Rapid Anionic Micelle-mediated α-Synuclein Fibrillization *in Vitro**

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Parkinson's disease is characterized by the aggregation of α-synuclein into filamentous forms within affected neurons of the basal ganglia. Fibrillization of purified recombinant α-synuclein is inefficient *in vitro* but can be enhanced by the addition of various agents including glycosaminoglycans and polycations. Here we report that fatty acids and structurally related anionic detergents greatly accelerate fibrillization of recombinant α-synuclein at low micromolar concentrations with lag times as short as 11 min and apparent first order growth rate constants as fast as $10.4 \ h^{-1}$. All detergents and fatty acids were micellar at active concentrations because of an α-synuclein-dependent depression of their critical micelle concentrations. Other anionic surfaces, such as those supplied by anionic phospholipid vesicles, also induced α-synuclein fibrillization, with resultant filaments originating from their surface. These data suggest that anionic surfaces presented as micelles or vesicles can serve to nucleate α-synuclein fibrillization, that this mechanism underlies the inducer activity of anionic surfactants, and that anionic membranes may serve this function *in vivo*.

Lewy bodies are hallmark lesions of Parkinson's disease that appear in dopaminergic neurons of the substantia nigra and other brain regions (1–4). They consist primarily of the protein α-synuclein aggregated into filaments having non-twisted (5–10-nm width) or twisted (5–18-nm width) morphology (5–8). Filamentous α-synuclein inclusions are also found in other synucleinopathies including dementia with Lewy bodies and multiple system atrophy (2, 8, 9). In addition, Lewy body pathology frequently overlaps with the lesions of other neurodegenerative diseases including Alzheimer's disease (10–16), Down's syndrome (17), and neurodegeneration with brain iron accumulation type 1 (Hallervorden-Spatz syndrome; Ref. 18). Because specific point mutations in the α-synuclein gene cause rare, early onset, autosomal-dominant inherited Parkinson's disease cases (19–21), it has been suggested that misfunction of α-synuclein plays a primary role in the pathogenesis of synucleinopathies.

Full-length, recombinant α-synuclein has been shown to aggregate *in vitro* near physiological pH into filaments morphologically indistinguishable from those found in Lewy bodies (7, 22, 23), but the reaction requires high protein concentrations to support measurable fibrillization over time courses ranging from days to weeks (23–25). Fibrillization can be accelerated by incubation of full-length recombinant protein at acidic pH with continuous shaking (25, 26), by changes in protein structure through truncation (27) or point mutation (28), or by the addition of exogenous aggregation inducers such as tubulin (29), glycosaminoglycans (*e.g.* heparin; Ref. 30), and polyamines (31).

The weak efficacy of the latter agents has led to a search for more efficient α-synuclein fibrillization inducers. Because anionic detergents, fatty acids, and lipids bind α-synuclein (32–36), stabilize elements of secondary structure (33), and induce oligomerization (37), they are candidate inducers of α-synuclein fibrillization. Here we show that anionic detergents, fatty acids, and phospholipids in micellar or vesicular forms are capable of inducing efficient fibrillization of full-length recombinant α-synuclein at near physiological pH, temperature, reducing environment, and ionic strength over a broad range of α-synuclein concentrations down to low micromolar. In addition to providing a useful tool for modeling α-synuclein fibrillization *in vitro*, these agents point toward intracellular anionic surfaces as potential nucleating agents *in vivo*.

EXPERIMENTAL PROCEDURES

Materials—Arachidonic acid was obtained from Cayman Chemicals (Ann Arbor, MI) and stored at −80 °C under argon. Alkyl sulfate detergents (12–20 carbons) were obtained from Mallinckrodt Chemical Works, Acros Organics (Morris Plains, NJ), Lancaster Synthesis (Pelham, NH), and Research Plus (Bayonne, NJ) as sodium salts. Non-ionic and cationic detergents (bromide salts) were purchased from Sigma ($C_{10}E_8$, $C_{12}E_{23}$, and $C_{14}E_8$) or Fluka (Milwaukee, WI; C_8E_4, $C_{10}E_6$, $C_{19}H_{42}BrN$, and $C_{15}H_{34}BrN$). Heparin sodium salt grade I-A (~18,000 Da) and heparin sodium salt (~6,000 Da) from porcine intestinal mucosa were obtained from Sigma, dissolved in water, and kept under argon. All detergent stock solutions were prepared in either water or a 1:1 water:isopropyl alcohol. Zwittergent 3–16 (Calbiochem) was dissolved in water and used the same day. *N*-Phenyl-1-naphthylamine and ThT[1] were from Sigma. Glutaraldehyde, uranyl acetate, and Formvar/carbon-coated 300 mesh grids were from Electron Microscopy Sciences (Ft. Washington, PA).

Liposome Preparations—PA (1-stearoyl-2-oleoyl-*sn*-glycerol-3-phosphate), L-α-PS (brain), L-α-PC (brain), L-α-PI (bovine liver), L-α-PIP (porcine brain), and L-α-PIP$_2$ (porcine brain) were obtained from Avanti Polar-Lipids (Alabaster, AL), dissolved in chloroform, and finally, dried under a stream of argon (10 min) followed by vacuum desiccation (45 min). Samples were hydrated (1 mM final concentration) in 10 mM HEPES, pH 7.4, 100 mM NaCl by bath sonication in a Parafilm-sealed glass test tube for 3 h. Liposomes were freshly prepared for each experiment, and their presence was confirmed by electron microscopy.

α-Synuclein Expression and Purification—A cDNA for α-synuclein was obtained from a human brain library (Stratagene, La Jolla, CA)

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[1] The abbreviations used are: ThT, thioflavin T; CMC, critical micelle concentration; PA, phosphatidic acid; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP$_2$, phosphatidylinositol 4,5-bisphosphate; PS, phosphatidylserine; PC, phosphatidylcholine; Tween 20, polyoxyethylene sorbitan monolaureate; zwittergent 3–16, *n*-hexadecyl*N,N*-dimethyl-3-ammonio-1-propanesulfonate.

using PCR and primers containing NdeI and HindIII restriction sites, 5′-AAA GAA TTC <u>CAT ATG</u> GAT GTA TTC ATG AAA GGA and 5′-AAA <u>AAG CTT</u> AGG CTT CAG GTT CGT AGT C (restriction sites are underlined). The PCR product was cloned directly into PCR-BluntII-TOPO vector (Invitrogen). The construct was then digested with NdeI and HindIII and ligated into expression vectors pT7c and pT7II. These vectors drive the expression of poly-His-tagged and non-tagged α-synuclein, respectively (38). His-tagged α-synuclein protein was expressed and purified as described previously for tau protein (38), whereas non-tagged α-synuclein was prepared as in Antony *et al.* (31) except that Mono Q chromatography was used as the anion exchange resin. Because these preparations behaved similarly in all assays described below, the His-tagged form was used for experimentation for ease of preparation.

α-Synuclein Aggregation—Under standard conditions α-synuclein (4–70 μM) was aggregated in assembly buffer (10 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM dithiothreitol) at 37 °C for up to 44 h without agitation unless noted otherwise. Reactions were performed in the presence and absence of various aggregation inducers prepared by serial dilution of stock solutions. Control reactions were normalized for 1:1 H$_2$O:isopropyl alcohol vehicle, which was limited to 5% in all reactions. Samples were processed for transmission electron microscopy and fluorescence spectroscopy assays as described below.

Electron Microscopy Assay—Aliquots of α-synuclein aggregation reactions (50 μl) were removed, treated with 2% glutaraldehyde (final concentration), adsorbed onto Formvar/carbon-coated 300 mesh grids (1 min), and negatively stained with 2% uranyl acetate (1–2 min) as described previously (39). Images were viewed in a Phillips CM 12 transmission electron microscope operated at 65 kV. Random images were captured on film at 22,000–100,000× magnification, digitized at 600 dots-per-inch resolution, and processed using Adobe Photoshop.

Fluorescence Assay—α-Synuclein (4–70 μM) was aggregated at 37 °C in assembly buffer as described above except that the reactions contained 20 μM ThT. Resultant changes in fluorescence were monitored at $\lambda_{ex} = 440$ nm and $\lambda_{em} = 485$ nm (40, 41). To assay the degree of α-synuclein fibrillization under near equilibrium conditions, fluorescence was measured after 16 h of incubation in a FlexStation plate reader (Molecular Devices, Sunnyvale CA). To assay fibrillization in real time, fluorescence was measured with continuous stirring at a constant rate for up to 44 h in a PTI fluorimeter (1-nm bandwidth, 800 V, gain 12.5, and slit 16). These reactions (4 ml) were initiated by the addition of 30–70 μl of C$_{12}$H$_{25}$SO$_4$Na (20 mM).

Kinetic Analysis—Fluorescence time courses were fit to the linear logistic differential equation (42) $\log(F_t/(F_\infty - F_t)) = mt + b$, where F_t is the ThT fluorescence at time t, F_∞ is the ThT fluorescence at equilibrium, and m and b are the slope and y intercept of each straight line. From these plots, the half-time to maximum fluorescence, t_{50}, was determined as the time when $\log(F_t/(F_\infty - F_t)) = 0$ (42). The apparent first order rate constant for growth of ThT fluorescence, k_{app}, was derived from the slope m, where $k_{app} = m(\ln 10)$, and reported in units of time^{-1}. Lag times, which were defined as the time point where the tangent of the point of maximum polymerization rate intersected the *abscissa* of sigmoidal reaction progress curves (43), were calculated as $t_{50} - 2\tau$, where $\tau = 1/k_{app}$ (44). Both k_{app} and lag time are comparable with values calculated using integral logistic equations (44). Although they are composite parameters unequal to underlying elementary rate constants, they are useful for purposes of comparison. Because α-synuclein fibrillization follows a nucleation-dependent mechanism (45), lag time was assumed to reflect filament nucleation, whereas k_{app} was taken as the macroscopic rate constant for the entire filament population during the exponential phase of growth.

CMC Measurements—Detergents or arachidonic acid suspended in assembly buffer at varying concentrations (1 μM–50 mM) were incubated 1 h in the presence of 10 μM N-phenyl-1-naphthylamine, after which fluorescence intensity was read directly in a PTI fluorimeter ($\lambda_{ex} = 346$ nm; $\lambda_{em} = 420$ nm; 1-nm bandwidth, 800 V, gain 12.5, and slit 16) jacketed at 37 °C. When present, α-synuclein was maintained at 4 μM for the positively charged detergents and at 15 μM for the anionic detergents and arachidonic acid. CMCs were estimated from *abscissa* intercepts after least squares linear regression as described previously (46) and were expressed as CMC ± S.E. of the estimate. Only data points within 5-fold of the CMC were used to calculate CMC.

Nomenclature—Polyoxyethylene detergents of formula CH$_3$(CH$_2$)$_y$-O(CH$_2$CH$_2$)$_x$-H are referred to as C$_{(y+1)}$E$_x$, where y and x are the number of methylene and oxyethylene groups, respectively. Sodium alkyl sulfate detergents of structure
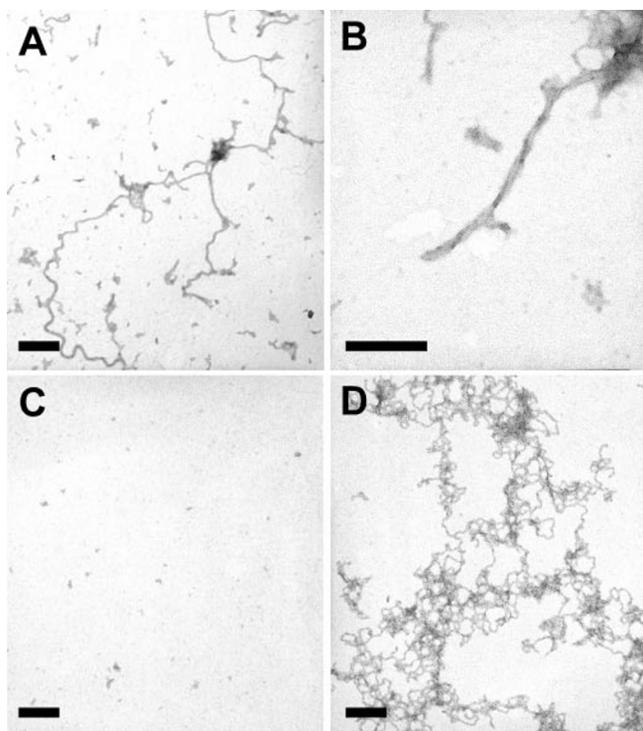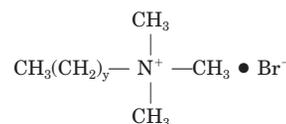

FIG. 1. **Induction of α-synuclein fibrillization with anionic detergents and fatty acid.** All aggregation assays were performed at 37 °C without agitation, after which time they were terminated with glutaraldehyde and subjected to transmission electron microscopy at 35,000–100,000-fold magnification. *A*, in the presence of arachidonic acid (200 μM), α-synuclein at 4 μM formed abundant filaments with non-twisted morphology in just 4 h. *B*, at higher α-synuclein concentrations (70 μM), filaments with twisted morphology were apparent within 4 h of incubation. *C*, in the presence of ethanol vehicle alone, α-synuclein (30 μM) formed few filaments even when incubated up to 16 h. *D*, anionic detergents such as C$_{14}$H$_{29}$SO$_4$Na (100 μM) also induced α-synuclein fibrillization (30 μM), yielding large masses of filaments after 16 h. All *bars* correspond to 200 nm.

$$\text{CH}_3(\text{CH}_2)_y\!-\!\text{O}\!-\!\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle O}{\|}}{\text{S}}}\!-\!\text{O}\!-\!\text{Na}$$
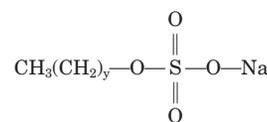
STRUCTURE I

and alkyl tetramethyl ammonium bromide detergents of structure

$$\text{CH}_3(\text{CH}_2)_y\!-\!\overset{\overset{\displaystyle CH_3}{|}}{\underset{\underset{\displaystyle CH_3}{|}}{\text{N}^+}}\!-\!\text{CH}_3 \bullet \text{Br}^-$$

STRUCTURE II

are referred to by their full chemical formulas. Examples within each of these classes differed only in the number of methylene groups present (*y*).

RESULTS

Efficient Induction of α-Synuclein Aggregation—Experimentation began with arachidonic acid because this fatty acid had been shown to induce α-synuclein oligomerization *in vitro* (37). Pilot electron microscopic assays showed that arachidonic acid did indeed induce the fibrillization of 4–70 μM recombinant α-synuclein after just 4 h of incubation at 37 °C without agitation (Fig. 1*A*). Filaments mostly had non-twisted morphology under these conditions, with widths varying from 5 to 14 nm (Fig. 1*A*). As α-synuclein concentrations were raised, however,
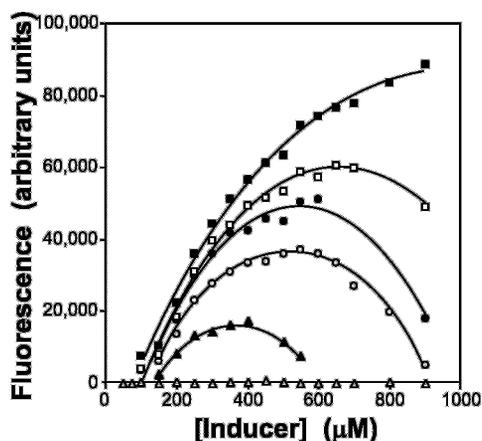
Micelle-dependent α-Synuclein Fibrillization

FIG. 2. **α-Synuclein fibrillization as a function of protein and inducer concentrations.** α-Synuclein at 5 μM (▲), 10 μM (○), 15 μM (●), 20 μM (□), or 30 μM (■, △) was incubated (16 h at 37 °C) in the presence of varying concentrations of $C_{12}H_{25}SO_4Na$ (▲, ○, ●, □, ■), or heparin (△), and resultant fibrillization was assayed using ThT fluorescence. Each point represents fluorescence in arbitrary units corrected using inducer-only and α-synuclein-only control reactions. Each line represents the best polynomial fit to each set of data points. Induction of α-synuclein fibrillization required a threshold of ~100 μM $C_{12}H_{25}SO_4Na$, above which fluorescence increased biphasically, with maximal levels dependent on α-synuclein concentration. In contrast, heparin treatment (shown in units of micromolar saccharide monomer) induced α-synuclein fibrillization only weakly in 16 h.

FIG. 3. **Anionic detergents and arachidonic acid induce optimal α-synuclein fibrillization at different mol/mol ratios.** The concentration of $C_{20}H_{41}SO_4Na$ (▲), $C_{18}H_{37}SO_4Na$ (○), $C_{17}H_{35}SO_4Na$ (●), $C_{16}H_{33}SO_4Na$ (□), $C_{14}H_{29}SO_4Na$ (■), and arachidonic acid (△) required for maximum ThT fluorescence determined from dose-response data (see Fig. 2) was plotted against α-synuclein concentration. Each line represents linear regression analysis of the data points for each inducer, with slopes corresponding to the detergent:α-synuclein molar ratio yielding maximal ThT fluorescence. The ratio varied for alkyl sulfate detergents from 18.2 ± 1.3 mol/mol for $C_{14}H_{29}SO_4Na$ to 6.9 ± 0.8 mol/mol for $C_{20}H_{41}SO_4Na$ and was 31.2 ± 1.5 mol/mol for the fatty acid arachidonic acid. All x axis intercepts were positive and in the range of 1–2 μM, suggesting that fibrillization was measurable down to these levels of α-synuclein.

significant numbers of twisted filaments with estimated widths of 12–20 nm were observed (Fig. 1*B*). In contrast, incubations with vehicle alone invariably produced few filaments over 16 h of incubation, even at 30 μM α-synuclein (Fig. 1*C*; see time course below). Together these data confirmed that arachidonic acid was an efficacious accelerator of α-synuclein fibrillization and that the resultant filaments were morphologically similar to both those obtained from protracted incubation of recombinant α-synuclein without inducer and to those found in disease tissue (6, 7, 22, 23).

Because arachidonic acid shares chemical properties with detergents, a series of ionic and nonionic synthetic detergents also were tested for the ability to induce recombinant α-synuclein polymerization. For purposes of comparison, the analysis included the established α-synuclein fibrillization inducer heparin (30). Nonionic detergents tested included the polyoxyethylenes Tween 20, C_8E_4, $C_{10}E_6$, $C_{10}E_8$, $C_{12}E_{23}$, and $C_{14}E_8$, cationic detergents included $C_{15}H_{34}BrN$ and $C_{19}H_{42}BrN$, zwitterionic detergents included zwittergent 3–16, and anionic detergents included alkyl sulfates containing 12–20 carbons. Of these only the anionic detergents were capable of inducing significant α-synuclein fibrillization (using both transmission electron microscopy and fluorescence aggregation assays) when tested at 25–5000 μM concentration for up to 16 h (Fig. 1*D*). Although heparin induced a small amount of fibrillization within 16 h as shown previously (30), the signal produced in the fluorescence assay was negligible relative to those obtained from anionic detergents and arachidonic acid (Fig. 2). Together these data showed that both straight alkyl chain anionic detergents and fatty acids (but not cationic or nonionic detergents) were strong inducers of α-synuclein fibrillization and far more efficacious than heparin.

Mechanism of Detergent-mediated α-Synuclein Fibrillization—To assess potency, fibrillization activity of fatty acids and alkyl sulfates was quantified as a function of both inducer and α-synuclein concentrations. All resultant dose-response curves were biphasic (representative data from fluorescence assays are shown only for the anionic detergent $C_{12}H_{25}SO_4Na$ in Fig. 2). Some of the curves showed a clear *abscissa* intercept, which
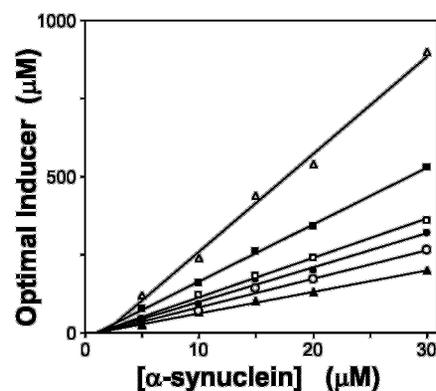
was especially pronounced for short-chain detergents. For $C_{12}H_{25}SO_4Na$, this threshold was ~100 μM, below which no fibrillization activity was detectable after 16 h of incubation (Fig. 2). Above this threshold concentration, maximal filament yield increased with increasing detergent concentration to an optimum and then decreased. The optimal detergent concentration depended on α-synuclein concentration (Fig. 2). Plots of optimal inducer concentration *versus* α-synuclein concentration were linear for arachidonic acid and all anionic detergents tested except $C_{12}H_{25}SO_4Na$, showing that fibrillization efficiency depends on the detergent:α-synuclein ratio (Fig. 3). Under optimal detergent/α-synuclein ratios, the minimum α-synuclein concentration required to support measurable detergent-induced fibrillization (*i.e.* the x axis intercept) was between 1 and 2 μM for all inducers. These data suggest that the minimal concentration of α-synuclein required to support fibrillization is no greater than low micromolar under these conditions.

Detergent-induced α-Synuclein Fibrillization Correlates with Micelle Formation—The molar ratio of detergent:α-synuclein yielding maximum activity differed for each detergent tested (Fig. 3). Replots of these data revealed a log-linear inverse relationship with alkyl chain length, suggesting that hydrophobic interactions were a major determinant of inducer potency (Fig. 4). To determine whether these hydrophobic interactions involved detergent micellization, CMC values for arachidonic acid and alkyl sulfate detergents were measured in the presence and absence of α-synuclein. Consistent with earlier observations (47) the CMC for arachidonic acid in assembly buffer alone was measured as 236 ± 12 μM, which was well above the concentration required for acceleration of α-synuclein fibrillization. When CMC was measured in assembly buffer complete with α-synuclein at 15 μM, however, the CMC decreased to 10.3 ± 4.8 μM. This magnitude of CMC depression is similar to that induced by tau protein (48). These data show that fatty acids such as arachidonic acid aggregate to form micelles at much lower concentrations in the presence of α-synuclein than in its absence.

These observations were extended to a series of alkyl sulfate detergents, which follow a log-linear relationship between
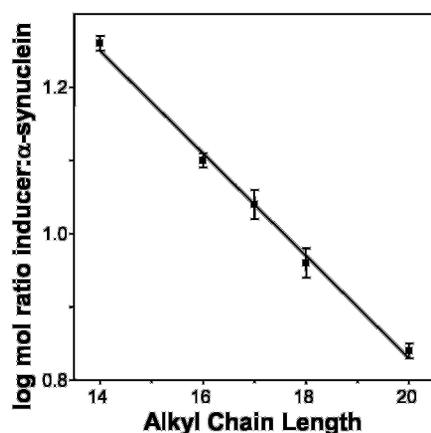
FIG. 4. **Detergents induce fibrillization at low α-synuclein concentrations.** The molar ratios of alkyl sulfate detergents to α-synuclein producing maximal ThT fluorescence were calculated from Fig. 3 and plotted as logarithms against detergent alkyl chain length. The line represents linear regression analysis of the data points. Optimal detergent:α-synuclein stoichiometry is inversely proportional to alkyl chain length.
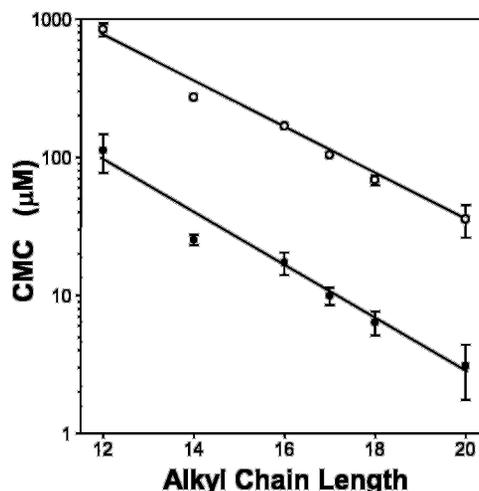

FIG. 5. **α-Synuclein depresses the CMC of alkyl sulfate detergents.** CMC values for alkyl sulfate detergents were determined (37 °C) in assembly buffer in the presence (●) and absence (○) of α-synuclein (15 μM). Although both curves show an inverse log-linear relationship between alkyl chain length and CMC (*solid lines*), the presence of α-synuclein greatly depressed measured CMC values relative to buffer alone.
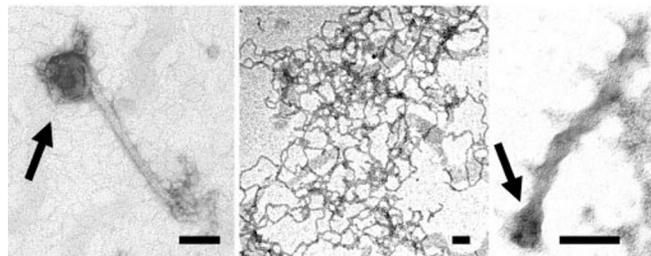

FIG. 6. **Induction of α-synuclein fibrillization by anionic lipids.** α-Synuclein (*A*, 5 μM; *B* and *C*, 50 μM) was incubated (4.5 h at 37 °C) with preformed PA (*A*, 200 μM), PS (*B*, 100 μM), or PIP (*C*, 300 μM) vesicles and then examined by transmission electron microscopy. Each of these lipids induced α-synuclein fibrillization. Vesicles associated with at least one filament end were a common observation for all the samples incubated in the presence of PA and PIP (*arrows*). *Bars* = 100 nm.

CMC and alkyl chain length when analyzed in water (49). The slope of this relationship is proportional to the free energy contribution of transferring methylene groups from solvent to micelles (49). When measured in the presence of assembly buffer (without α-synuclein), the relationship between log CMC and alkyl chain length remained linear but was depressed (relative to values measured in water) because of the presence of neutral electrolyte (100 mM NaCl) in the buffer (50). The free energy of micelle formation per methylene group determined from the slope of this plot was 264 ± 65 kcal/mol (Fig. 5). When measured in assembly buffer complete with α-synuclein, however, CMC values depressed still further, so that they were one order of magnitude below values observed in buffer alone (Fig. 5). Yet the free energy of micelle formation per methylene group determined from the slope of this plot (237 ± 38 kcal/mol) was statistically indistinguishable (*p* < 0.05) from the value determined for assembly buffer alone.

These data show that α-synuclein exerted a profound effect on the micellization of anionic detergents, that the micelles formed in the presence of α-synuclein resembled authentic micelles with respect to their free energies of formation, and that anionic micelles were present under all assembly conditions that yielded α-synuclein filaments (*e.g.* Fig. 1).

Anionic Liposomes Induce α-Synuclein Fibrillization—To determine whether lipids could substitute for anionic detergents as fibrillization inducers, α-synuclein (5–50 μM) was incubated (4.5 h at 37 °C) with liposomes freshly prepared from 25–400 μM PS, PA, PC, PI, PIP, or PIP$_2$ and analyzed using the electron microscopy assay. CMCs for these lipids in aqueous solution have been estimated in the nanomolar range (51), assuring that they were almost completely vesicular before incubation with α-synuclein protein. Examination of reaction products by electron microscopy showed the presence of vesicles and abundant filaments and/or filamentous conglomerates with the anionic liposomes composed of PS, PA, PI, PIP, and PIP$_2$ (data for PS, PA, and PIP are shown in Fig. 6). At high magnification, a portion of well resolved filaments could be seen associated endwise with vesicles (Fig. 6). In contrast, PC liposomes yielded extremely few filaments (data not shown). These data show that anionic but not zwitterionic liposomes accelerate α-synuclein fibrillization and suggest that the mechanism of polymerization induction may involve nucleation of filaments at the vesicle surface.

Kinetics of Detergent Induced α-Synuclein Fibrillization—To determine whether anionic detergents influence filament nucleation, the kinetics of micelle-mediated α-synuclein assembly was followed by ThT fluorescence over a period of up to 44 h in the presence of 0, 150, 200, 250, 300, and 350 μM C$_{12}$H$_{25}$SO$_4$Na at 37 °C with constant agitation. Reactions were initiated by the addition of C$_{12}$H$_{25}$SO$_4$Na (when present), which produced a small immediate rise in fluorescence followed by a much larger sigmoidal increase in fluorescence over time (shown for 350 μM C$_{12}$H$_{25}$SO$_4$Na in Fig. 7, *top*). The final equilibrium level achieved was roughly proportional to C$_{12}$H$_{25}$SO$_4$Na concentration (see Fig. 2). All curves were consistent with nucleation-dependent kinetics (52). Replotting these data assuming a logistic process (53) yielded straight lines of varying slopes, showing that all reaction progress curves remained logistic even when accelerated in the presence of detergent (Fig. 7, *bottom*). Kinetic parameters of lag time and k_{app} were derived from each of these curves as described under "Experimental Procedures." In the absence of detergent, 5 μM α-synuclein aggregated with a lag time of 16.1 h and k_{app} of 0.32 h^{-1} (Fig. 7, *top inset*). Similar values had been obtained previously using non-His tagged α-synuclein (30). Plotting these parameters *versus* inducer concentration (Fig. 8) revealed that as C$_{12}$H$_{25}$SO$_4$Na approached optimal concentrations (~350 μM at 5 μM α-synuclein; Fig. 2), fibrillization lag time decreased to as low as ~11 min, whereas k_{app} increased to as high as 10.4 h^{-1}. Together these data show that incubation of α-synuclein with anionic detergent micelles

FIG. 8. **Detergent fibrillization inducers modulate α-synuclein nucleation.** Lag times (□) and apparent growth rate constants (k_{app}; ■) were determined from data in Fig. 7 and plotted against $C_{12}H_{25}SO_4Na$ inducer concentration. In the absence of detergent, α-synuclein fibrillization proceeded with a lag time 16.2 h (not plotted for clarity) and a k_{app} of 0.32 h^{-1}. Increasing concentrations of inducer were accompanied by large decreases in lag times and large increases in k_{app}.
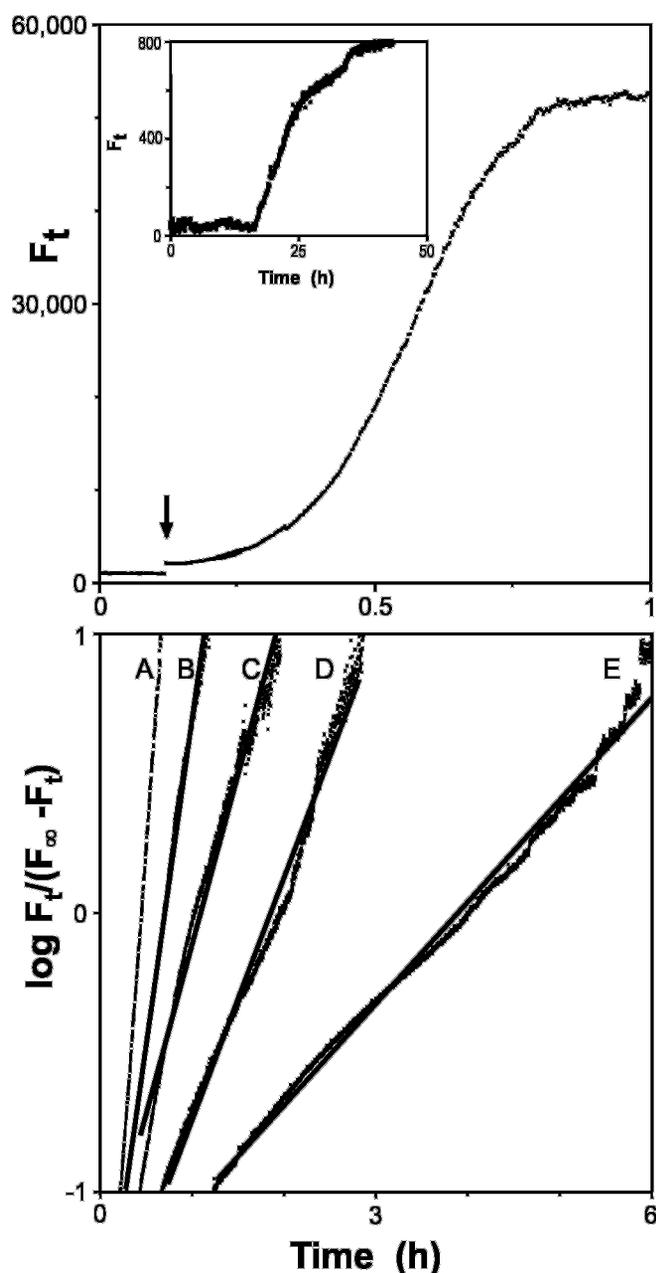
FIG. 7. **Time course of α-synuclein fibrillization.** *Top*, fibrillization of α-synuclein (5 μM) in the absence (*inset*) or presence of 350 μM $C_{12}H_{25}SO_4Na$ was followed using ThT fluorescence (F_t) as a function of incubation time at 37 °C with constant agitation. The *arrow* marks the time of detergent addition. In the absence of detergent, α-synuclein aggregated after a pronounced lag phase. The reaction was much faster and yielded higher levels of fluorescence at equilibrium in the presence of detergent. *Bottom*, α-synuclein fibrillization time courses were plotted in linear form assuming logistic growth kinetics. Each point represents the value $\log (F_t/(F_\infty - F_t))$ *versus* incubation time, where F_∞ represents an estimation of fluorescence at equilibrium, and where time is marked from the addition of $C_{12}H_{25}SO_4Na$ inducer. Each line represents a linear least squares fit of the data. Final concentrations of $C_{12}H_{25}SO_4Na$ were 350 μM (data from *top panel*) (*A*), 300 μM (*B*), 250 μM (*C*), 200 μM (*D*), and 150 μM (*E*). The time at which $\log (F_t/(F_\infty - F_t)) = 0$ was taken as t_{50} and used to calculate lag time as described under "Experimental Procedures."

leads to the greatest acceleration of α-synuclein fibrillization yet described *in vitro* and suggest that it results in part from greatly enhancing filament nucleation.

DISCUSSION

Fatty acids such as arachidonic acid, anionic detergents, and anionic lipids form a class of powerful α-synuclein fibrillization
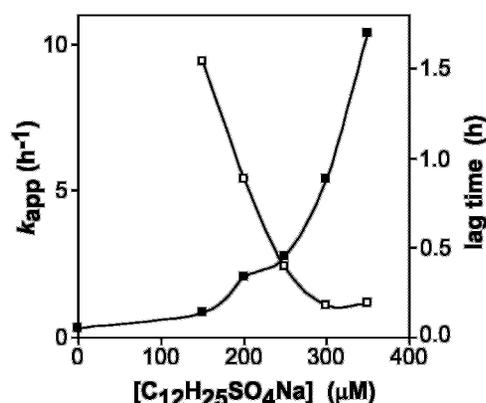
inducers active over a broad range of α-synuclein concentrations (4–70 μM) at physiological conditions of ionic strength, temperature, pH, and reducing environment. Each member of the class shares two key structural features, an alkyl chain and an anionic head group. The alkyl chain may be saturated, unsaturated, or part of fatty esters such as in phospholipids. Although α-synuclein appears to contain a degenerate binding site for fatty acid monomers (54), the data presented here suggest that the alkyl moieties of fatty acids support fibrillization by interacting to form micelles rather than by direct protein binding through these sites. First, the presence of α-synuclein greatly depressed inducer CMCs compared with values determined in water or buffer alone, so that all inducers were mostly micellar at concentrations that accelerated fibrillization. This was most clearly observed in the case of $C_{12}H_{25}SO_4Na$, where fibrillization-inducing activity was not detectable until the CMC threshold (~100 μM at 15 μM α-synuclein) was exceeded (Fig. 2). Micelles formed under these conditions were energetically indistinguishable from authentic micelles formed in assembly buffer alone. Second, filament yield as detected by electron microscopy or ThT fluorescence initially increased above the CMC, where increases in detergent concentrations are accompanied by increases in micelle but not monomer concentrations (55). Finally, preformed anionic liposomes (*i.e.* having extremely low CMCs in the absence of α-synuclein) such as PS or PA were fibrillization inducers.

The requirement for an anionic head group could be met with sulfate or carboxylate moieties and could be presented as part of a phospholipid head group as in the case of PS. The activity of PA, PI, PIP, and PIP$_2$ suggests that phosphate head groups also support α-synuclein fibrillization. The negative charges appear to supply more than just hydrophilic character to support micelle formation, because neither cationic nor nonionic detergents of similar alkyl chain length or CMC induced α-synuclein fibrillization. Moreover, zwitterionic micelles or vesicles composed of zwittergent 3–16 or PC did not accelerate α-synuclein fibrillization. The reported modest binding of α-synuclein to zwitterionic phospholipids like PC (56, 57) is consistent with the observations reported here. Therefore, it appears that the key role of ionizable groups is to present a negatively charged surface on the micelle or vesicle.

Anionic surfaces have been shown to promote protein aggregation and fibrillization at low protein concentrations (58). Binding to these surfaces may concentrate α-synuclein mole-

cules such that the energy barrier for nucleation is overcome, as suggested for other aggregating proteins (39, 47). Consistent with this hypothesis, α-synuclein filaments occasionally could be observed extending from the surface of vesicles composed of pure anionic lipid (Fig. 6). This observation does not, however, rule out the possibility that α-synuclein partially inserts into the hydrophobic core of the vesicles in these complexes. Association of α-synuclein with anionic surfaces can also lead to stabilization of helical secondary structure (33, 36), which could be an intermediate on the pathway to fibrillization as it is with β-amyloid (59) and insulin (60). In these cases, fibrillization proceeds after transition of the partially folded intermediate from α-helix to β-sheet (61).

The fibrillization of α-synuclein has been modeled as a nucleation dependent process because it is accompanied by a lag phase, shows a seeding effect, and is supported by minimal concentration of monomer at equilibrium (45). Like other amyloids, α-synuclein is thought to fibrillize according to the general scheme,

$$U \leftrightarrow I \begin{array}{c} \nearrow N \\ \downarrow \\ \searrow F \end{array}$$
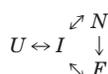
SCHEME I

where U represents the unfolded state, I represents intermediate forms that may contain secondary or quaternary structure and consist of multiple alternatives, N represents the nucleus, the formation of which is rate-limiting, and F represents filamentous forms, which again may be multiple and include protofilaments. Mature filaments eventually reach equilibrium with soluble protein, presumably in its U and I forms. On the basis of results with $C_{12}H_{25}SO_4Na$, anionic micelles appear to accelerate fibrillization by greatly increasing nucleation as reflected in a nearly ~90-fold decrease in lag time (when assayed at 5 μM α-synuclein) and a ~30-fold increase in k_{app}. Moreover, the final equilibrium levels of ThT fluorescence increases in the presence of inducer, suggesting an decrease in the proportion of α-synuclein in non-fibrillar form and, hence, a decrease in the minimal concentration required to support fibrillization. These properties, seen here with anionic detergents but also previously to a less dramatic extent with heparin (30), metals (62), and extremes of pH (41), suggest that it is the stabilization of an intermediate with greater ability to nucleate and with higher affinity for the ends of mature filaments that leads to the observed changes in kinetic and equilibrium properties. This model assumes that the interaction between α-synuclein and detergent micelles is reversible and that measured ThT fluorescence is proportional to total filament mass. In contrast, inducers that act primarily by inducing amorphous aggregates of α-synuclein increase fibrillization kinetics without affecting the minimal concentration of assembly (31).

Micelle-mediated induction of α-synuclein fibrillization is biphasic with a clear optimum defined by a specific inducer: protein molar ratio for each inducer. Fibrillization efficiency steadily decreases above this level, which is consistent with the inhibition of fibrillization seen previously at high concentrations of anionic lipid (56). Although all anionic detergents and fatty acids tested promoted fibrillization, their biphasic induction curves were shifted to lower inducer concentrations as the length of the alkyl chain increased. Because the degree of ionization of alkyl sulfate micelles is independent of aggregation number and salt concentration, it is unlikely that differences in anionic detergent potency stem from chain-length-dependent variations in micelle surface charge (64). Rather, a relationship between potency and CMC is suggested from the alkyl sulfate series. But the slope of the inverse relationship

between detergent:α-synuclein molar ratio and alkyl chain length is inconsistent with CMC being the sole determinant of detergent potency outside of providing a threshold concentration for activity. Although the relationship between CMC, detergent aggregation number, and hence, micelle concentration is complicated by CMC depression induced by varying protein concentrations, shorter chain detergents typically yield higher concentrations of smaller micelles than do longer chain detergents (65). The lower potency of short chain detergents suggests that it is not just micelle number concentration but micelle size or surface area that defines potency in terms of detergent:α-synuclein molar ratios. Additional experimentation will be required to resolve this point.

The results reported here again show the importance of measuring detergent and fatty acid CMC under relevant assay conditions instead of relying on tabular data collected in water or other solvents. Depression of the CMC for anionic detergents is regularly observed in the presence of cationic electrolytes (66) including proteins such as tau (48). But whereas tau protein contains a net positive charge at assay pH, α-synuclein is negatively charged. It is likely, therefore, that for amphoteric polyelectrolytes such as proteins, depression of anionic surfactant CMC is mediated by clustered charge rather than net charge and in this sense resembles association between proteins and ion exchange resins. But hydrophobic interactions can also contribute to CMC depression (as shown for nonprotein analytes; Ref. 66), and this may be the reason that even short peptides can depress CMC (67). Conclusions regarding the micellar state of anionic surfactant required to induce conformational change and subsequent aggregation of proteins as diverse as β2-glycoprotein I (68), complement receptor 1-derived peptide (69), and prion protein (70) must be reassessed in light of these considerations.

Although other polyanions such as glycosaminoglycans have emerged as α-synuclein polymerization inducers *in vitro*, their structures have not pointed toward a clear cellular agent that would serve as a substrate for α-synuclein fibrillization in disease. For example, heparan sulfate proteoglycans are extracellular and sequestered from the bulk of physiological α-synuclein protein. In contrast, the activity of anionic micelles and vesicles points toward cellular membranes as naturally abundant intracellular sources of clustered negative charge. Indeed, interaction with cellular membranes is thought to be part of the normal function of α-synuclein. In addition to colocalizing with synaptic vesicles (71–75), aggregates of α-synuclein can be found on the surface of the rough endoplasmic reticulum (76). Expression of α-synuclein in neuronal culture leads to membrane association, which may serve to trigger aggregation (77, 78). Consistent with this hypothesis, lipids have been found associated with authentic human brain Lewy bodies (79, 80). Together these data suggest that events that enhance or prolong the association between small amounts of α-synuclein and cellular membranes with significant anionic content, which normally appears to be transient and reversible (36, 63), may nucleate fibrillization *in vivo*.

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Micelle-dependent α-Synuclein Fibrillization

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