-nitrobenzofurazan)-labeled PA, PG, and PC were purchased from Avanti Polar Lipids as chloroform solutions. Laurdan was obtained from Molecular Probes, Inc. Thioflavin T and calcein (2,4-bis[N-(2-carboxyethyl)-N-(2-carboxyethyl)aminoethyl]fluorescein) were purchased from Sigma, and were used without further purification.

**Preparation of SUVs by Sonication—**SUVs of PA/PC, PS/PC, PG/PC, and PG/PC (all at a molar ratio of 1:1), and PC alone were prepared by sonication. Lipids, dissolved in chloroform stock solutions, were mixed, and dried under N2 while shaking gently to form a thin film on the wall of the vial. The thin film was hydrated in phosphate-buffered saline buffer, pH 7.4, and then vortexed. The hydrated sample was sonicated at 4 °C with a probe sonicator with the microprobe energy at level 3. The sonicator program was set at 1-min pauses after a 1-min sonication period to prevent overheating. The sonication time varied from 2.5 to 10 min depending on the size of the samples. This procedure routinely produced small unilamellar vesicles with diameters of 20–25 nm (10).

**Preparation of LUVs—**Hydrated vesicles were obtained as described above, frozen at −80 °C, and then thawed at 70 °C (above the phase transition temperature). The freeze-thaw procedure was repeated 10 times, and the solution was then extruded through two stacked polycarbonate filters of 100-nm pore size for 12 times. The lipid concentration of the final vesicle solution was determined by a lipid phosphate assay (25). The sizes of vesicles were examined by EM imaging. Protein was mixed with pre-formed SUVs or LUVs, and incubated for 20 min before circular dichroism, fluorescence, or kinetic measurements.

**Encapsulation of α-Synuclein in Lipid Vesicles—**Encapsulated α-synuclein was prepared as described previously (26, 27), simply by slowly adding the lipids to the α-synuclein, while mixing constantly. The mixture was then sonicated or subjected to freeze-thaw cycling to obtain SUVs and LUVs, respectively.

**Intrinsic Fluorescence—**Steady-state emission and excitation spectra were measured with a Fluoromax-3 spectrofluorometer. The fluorescence of the four α-synuclein tyrosine side chains was measured to determine lipid binding to encapsulated α-synuclein. Emission spectra were recorded from 300 to 400 nm with excitation at 281 nm, with or without lipid vesicles. Spectra were corrected by subtracting the contributions of buffer and vesicle scattering.

**Laurdan Fluorescence—**Stock solutions of Laurdan (0.05%) and 0.03 mM PA/PC (1:1), PG/PC (1:1), or PC were made in chloroform. SUVs of Laurdan-labeled lipid were prepared as described above. Excitation spectra from 350 to 410 nm were recorded with emission wavelength at 434 nm. Spectra were corrected for background scattering produced from buffer or vesicles. The generalized polarization, Gp, values for Laurdan were calculated by using 434 nm emission wavelength and 375 (λ1) and 395 nm (λ2) wavelengths for the excitation intensities according to the following expression.

\[
G_{Pa} = (I_{\lambda_2} - 0.15I_{\lambda_1})/I_{\lambda_1} - 2I_{\lambda_2}I_{\lambda_1}/I_{\lambda_1} (\text{Eq. 1})
\]

For lipid titration experiments, aliquots of α-synuclein, protofibrils, or fibrils of α-synuclein were titrated directly into the fluorescence cuvette, and measurements were taken after incubating the solution for 20 min.

**NBD Fluorescence—**SUVs of NBD-incorporated lipid were prepared as described above, with 5 mol % NBD-PA or NBD-PG incorporated into 0.03 mM lipid vesicles. Emission spectra of NBD were recorded from 490 to 560 nm with excitation at 485 nm. Anisotropy (r) was calculated as follows,

\[
r = (I_{\lambda_C} - g_{\lambda_B})/I_{\lambda_C} - 2g_{\lambda_B} (\text{Eq. 2})
\]

where I_{\lambda_C} and I_{\lambda_B} are parallel and perpendicular fluorescence intensities, respectively, and g factors were calculated as I_{\lambda_B}/I_{\lambda_C}.

**Vesicle Membrane Permeability—**Calcine-loaded LUVs were prepared by hydrating lipid films in the presence of 40 mM calcine, and separated from free dye using a Sepharose 4B column (Sigma) (1.5 × 12 cm). The encapsulated fluorophore has a low fluorescence intensity because of self-quenching by the high concentration of calcine in the vesicle. The fluorescence intensity increases on release of the dye from the vesicle core by the addition of an aliquot of a membrane-disruptive agent. The change in the fluorescence intensity was monitored at 510 nm (excitation 490 nm) after incubating α-synuclein and dye-containing vesicles at 25 °C in 20 mM Tris-HCl buffer, 100 mM NaCl, pH 7.4, until the dye release reached its end point, around 30 min. Total dye release was completed by the addition of 0.2 vol% Triton X-100. Vesicles containing dye were diluted to 10 μM before the dye release to avoid probe-molecule interaction. For the same reason, the probe concentrations were checked before starting the experiments, and were less than 0.5 μM after complete release in all experiments. The percentage of probe release was calculated as follows,

\[
\% \text{Dye release} = \left( I_{\text{d}} - I_{\text{f}} \right) \times 100/(I_{\text{c}} - I_{\text{f}}) (\text{Eq. 3})
\]

where I_{\text{c}}, I_{\text{d}}, and I_{\text{f}} are the fluorescence intensity of the dye released by the protein, total dye released, and control blank. The percentage released was ignored if it was less than 15%. The percent release was reproducible within ±10%.

**Circular Dichroism Measurements—**The concentrations of protein were kept at 14 μM, with the mass ratio of protein to phospholipid varied from 5 to 1:10. Far-UV CD spectra were collected from 195 to 250 nm at a step resolution of 1 nm, band width 1.5 nm, and averaging time 5 s using an Aviv model 60DS (Lakewood, NJ) spectrometer. The final spectra were obtained by calculating the mean of five individual scans and subtracting the background consisting of buffer or vesicles without protein. The percentage of α-helix for α-synuclein was estimated from the molar ellipticity at 222 nm, as described (28).

**Preparation of Planar Phospholipid Bilayer for AFM Measurements—**Freshly cleaved mica was hydroxylated by submerging it in a solution consisting of concentrated H2SO4 (95 vol %) and 50 mM potassium dichromate for 20 min, rinsed with water, and dried with a stream of nitrogen. Samples of planar unilamellar bilayer were prepared by the vesicle fusion as previously reported (29). Briefly, mica was submerged into 2.5 mg/ml LUVs lipid solution at 4 °C for 4 h to form a self-assembled bilayer. The excess vesicles were removed by rinsing the mica surface with buffer. The prepared bilayer was incubated overnight at 37 °C and then stored in buffer (50 mM phosphate buffer at pH 7.5 containing 100 mM NaCl) at 4 °C prior to use. The properties of the planar bilayers were checked by AFM imaging.

**AFM Measurements—**Mica-adsorbed samples were washed with water and blown dry with N2. AFM imaging was done using an Autoprobe.
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RESULTS

Binding of Different Forms of α-Synuclein to Unilamellar Bilayers Characterized by Laurdan Fluorescence Spectra—

Laurdan (Fig. 1) is a fluorescence dye that incorporates into membranes and is sensitive to lipid packing density, which is manifest by changes in both fluorescence intensity and polarization. Membrane binding of soluble monomeric, protofibrillar, and fibrillar α-synuclein was monitored as a function of protein concentration to vesicles containing Laurdan. The fluorescence excitation spectra of Laurdan in PA/PC vesicles with or without α-synuclein or α-synuclein protofilaments and fibrils are shown in Fig. 2, A and B. The intensity of the excitation spectra substantially increases as a function of protein concentration. For a fixed concentration of α-synuclein (0.025 mg/ml) the effect on the spectrum for protofibrillar and fibrillar α-synuclein are much more pronounced (Fig. 2B), suggesting much stronger interactions with the membrane, compared with the soluble monomeric protein. Similar effects are seen for other lipid compositions, e.g. Fig. 2C for PA/PC and Fig. 2D for PG/PC vesicles, in which the changes in fluorescence intensity are shown as a function of protein concentration for monomeric, protofibrillar, and fibrillar α-synuclein. The change in the fluorescence intensity reflects the interactions between α-synuclein and the membrane (31). The much larger effects of the protofibrillar and fibrillar forms of α-synuclein indicate that these have a much greater effect on the membrane structure than the monomeric form. Both protofibrils and fibrils appear to have very similar effects on the membrane structure, based on the almost identical effects on Laurdan.

Fig. 2A also shows that the excitation spectrum of Laurdan in PA/PC vesicles has well separated electronic transition maxima at 375 and 395 nm. Generalized polarization, GP_{em}, values were calculated from the relative intensity of the two bands. Increasing protein concentration leads to increased mobility of the membranes, together with increased water penetration in the lipid bilayer, as indicated by the increase in intensity of the excitation bands of Laurdan, especially the blue excitation (375 nm). The ratio of blue band intensity to red band intensity increases, indicating the the Laurdan environment is more hydrophobic after interaction of the protein with the membrane. Fig. 2, E and F, shows that the relative intensities of the red and blue excitation bands of Laurdan-labeled PA/PC and PG/PC, indicated as GP_{em} values, decrease with increasing protein concentration. All three forms of α-synuclein induce significant changes in GP_{em}, but fibrillar and protofibrillar protein had a much stronger effect. Equilibrium measurements showed that the fibrillar form of α-synuclein binds to the lipid bilayer immediately following the addition of protein, and binding (and associated effects) is complete within 5 min. However, binding of soluble α-synuclein monomer required prolonged incubation times, typically from 20 to 30 min.

**Fig. 1.** Top, structure and location in a model PC monolayer of the fluorescent dye Laurdan, and NBD-labeled PC. The dimethylamino group of Laurdan is reported to be located about 5 Å from the bilayer surface, whereas the NBD moiety “loops back” toward the head group region (31). Bottom, structures of the phospholipids used in this study.
Binding of α-Synuclein to PA/PC and PG/PC Vesicles as Characterized by NBD Fluorescence—NBD-PA and NBD-PG are acyl-modified lipid probes that can be incorporated into the lipid bilayer (Fig. 1). The intensity of the spectra increases with increasing concentration of soluble monomeric α-synuclein concentration; concentrations were: 0.025, 0.05, 0.075, and 0.100 mg/ml from lowest to highest fluorescence intensity. B, the intensity of the spectra increases in the presence of protofibrils (dashed line) and fibrils (dash-dot line) relative to soluble monomeric α-synuclein (dotted line); protein concentration was 0.025 mg/ml in each case. Panels C and D show the fluorescence increases as a function of the concentration of monomeric α-synuclein (squares), protofibrils (filled circles), and fibrils (open circles) for PA/PC (C) and PG/PC (D). Panels E and F illustrate that the generalized polarization (GPem) (see text) shows a linear decrease as a function of concentration of α-synuclein (squares), protofibrils (filled circles), and fibrils (open circles) for PA/PC (E) and PG/PC (F).
presumably reflecting the interaction of the protein with the lipid (especially the head groups) leading to quenching of the intrinsic fluorescence of $\alpha$-synuclein. Because association of $\alpha$-synuclein typically leads to significant increases in the intrinsic fluorescence of $\alpha$-synuclein,$^2$ the observed decrease on interaction with the vesicles is unlikely to arise from association of the protein in the membrane. In addition, changes in secondary structure of $\alpha$-synuclein from random coil to $\alpha$-helix were observed upon binding to lipid (see below and Ref. 21). Thus, although it is possible that the formation of helical secondary structure is accompanied by collapse of the $\alpha$-synuclein to a relatively compact state in which quenching of the tyrosine residues occurs, the fact that the fluorescence intensity increases in helical oligomers induced by hexafluoroisopropyl alcohol (33), suggests that this is not the case. This conclusion is also supported by the absence of helical structure with PC vesicles, which show the change in Tyr fluorescence.

Binding of $\alpha$-Synuclein to Vesicles Induces Conformational Changes—The effect of lipid binding on the secondary structure of $\alpha$-synuclein was monitored by circular dichroism. Fig. 5 shows the spectrum of $\alpha$-synuclein in the absence of lipid, which is characteristic of an unfolded protein, with a minimum in ellipticity at 198 nm. The calculated $\alpha$-helical content is less than 3%. Upon binding to lipid, $\alpha$-synuclein undergoes a conformational change as evidenced by the ellipticity changes. Interestingly, significantly different effects were observed, depending on the mass ratio of $\alpha$-synuclein to lipid. At relatively high ratios of $\alpha$-synuclein (5:1) the vesicles induce a conformation whose CD spectrum is similar to that previously observed for the critical amyloidogenic intermediate of $\alpha$-synuclein (5).

$^2$ M. Zhu, J. Li, and A. L. Fink, unpublished observations.
ample, in the case of 1:5 α-synuclein/lipid ratio, over 70 and 60% helix was observed with PA/PC and PG/PC vesicles, respectively.

Previous studies (10) reported that α-synuclein bound only to acidic phospholipids, such as PA and PG, and not to neutral lipids, such as PC. To determine whether the presence of a positively charged phospholipid head group is responsible for the preferential binding of the protein, we examined a series of vesicles including PA, PG, PS, PE, PC, and binary mixtures of these. Similar changes in conformation from random coil to helical structure were observed on the addition of the acidic phospholipids PA, PG, and PS (data not shown) to either PC or PE, Fig. 5 (see also Ref. 21). The amount of helix induced was not as large for PA/PC compared with the same ratio of protein for PG and PS alone or mixed with PC or PE. Our results show that the ellipticity at 198 nm increased somewhat with PC vesicles (Fig. 5C), but no helical structure is observed. This result suggests that α-synuclein binds at least weakly to phospholipids with neutral head groups, such as PC, presumably by electrostatic interactions between the negatively charged C-terminal region of α-synuclein and the positively charged chol

Fig. 5. The effect of membrane composition, protein concentration, and ionic strength on the secondary structure of α-synuclein. Circular dichroism spectra of α-synuclein (solid line) bound to PA/PC (A), PG/PC (B), and PC (C) at protein/lipid mass ratios of 5:1 (dotted lines), 1:1 (dashed lines), and 1:5 (dash-dot lines) for PA/PC and PG/PC, except for 1:20 (dotted line) for PC. Panel D shows the dependence of the ellipticity at 222 nm of α-synuclein-PA/PC SUVs (1:5) on ionic strength.

Effect of Ionic Strength on α-Synuclein Binding to Membranes—Because α-synuclein preferentially binds to acidic phospholipids, and the N-terminal region of α-synuclein is rich in positively charged residues, we examined the effect of ionic strength on the association with lipid to determine the importance of electrostatic interactions. If such electrostatic interactions are important for the association one would expect that increasing ionic strength would weaken the binding of α-synuclein to the negatively charged membrane surface.
5D shows the ellipticity at 222 nm, a measure of the helix content, as a function of increasing NaCl concentration. The amount of /H9251/-helix decreases with increasing salt concentration, but binding is not abolished even at 1.5 M NaCl, conditions under which the lipid charge should be totally screened. Thus, although electrostatic forces may play an important role in the binding of protein to lipid, additional forces, presumably hydrophobic, contribute to the interaction of protein and the lipid.

Kinetics of /H9251/-Synuclein Fibril Formation in the Presence of Vesicles—/H9251/-Synuclein was incubated with SUVs (average diameter of 20–25 nm) of PA/PC, PG/PC, PA/PE, PG/PE, or PC at various concentrations of protein. Fibril formation was monitored by the increase in thioflavin T fluorescence (Fig. 6), and then verified by EM and AFM images. Table I shows the results for 0.5 mg/ml /H9251/-synuclein in the presence and absence of lipid.

The fibrillation of /H9251/-synuclein was very significantly affected by acidic phospholipids, such as PA/PC, PG/PC, PA/PE, and PG/PE vesicles in a concentration-dependent fashion (Fig. 6). However, PC vesicles, with neutral head groups, have only a small inhibitory effect on the fibrillation of /H9251/-synuclein (21). CD spectra (Fig. 5) indicate that at high lipid/protein ratios, PA/PC and PG/PC vesicles induce /H9251/-helical structure in /H9251/-synuclein, whereas lower ratios induce a partially folded /H9251/-synuclein intermediate, which has previously been shown to accelerate fibril formation (5). Consistent with the CD results, fibril formation of /H9251/-synuclein is significantly accelerated by binding at a 5:1 mass ratio of protein to lipid. EM and AFM images show that the size and shape of fibrils, formed in the presence of PA/PC, have a typical height of 7.6 nm, and a 60-nm periodic left-hand twisted morphology, indicating similarly morphology to fibrils grown in lipid-free solution (data not shown). As the ratio of lipid to protein increases, the amount of helical protein increases: at a lipid/protein mass ratio of 1:1 30% of the protein

TABLE I

| Phospholipid | Protein/lipid | Lag time  |
|--------------|---------------|-----------|
| None         | mass ratio    | h         |
| PA/PC        | 5:1           | 4 ± 2     |
|              | 1:1           | 15        |
|              | 1:5           | >4 weeks  |
| PG/PC        | 5:1           | 9 ± 2     |
|              | 1:1           | 18        |
|              | 1:5           | >4 weeks  |
| PA/PE        | 5:1           | 3 ± 1     |
|              | 1:1           | 39 ± 5    |
|              | 1:5           | >4 weeks  |
| PG/PE        | 5:1           | 2 ± 1     |
|              | 1:1           | 28 ± 3    |
|              | 1:5           | >4 weeks  |
| PC           | 5:1           | 14 ± 2    |
|              | 1:1           | 17 ± 2    |
|              | 1:5           | 19 ± 2    |

The kinetics of fibrillation were monitored using the thioflavin T fluorescence assay (see Fig. 6). The lag times for fibrillation in the presence of SUVs are shown.
is helical, and fibril formation is markedly slowed down. At a mass ratio of 1:5 (protein/lipid), the helical fraction increases to around 70–80% of the α-synuclein, and fibril formation is completely inhibited (Table I).

 α-Synuclein-induced Membrane Permeability—The ability of α-synuclein to disrupt lipid membranes was investigated using a dye release assay. A self-quenching fluorescent dye, calcein, was encapsulated in the vesicle. Monomeric, fibrillar, or protofibrillar α-synuclein at various concentrations were incubated for 20 min with PA/PC, PA/PE, PG/PC, PG/PE, or PC vesicles loaded with calcein. The increase in fluorescence intensity because of the calcein release from the vesicle was observed. Table II shows the extent of calcein leakage induced by monomeric α-synuclein, its protofibrils, and fibrils. Whereas 0.0125 mg/ml α-synuclein resulted in 12–15% release of dye for PA or PG vesicles, the same amount of fibrillar or protofibrillar α-synuclein led to two to three times as much of the vesicle-encapsulated dye being released, indicating that protofibrils and fibrils of α-synuclein were significantly more effective in membrane disruption than soluble α-synuclein monomer. Interestingly, the protofibrils and fibrils had comparable effects on permeability. Increasing concentrations of soluble α-synuclein resulted in an increase in dye leakage (Table II). We assume that when the vesicles are disrupted, α-synuclein still binds to the vesicles, thus further addition of α-synuclein induces further disruption. The α-synuclein concentration is so low, and the time of incubation so short, that there will be negligible formation of oligomers (protofibrils) or fibrils under these conditions. Based on the lack of dye release, α-synuclein did not disrupt PC vesicles, consistent with the Laurdan and NBD results. The error in measurements for dye leakage comes from light scattering because of the insoluble aggregates. However, in the case of fibrillar α-synuclein, we observed that the fibrils bind to calcein and cause a 10-fold increase in the background fluorescence intensity of calcein; thus an additional error of around 7% is also introduced in this case from subtraction of a large background. We observed a strong correlation between the ability of α-synuclein to disrupt membranes and the binding affinity of α-synuclein for the particular membrane composition.

Preparation and Stability of Planar Phospholipid Bilayers—In addition to SUVs and LUVs, planar lipid bilayers were also prepared to measure membrane disruption using AFM imaging. Planar bilayers were formed by fusing LUVs at lipid concentrations of 2.5 mg/ml on the hydrophilic surface of mica. EM images showed that LUVs in solution had sizes in the range of 60–200 nm, with an average of 120 nm (Fig. 7A). The procedure for preparing stable flat bilayers from LUVs has been well investigated (29, 35–37). Several important factors, such as vesicle concentration, temperature, and incubation time, which affect the process, were studied. Bilayer formation, including subsequent adsorption, fusion, and rupture on the mica surface, were monitored by time-dependent sequences of AFM imaging (Fig. 7). These images show the initial fusion of vesicles on the mica surface, with a mean radius from 160 to 250 nm during the early adsorption stage of bilayer formation (Fig. 7B). Subsequently the vesicle size increased to 380–460 nm after incubation for 4 h, indicating the rupture of the vesicles (Fig. 7C). The disks exhibit a height of 3.8–4.5 nm at the first adsorption stage, which decreases after rupture and fusion to 0.5–1.8 nm. The larger vesicles finally coalesce to form the intact bilayer after overnight incubation in buffer (Fig. 7D).

We estimate that over 90% of the mica surface was covered by fused vesicles with an average roughness of 0.3 nm, which indicates well packed bilayers. The stability of the bilayers formed on mica was tested by incubating them with a large volume of lipid-free buffer solution at 37 °C for up to 20 h, because incubation of planar bilayer in buffer may cause the loss of lipid (29). The lipid loss was examined by measuring the increase in area of bilayer defects using AFM. The existence of exposed mica in the defects was verified by measuring the roughness of the exposed dark regions in AFM images. We find that lipid loss is much faster at temperatures above the phase transition temperature, which is around 65 °C in this lipid system, and after incubation for more than 20 h. The bilayer is stable for up to 20 h when incubated at 50 °C (less than 10% defects at 20 h). It took more time to form bilayers from 2.5 mg/ml lipid solutions, and the roughness of the bilayer was larger than for bilayers prepared from 1 mg/ml solution (roughness = 0.2 nm). However, bilayers prepared from 2.5 mg/ml solutions were much more stable at high temperature and in long term incubations than bilayers prepared from lower concentrations of lipid. If the concentration of the original lipid solution was increased to 5 mg/ml, the increase in roughness of the resulting bilayer was up to 1.0 nm, which was unacceptable. Higher NaCl concentrations favored the adsorption of vesicles to the mica. The addition of calcium to the lipid solution did not contribute to the stability and roughness of the bilayer. Thus, 2.5 mg/ml phospholipid vesicles in 20 mM phosphate buffer, pH 7.4, containing 100 mM NaCl and no calcium were used to prepare the lipid bilayers. At this concentration multiple layers of bilayers were formed on the mica. The height of a single bilayer was measured with a few defects as a background (Fig. 7A), and was ~3.8 ± 0.2 nm high. This size is consistent with the size of a PA/PC bilayer, which is estimated as 3.6 nm. Repeated AFM scans of the same area (5 μm) reproducibly created the same image and no evidence for tip-induced damaged to the membrane was observed.

Penetration and Disruption of Bilayers by Monomeric and Fibrillar α-Synuclein—We investigated whether α-synuclein penetrated into the lipid membrane or aggregated on the membrane surface using AFM imaging. We characterized the bilayer defects by randomly sampling at more than four locations on the surface and more than 10 images were taken for each instance.
sample. In these experiments the planar bilayer was prepared on a small sheet of mica that was then placed in a solution containing \( \alpha \)-synuclein, as described under “Experimental Procedures.”

A planar PA/PC bilayer on mica was incubated with 0.3 mg/ml soluble \( \alpha \)-synuclein. Freshly prepared planar vesicles showed the intact bilayer surface with a few defects. Small particles on the surface were observed when the bilayer-coated mica was immersed in the protein solution (Fig. 8A). We assume that the particles are \( \alpha \)-synuclein molecules adsorbed on the bilayer. No attempt was made to image the fine structure of the small particles of 2–6 nm height, which may represent monomers or dimers of \( \alpha \)-synuclein. After incubating for 1.5 h, the small particles disappeared, indicating that \( \alpha \)-synuclein was incorporated into the lipid membrane, whereas the loss of lipid molecules was observed by the appearance of some circular defects (Fig. 8B). The depths of the defects beneath the surrounding surface were estimated, and varied from 1.8 to 2.1 nm (red arrows), 3.5–4.5 nm (green arrow), 7.8–8.5 nm (yellow arrows), and 20.7–36.8 nm (pink arrows); the sizes correspond to the monolayer, as well as one, two, or multiple unilamellar bilayers composed of an upper and bottom leaflet, respectively. The images suggest that multiple bilayers were gradually destroyed by \( \alpha \)-synuclein, layer by layer. The defects have diameters mostly in the range of 190–260 nm, with a few of them around 1 \( \mu \)m, and cover 26% of the surface by a grain analysis using SPIP applied on 5 images. After incubating the bilayers for 2 h, the loss of bilayer increased, along with the number and area of defects, which now covered 58% of the surface (Fig. 8C). As the incubation time increased to 4 h the bilayer defects gradually became larger (Fig. 8D). Circular defects combined with each other, and finally formed large defects so that 79% of the surface was covered by defects. No fibrils were observed when imaging the sample from the longer incubation times, nor when the protein concentration was increased to 0.6 mg/ml. The most likely explanation for the “holes” in the membranes is that the lipids became solubilized because of the membrane disruption, and it is also possible that a coating of \( \alpha \)-synuclein formed on the mica surface, which prevented lipid deposition.

We also examined the effects of fibrillar \( \alpha \)-synuclein on the disruption of bilayers by incubating preformed fibrils with a planar lipid bilayer. Fibrils were prepared by agitating 0.5 mg/ml \( \alpha \)-synuclein solution at 37 °C for 3 days. The solution was centrifuged at 14,000 rpm for 15 min, the supernatant was removed carefully, and the pellet was washed twice before resuspending in buffer solution (Tris-HCl, pH 7.5). The concentration of fibrillar \( \alpha \)-synuclein was estimated by subtracting the protein concentration in the supernatant from the total amount. A freshly prepared PA/PC planar bilayer was incubated in 0.2 mg/ml fibril solution, using freshly cleaved mica in the same solution as a control. Fibrils were clearly seen on the vesicle surface in the early stages in AFM images (Fig. 9A). However, the heights of fibrils were in the range of 3.2–6.0 nm, which is lower than for the same fibrils measured on the mica (typically 7.8 nm), indicating that the fibrils were partially incorporated into the bilayer. After 0.5 h, over 80% of the lipid was lost from the surface, and only a typical supporting substrate, the mica surface, was observed after incubating for 2 h (Fig. 9C). The results indicate that fibrillar \( \alpha \)-synuclein disrupts PA/PC membrane bilayers more readily than soluble monomeric \( \alpha \)-synuclein. The results are consistent with those from NBD fluorescence, Laurdan fluorescence, and dye permeability methods.

A number of interesting observations were made when PE was combined with PA or PG to form planar bilayers (Fig. 9, D–I). Fig. 9, D and G, shows similar small particles to those
observed with PA/PC when the PA/PE and PG/PE layers were initially exposed to α-synuclein. On incubation at 37 °C disruption of the lipid layers occurs over a period of 4–16 h. The membrane defects have regular shapes with both PA/PE and PG/PE layers as a result of the α-synuclein-induced disruption. For the PA/PE layers the disruption is completed quite rapidly, preventing acquisition of images at the earliest stages of the process. The slower disruption with PG/PE layers allowed us to observe the intermediate stages of membrane collapse. Fig. 9, H and I, clearly show that linear defects develop gradually leading to the final breakdown of the bilayer.

Comparison of the upper row in Fig. 9 with Fig. 8 shows the marked differences in the effects of α-synuclein monomers (at 0.3 mg/ml) versus fibrils (at 0.2 mg/ml) on the surface area covered by defects in PA/PC bilayers. The rates of bilayer disruption are dramatically faster with the fibrils: for example, 80% of the bilayer surface is degraded by 30 min; in contrast, starting with monomers only 26% has been disrupted by 1.5 h, 58% at 2 h, and 79% after 4 h.

We also examined the ability of soluble and fibrillar α-synuclein to disrupt PC vesicles. Neither sample showed any effect on the bilayers after incubation for 16 h (data not shown).

DISCUSSION

α-Synuclein Binds to Phospholipid Vesicles—Previous work has suggested that α-synuclein binds to curved, negatively charged lipid vesicles, such as SUVs of PA/PC and PG/PC (21), as well as to the relatively flat surfaces of LUVs, even when the surface involves neutral head groups, such as PC (14). In the present work, membrane binding was studied with a variety of techniques, including fluorescence, circular dichroism, and microscopy. We used the fluorescence dyes, NBD and Laurdan, which are localized at different depths in the bilayer, to probe the protein-lipid interaction.

Laurdan is a polarity-sensitive, amphiphilic fluorophore, which can embed into a membrane (31, 38). Its lauric acid chain serves as a hydrophobic anchor into the membrane bilayer core, pulling the fluorophore into the bilayer 5 Å beneath the lipid/water interface (Fig. 1). There are about 20 water molecules per lipid head group contained in a PC/water interface (40). Laurdan is relatively insensitive to the structure of the head groups, and differences in the fluorescence properties, such as fluorescence intensity and generalized polarization, are a manifestation of different depths of penetration of water into the membrane, thus an index for membrane disruption (31). Our data show differences in the excitation spectra, and in the GPem values, when α-synuclein is incubated with PA/PC or PG/PC vesicles, suggesting significant interaction between the protein and vesicles, in particular, significant penetration into the bilayer. The greater extent of perturbation of the membrane structure sensed by Laurdan in the presence of fibrillar and protofibrillar α-synuclein suggests a more disruptive effect of protofibrils and fibrils compared with soluble monomer on the membrane. Considering that the fluorophore of the dye is located 5 Å beneath the surface, the results suggest that the protein is able to insert significantly into the core of the bilayer, leading to changes in the fluidity, and eventually destruction of the bilayer. α-Synuclein has no effect on the fluorescence spectra of Laurdan-labeled PC vesicles, indicating a lack of significant penetration of the protein into the bilayer of PC SUVs. Because the intrinsic tyrosine fluorescence and thioflavin T data indicate some interaction between α-synuclein and PC SUVs, we assume that with PC SUVs the protein is only bound to the surface of the membrane, and does not penetrate into the bilayer, and thus has no effect on membrane structure.

The long chain acyl-modified NBD lipid probe is incorporated in the vesicle bilayer near the head group region (Fig. 1). The increase in both fluorescence intensity and anisotropy on the addition of soluble monomeric and fibrillar α-synuclein to acidic PA/PC and PG/PC vesicles indicates interactions between acidic lipid vesicles and the protein, which involve penetration of the protein into the core of the bilayer. This suggests that the binding involves hydrophobic interactions between hydrophobic patches on α-synuclein (possibly the amphiphilic helices induced by the lipid) and the hydrophobic core of the lipid bilayer. This changes the membrane fluidity, resulting in the change in fluorescence properties of the probe. This hydrophobic interaction is independent of any concurrent electrostatic interactions. Analogous to the situation with Laurdan-labeled lipid vesicles, fibrillar and protofibrillar α-synuclein required lower concentrations and shorter times for the same reaction than soluble monomeric α-synuclein, indicating stronger interactions (and presumably greater toxicity) than the monomer. Little or no change in fluorescence properties was observed when α-synuclein was added to NBD-containing PC vesicles, indicating the lack of a significant effect of the protein on the PL bilayer.

The data with Laurdan and NBD-containing vesicles indicate that protofibrils and fibrils of α-synuclein have a much larger and more rapid disruptive effect on membranes than the soluble monomeric protein, which was confirmed in the membrane permeability experiments. Because the structure of the fibrils and the protofibrils appears to be quite different, and the protofibrils are suspected of forming pores in the membrane...
Interaction of α-Synuclein with Membranes

(23), it is somewhat surprising that both forms of aggregated α-synuclein have very similar effects on the structural probes. This could be because the probes are reflecting the general perturbation of the membrane because of the presence of aggregated α-synuclein, or it could mean that there is some other key interaction with the membrane that is common to both protofibrils and fibrils. An additional significant difference between the interactions of the soluble monomeric α-synuclein and its protofibrillar or fibrillar forms is that the former involves a predominant helical conformation, whereas the latter involve a predominantly β-sheet conformation.

The intrinsic tyrosine fluorescence spectra of α-synuclein showed differences in the presence of the different charged SUVs, including PC vesicles, suggesting that α-synuclein binds to both negatively charged and neutral vesicles. The binding to PC SUVs was probably mediated through electrostatic interactions between the protein and the bilayer surface. Thus, α-synuclein can bind to the surface of PC vesicles, but not as strongly as with PA/PC, and does not penetrate the bilayer, presumably because the helical conformation is not induced because of the weak interaction. The binding affinity of α-synuclein is in the order of PA > PG > PC. The circular dichroism data indicate that at relatively high concentrations of α-synuclein interaction with vesicles leads to formation of an amyloidogenic conformation and accelerated fibrillation. At lower ratios of α-synuclein the interaction with the membrane leads to formation of helical structure and inhibition of fibrillation. Because the amount of α-helix decreases with increasing salt concentration, it is clear that electrostatic interactions are very important in the α-synuclein-membrane interaction. However, because binding is still present at very high ionic strengths, conditions under which the lipid charge should be totally screened, additional forces, presumably hydrophobic, must also contribute significantly to the interaction of protein and the lipid. It is the hydrophobic interactions, because of the helical conformation, that lead to the critical penetration of the bilayer.

The Effects of Lipid Binding on α-Synuclein Fibril Formation—As an intrinsically unstructured protein, α-synuclein is a conformationally dynamic molecule whose secondary structure is very dependent on its environment. The circular dichroism data indicate that the effect of membranes on α-synuclein conformation is highly sensitive to the ratio of lipid to protein. At relatively high ratios of protein, where the conformation is that of the amyloidogenic intermediate, fibrillation is accelerated significantly. On the other hand, at relatively high ratios of PL, α-helix is induced by acidic, but not by neutral, phospholipids, and leads to inhibition of fibrillation, as previously reported for a more limited set of vesicle compositions (21). Our data show that the effect of membranes on α-synuclein aggregation is very sensitive to both relative concentrations and membrane composition, thus, it is difficult to predict the effects in neurons. However, it is clear that changes in lipid composition because of cellular stress, for example, could have significant effects on α-synuclein fibrillation.

Although binding of α-synuclein monomer to membranes may stabilize the protein from fibrillation, membrane binding also may increase the effective concentration of the protein by a factor of 10 (41). Higher concentrations of α-synuclein enhance the rate of fibrillation. Thus there is a potential competition between acceleration and inhibition of fibrillation, which will be determined by the conformation of the protein, if it is helical, even at high concentration, fibrillation will be inhibited. Furthermore, increased non-helical protein concentration will increase the level of protofibrils and fibrils, which may destroy the membrane after prolonged incubation.

Insertion and Disruption of Vesicles by Monomeric and Fibrillar α-Synuclein—Lipid bilayers were used as a model for cell membranes, either as unilamellar vesicles or as planar supported bilayers. The surface tension of the bilayer membrane is very large (29). Consequently, a large driving force is required to disrupt it. In addition to binding to vesicles, initial experiments with NBD- and Laurdan-labeled vesicles demonstrated that soluble monomeric α-synuclein and fibrillar α-synuclein can insert into the bilayer to a depth of several angstroms, and affect the membrane properties. The results from the dye leakage experiments further confirm the ability of soluble and protofibrillar or fibrillar α-synuclein to insert into or penetrate the bilayer, leading to the leakage of dye from the vesicle lumen. A well packed and heat-stable membrane bilayer was formed on the surface of mica, which served as a planar bilayer for the membrane disruption experiments by means of AFM. AFM allows direct “visual” measurement of the interaction of α-synuclein with membranes. Our results clearly show that fibrillar α-synuclein has a much higher ability to disrupt membrane bilayers than soluble monomeric α-synuclein, and the order of effectiveness in disruption is PA > PG > PC, which is consistent with the membrane affinity. In the case of soluble monomeric α-synuclein, it is only under conditions (e.g. relatively low protein concentration) where the protein is in an α-helical conformation that membrane disruption is observed. This strongly suggests that it is the exposed, contiguous hydrophobic surfaces along the amphiphilic helices that lead to membrane penetration.

The results demonstrating that the addition of fibrils of α-synuclein to bilayers leads to the relatively rapid disintegration of the membranes, including penetration into multiple bilayers and disappearance of the fibrils into the lipid, dramatically illustrate the potential damage that fibrillar α-synuclein can do to membranes. The data indicate that although both monomeric soluble α-synuclein and fibrillar α-synuclein can induce membrane disruption, the fibrillar form is significantly more effective. The mechanism of membrane disruption by fibrils and protofibrils could be significantly different, in that the AFM images of fibril interactions (Fig. 9) show fibrils being “swallowed” by membranes, whereas it is assumed that protofibrils act by forming annular pores in the membrane (39). Interestingly, spherical protofibrils of α-synuclein have been observed to bind to brain-derived membrane fractions much more tightly than monomeric or fibrillar α-synuclein (6). Although it may seem contradictory that in addition to protofibrils and fibrils conditions that lead to helical formation in α-synuclein also lead to membrane disruption, this is readily explained as follows. We associate increased α-helix formation with strong interactions between α-synuclein and lipid. Thus it is not surprising that conditions that lead to helical formation correlate with an increase in bilayer disruption. On the other hand, as discussed above, protofibrils and fibrils have quite different mechanisms of membrane disruption.

These observations suggest a potential source of neurotoxicity for α-synuclein, namely via disruption of membranes, leading to ion fluxes that would be lethal to the cell. As noted, there have been previous suggestions that the cytotoxicity of α-synuclein results from protofibrils, including the idea that they render membranes leaky. Our results are consistent with both fibrillar and protofibrillar α-synuclein being much more toxic than the soluble monomer because of membrane destruction at much lower concentrations of α-synuclein and shorter times of exposure. Thus the association of α-synuclein protofibrils and fibrils with cell membranes may play a critical role in Parkinson's disease and other synucleinopathies.
