α-Synuclein Can Function as an Antioxidant Preventing Oxidation of Unsaturated Lipid in Vesicles†

Min Zhu, Zhi-Jie Qin, Dongmei Hu, Larissa A. Munishkina, and Anthony L. Fink*

Department of Chemistry and Biochemistry, University of California, Santa Cruz, California 95064

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ABSTRACT: α-Synuclein, a presynaptic protein associated with Parkinson's disease, is found as both soluble cytosolic and membrane-bound forms. Although the function of α-synuclein is unknown, several observations suggest that its association with membranes is important. In the present study we investigated the effect of α-synuclein on lipid oxidation in membranes containing phospholipids with unsaturated fatty acids. The kinetics of lipid oxidation were monitored by the change in fluorescence intensity of the dye C11-BODIPY. We find that monomeric α-synuclein efficiently prevented lipid oxidation, whereas fibrillar α-synuclein had no such effect. Our data suggest that the prevention of unsaturated lipid oxidation by α-synuclein requires that it bind to the lipid membrane. The antioxidant function of α-synuclein is attributed to its facile oxidation via the formation of methionine sulfoxide, as shown by mass spectrometry. These findings suggest that the inhibition of lipid oxidation by α-synuclein may be a physiological function of the protein.

Deposition of fibrillar α-synuclein is involved in several neurodegenerative diseases, including Parkinson's disease (PD)[1] (*1*−*4*). α-Synuclein is an abundant 140 amino acid neuronal protein that is intrinsically disordered (*5*). Its function is currently unknown. A significant fraction of α-synuclein is localized with membrane fractions, especially synaptic vesicles (*6*−*11*), and is associated with vesicular transport processes (*12*−*14*). These findings, together with in vitro studies, indicate that α-synuclein plays a key role in vesicle function (*15*−*20*).

Oxidative stress leads to oxidation of unsaturated phospholipids, and the resulting breakdown of the plasma or intracellular membranes may be a key pathogenic event in PD. Lipid oxidation increases with aging, and a role for lipid peroxidation in PD has been demonstrated (*21*). The intrinsically high potential oxidative stress levels in dopaminergic neurons, due to the sensitivity of dopamine to oxidation and generation of H_2O_2, and the high level of unsaturated fatty acids in their membranes mean that these neurons will be subject to increased levels of oxidation of unsaturated lipids in the presynaptic vesicles and plasma membrane. α-Synuclein has four methionine residues which are particularly sensitive to oxidation, probably due to their solvent exposure (*22*, *23*). In the presence of a methionine sulfoxide reductase, a cycle of α-synuclein Met oxidation and reduction can be envisaged, which, in conjunction with membrane-associated α-synuclein, would help to protect the highly unsaturated lipids in the neuronal membranes from oxidative damage (*22*). Thus, we hypothesize that one of the normal functions of α-synuclein involves the protein acting as an antioxidant to protect unsaturated lipids from oxidation. In the present study, we show that lipid oxidation is inhibited by monomeric, but not fibrillar, α-synuclein and that α-synuclein is indeed oxidized in this process, confirming that α-synuclein acts as an antioxidant for membrane lipid oxidation.

MATERIALS AND METHODS

Expression, Purification, and Treatment of α*-Synuclein for Kinetics and Lipid Oxidation Experiments.* Recombinant α-synuclein was expressed in the *Escherichia coli* BL21 cell line transfected with pRK172/α-synuclein plasmid (gift of M. Goedert, MRC, Cambridge) and purified as described previously (*24*). A stock solution of purified α-synuclein was treated with NaOH (final concentration 0.001 M), adjusted to pH 7.4, and centrifuged for 20 min at 14000 rpm to remove insoluble aggregated species, followed by treatment with Chelex resin to remove any existing metals such as copper and iron, which may catalyze lipid oxidation, and used within 1 day. Iron and copper content was measured after Chelex treatment.

Chemicals. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidic acid (DPPA), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidic acid (POPA), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol) (POPG), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), and 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (SAPC) were purchased from Avanti Polar Lipids as chloroform solutions. C11-BODIPY

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* Correspondence should be addressed to this author. Tel: (831) 459-2744. Fax: (831) 459-2935. E-mail: enzyme@cats.ucsc.edu.

[1] Abbreviations: PD, Parkinson's disease; ThT, thioflavin T; C11-BODIPY, 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-undecanoic acid; SUV, small unilamellar vesicle; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPA, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidic acid; POPA, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidic acid; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol); POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; SAPC, 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine.

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581/591 was obtained from Molecular Probes, Inc., and stored at −20 °C under nitrogen in the dark. Thioflavin T (ThT), hemin, and Chelex were purchased from Sigma and were used without further purification.

Preparation of SUVs. SUVs of POPA/POPC, POPG/POPC, and POPG/POPE (molar ratio, 1:1), as well as POPC and SAPC, were prepared under nitrogen by sonication as described previously (*16*). This procedure routinely produced SUVs with diameters of 20−25 nm (*25*).

Oxidation of SUVs. The molar ratio of C11-BODIPY 581/591 and phospholipid was 1:200. Oxidation was initiated by autoxidation or adding 2 mM H_2O_2 and 0−50 μM hemin or 0−60 μM Fe^{2+} with stirring at 600 rpm in the presence of 100 mM NaCl and 20 mM Tris-HCl buffer (pH 7.4) at 37 °C. Lipid oxidation was determined by monitoring the fluorescence increase of the oxidation product as described below and confirmed by the thiobarbituric acid assay (*26*). The total phospholipid concentration varied from 0.1 to 2.5 mg/mL, and total protein concentrations varied from 0.005 to 2.5 mg/mL. The C11-BODIPY concentration of the stock solutions was determined by measuring the absorbance at 581 nm using a molar extinction coefficient of 139444 L/mol.

Fluorescence Monitoring of Lipid Oxidation. Fluorescence measurements were performed at ambient temperature using a Fluoromax-3 spectrofluorometer (Jobin Yvon Horiba). Emission spectra of 0.05 μM C11-BODIPY in DPPC were recorded from 490 to 640 nm after exciting at 480 nm. The spectra were collected every 10 min after the oxidation was initiated by the addition of 2 mM H_2O_2 and 5 μM hemin.

Lipid Oxidation Monitored Using a 96-Well Plate Reader. The vesicles with C11-BODIPY (1:200) were dispensed into a 96-well plate. A plate reader (Fluoroskan ascent CF, Labsystems Inc.) was used to monitor the increase in fluorescence due to oxidation of C11-BODIPY. The excitation and emission band-pass filters were 485 and 510 nm, respectively. The plate was shaken at 600 rpm with a diameter of 1 mm at 37 °C. Fluorescence intensities were recorded for 10−30 h at intervals of 10 min.

Circular Dichroism (CD) Measurements. Far-UV CD spectra were collected using an Aviv CD spectrometer, model 60DS (Lakewood, NJ). Spectra were obtained by scanning from 195 to 250 nm at a step resolution of 1 nm, bandwidth of 1.5 nm, and average time of 5 s. The final spectra were the average of five individual scans.

Mass Spectrometry. Freshly prepared α-synuclein was mixed with SUVs of POPG/POPE (molar ratio, 1:1) in the presence of 2 mM H_2O_2 and 50 μM hemin in phosphate buffer with 100 mM NaCl, pH 7.4. The final concentration of α-synuclein was 0.4 mg/mL and 2 mg/mL SUV. One milliliter of this solution in a 2 mL glass vial was incubated at 37 °C for 24 h with stirring at 600 rpm. The total solution incubated above (1 mL) was mixed with 4 mL of a mixture of chloroform/methanol (2:1 v/v) and vortexed for 30 s. The mixture was then centrifuged at 10000*g* for 5 min at room temperature. The resulting aqueous (upper) and organic (lower) phases were removed. The interface, containing the protein, was lyophilized and resuspended in water. The suspended protein was run through a mini C18 column (Pharmacia) and eluted with 80% acetonitrile containing 1% formic acid before performing ESI MS analysis (MicroMass ZMD).

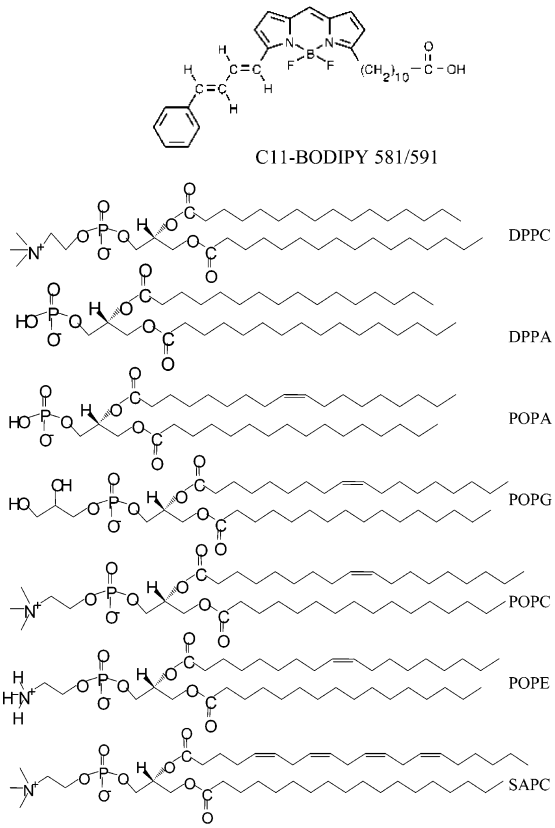

FIGURE 1: Structures of C11-BODIPY 581/591 and various lipids (see text for full names).

Determination of Iron Content of α*-Synuclein.* The standard colorimetric assay for dissolved Fe^{2+} using 1,10-phenanthroline was used to measure the iron content of the α-synuclein samples. Absorbance was measured at 510 nm. A standard curve was used for calibration.

RESULTS

C11-BODIPY 581/591 as a Probe of Lipid Oxidation. C11-BODIPY 581/591 [4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-undecanoic acid] is an oxidation-sensitive fluorescence dye (Figure 1). The dye can be incorporated readily into lipid bilayers by its fatty acyl chain and monitors the oxidation of unsaturated fatty acids in the lipid bilayer (*27*−*30*). C11-BODIPY is insensitive to superoxide and transition metal ions, and its sensitivity to oxidation is similar to that of endogenous PUFAs (*29*). To confirm that C11-BODIPY was specific and sensitive to lipid oxidation, the dye (0.5 mol %) was incorporated into several different phospholipid vesicles under various oxidation conditions. Stable SUVs are readily made from 1:1 molar ratios of many phospholipids with phosphatidylcholine. Lipid oxidation was initiated by the addition of 2 mM H_2O_2 with or without 50 μM hemin, and fluorescence spectra were collected every 10 min at 23 °C. The fluorescence of C11-BODIPY was compared in SUVs of saturated DPPA/DPPC and unsaturated POPA/POPC, POPG/POPC (containing one unsaturated bond), or SAPC (containing four unsaturated bonds) (see Figure 1 for structures). No increase in fluorescence was observed in the presence of saturated DPPA/DPPC (Figure 2A), indicating that the fluorescence signal of the dye was stable in vesicles in the absence of unsaturated fatty acid oxidation. On the other hand, as shown in Figure 2A,

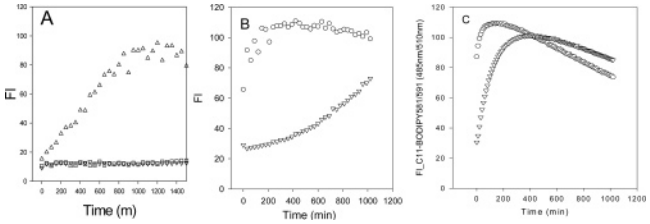

FIGURE 2: C11-BODIPY as a probe for the oxidation of unsaturated phospholipids. (A) No fluorescence increase was observed for the dye alone (squares) or for the dye incorporated into vesicles containing saturated DPPA/DPPC (inverted triangles) in the presence of the oxidant H_2O_2 (2 mM). Oxidation of monounsaturated POPA/POPC with 2 mM H_2O_2 (triangles) led to an increase in fluorescence signal of the dye. (B) POPG/POPC in the absence of H_2O_2 (inverted triangles) and in the presence of 2 mM H_2O_2 plus 50 μM hemin (circles). The addition of the hemin significantly increases the rate of oxidation. (C) SAPC (four double bonds) in the absence of H_2O_2 (inverted triangles) and in the presence of 2 mM H_2O_2/50 μM hemin (circles).

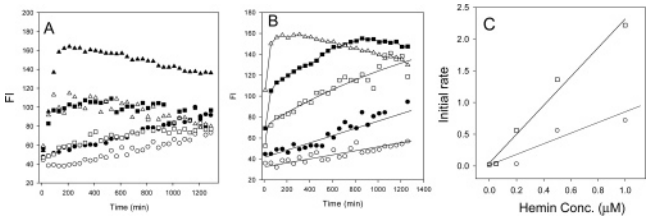

FIGURE 3: Iron catalyzes the oxidation of unsaturated phospholipids. (A) POPG/POPC and (B) POPG/POPE, with 2 mM H_2O_2 in the presence of 0 (open circles), 50 nM (solid circles), 200 nM (open squares), 500 nM (solid squares), 1 μM (open triangles), and 5 μM (solid triangles) hemin. (C) Initial oxidation rates of POPG/POPC (circles) and POPG/POPE (squares) vs hemin concentration.

when unsaturated lipid was present, the oxidation rate as measured by the dye was significant. A very similar time-dependent increase in fluorescence was seen with monounsaturated POPG/POPC, demonstrating that the nature of the headgroup did not affect the rate of oxidation of the unsaturated fatty acid. The increase in fluorescence was linear with time, indicating that the rate of oxidation was constant during the period measured. At longer time periods the fluorescence signal showed a plateau, after which the fluorescence decreased slightly due to photobleaching. When the oxidation was triggered by the addition of 2 mM H_2O_2 in the presence 50 μM hemin, higher oxidation rates were observed compared to oxidation with H_2O_2 alone (Figure 2B), indicating that hemin catalyzed the lipid oxidation. Furthermore, when C11-BODIPY was mixed with vesicles of SAPC, containing the polyunsaturated arachidonoyl chain with four double bonds, the initial oxidation rate was higher (Figure 2C) than that with POPA/POPC or POPG/POPC, which have just one double bond. Therefore, the oxidation rates measured with C11-BODIPY are consistent with the number of unsaturated groups contained in the lipids. We attribute the higher rate of oxidation of BODIPY in the presence of polyunsaturated lipids to the increased mobility of the lipids, allowing faster diffusion of oxygen to the lipid phase. Similar data but with higher oxidation rate were also observed on the addition of 2 mM H_2O_2 and 50 μM hemin to SAPC (Figure 2C).

Kinetic data were also obtained for the sequential addition of hemin or Fe^{2+} (0, 50, 200, 500, 1000 nM) for the H_2O_2-catalyzed oxidation of vesicles of both POPG/POPC and POPG/POPE (Figure 3) and POPA/POPC (data not shown). The initial rates as a function of hemin concentration are presented in Figure 3C. A linear correlation is found between initial rates and catalyst levels, at relatively low catalyst concentrations. Similar data were obtained for Fe^{2+} as catalyst (see Figure 7).

As further controls, lysozyme and polyglutamic acid were mixed with POPA/POPC SUVs (1.0 mg/mL) at 0.1, 0.5, and 1.5 mg/mL in the presence of 2 mM H_2O_2 and 50 μM hemin. No significant effect on the oxidation of POPA/POPC was observed, demonstrating that the presence of a typical protein or polyamino acid had no effect on the dye fluorescence signal or the oxidation of unsaturated lipids.

α-Synuclein Inhibits the Oxidation of POPA/POPC and POPG/POPC Vesicles. α-Synuclein is known to bind to membranes with acidic headgroups, such as PA, PS, and PG, and results in the induction of α-helical conformation (*16*, *31*−*34*). When POPA/POPC SUVs (1.0 mg/mL) were mixed with increasing concentrations of α-synuclein, a dose-dependent decrease in POPA/POPC lipid oxidation was observed with increasing α-synuclein concentration from 0.005 to 2.5 mg/mL (Figure 4A). The initial oxidation rates showed a first-order decrease as protein concentration increased (Figure 4B). We also examined SUVs of POPA/POPC at various concentrations of vesicles, from 0.02 to 2.5 mg/mL, mixed with 0, 0.1, or 0.5 mg/mL α-synuclein to form protein−lipid mixtures at ratios over the range of 0−5. The initial lipid oxidation rates (as fluorescence change per minute per mg/mL lipid) are shown in Figure 4C. The presence of α-synuclein clearly inhibits the lipid oxidation under all the conditions examined, including with protein concentration as low as 0.005 mg/mL (350 nM). As shown in Figure 4C with low concentrations of both α-synuclein and lipid very large decreases in oxidation rate are observed, indicating that in the absence of metal contamination (see below) α-synuclein is an extremely effective inhibitor of lipid oxidation.

In the case of POPG/POPC (Figure 5A) and POPG/POPE (data not shown), lipid oxidation was inhibited with increasing α-synuclein concentrations in the range of 0.005 (0.02 for POPG/POPC) to 0.1 mg/mL. However, subsequent increases in protein concentration led to decreased inhibition of oxidation: this is attributed to catalysis by contaminating Fe^{2+} in the α-synuclein (see below). The maximal inhibition was observed at 0.1 mg/mL, with less inhibition at 0.5 mg/mL; subsequently, lipid oxidation was enhanced by α-synuclein at a high concentration of 1.5 mg/mL (Figures 5B and 6A). We also examined POPG/POPC and POPG/POPE SUVs at various lipid concentrations (0.05−1.25 mg/mL) in the presence of 0.1 or 0.5 mg/mL α-synuclein (Figures 5C and 6B). Under all conditions, α-synuclein inhibited the lipid oxidation. It is noteworthy that, for 0.1 mg/mL POPG/POPC or POPG/POPE combined with 0.5 mg/mL α-synuclein, the protein to lipid ratio is 5, which was much higher than the condition with 1.5 mg/mL α-synuclein in 1 mg/mL lipid. This means the enhanced oxidation rate was not due to the higher protein to lipid ratio but rather to the higher α-synuclein concentration itself. This was confirmed in experiments described below. Such effects were not observed with the POPA/POPC vesicles (Figure 3) due to the tighter binding of α-synuclein to POPA.

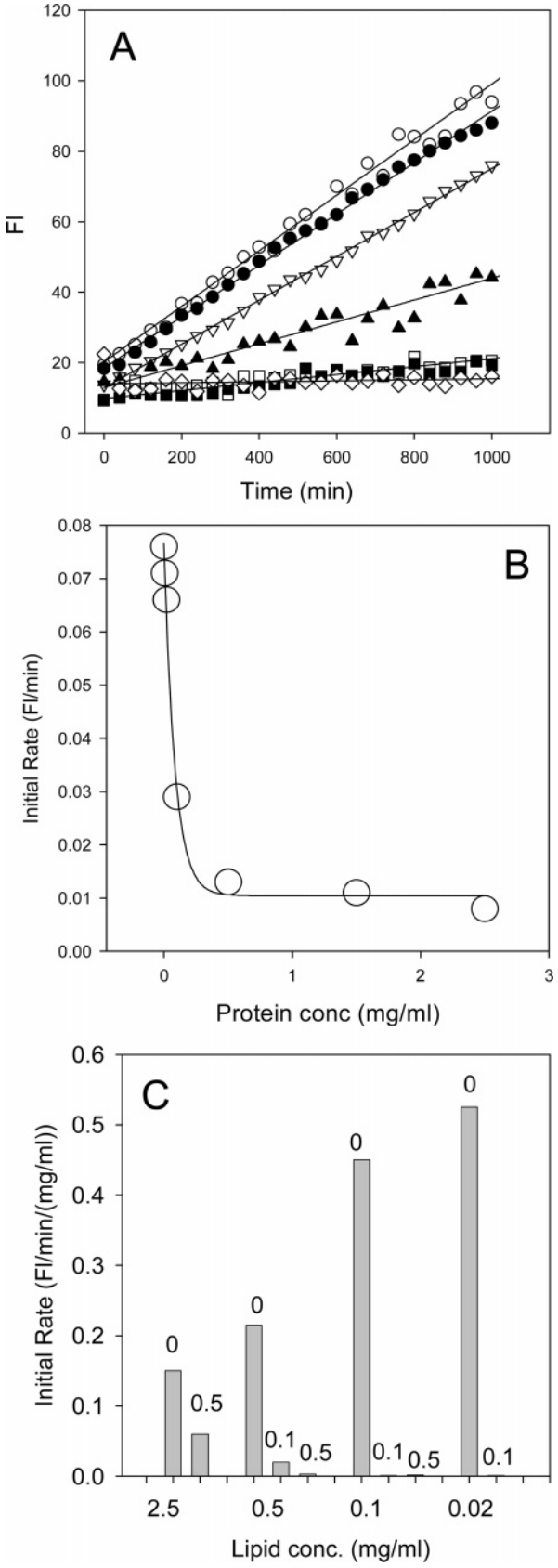

FIGURE 4: α-Synuclein inhibits lipid oxidation. (A) Oxidation of POPA/POPC vesicles by H_2O_2 in the presence of increasing concentrations of α-synuclein: 0 (open circles), 0.005 (filled circles), 0.02 (open triangles), 0.1 (filled triangles), 0.5 (open squares), 1.5 (filled squares) and 2.5 (diamonds) mg/mL. (B) Initial oxidation rate as a function of α-synuclein concentration. (C) Initial oxidation rates as a function of both α-synuclein (numbers above bars, in mg/mL) and lipid concentration.

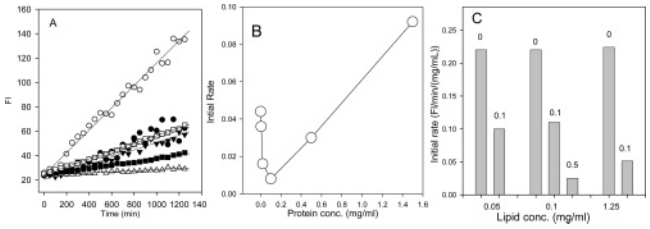

FIGURE 5: α-Synuclein inhibition of lipid oxidation is concentration dependent. (A) Oxidation of POPG/POPC SUVs in the presence of α-synuclein: 0 (open circles), 0.005 (filled circles), 0.02 (filled triangles), 0.1 (open triangles), 0.5 (filled squares), and 1.5 (open squares) mg/mL. (B) Initial oxidation rate as a function of α-synuclein concentration for POPG/POPC. (C) Initial oxidation rates as a function of lipid and α-synuclein concentration for POPG/POPC.

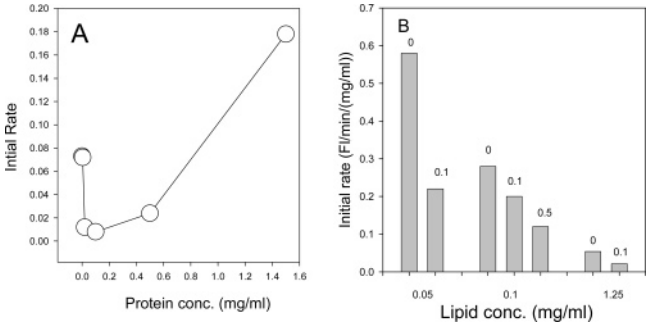

FIGURE 6: α-Synuclein inhibition of lipid oxidation is concentration dependent and lipid specific. (A) Initial oxidation rate as a function of α-synuclein concentration for POPG/POPE. (B) Initial oxidation rates as a function of lipid and α-synuclein concentration for POPG/POPE.

To demonstrate that the inhibitory effect of α-synuclein on lipid oxidation required α-synuclein bound to the vesicle, we investigated the effect of α-synuclein on lipid oxidation in vesicles of POPC, which is known not to bind α-synuclein significantly due to the lack of an acidic headgroup. α-Synuclein did not inhibit lipid oxidation in SUVs of POPC over a wide range of concentrations (0.1−2.0 mg/mL). Instead, the presence of α-synuclein led to enhanced oxidation (Figure 7A). The initial oxidation rates versus α-synuclein concentration displayed a linear relationship (Figure 7B). We hypothesized that trace amounts of metals, such as iron, tightly bound to α-synuclein are responsible for the enhanced lipid oxidation. α-Synuclein used in this experiment was treated with Chelex resin to remove iron (and other metal) ions. However, Chelex treatment was not able to remove all of the iron completely, and a trace amount of iron, an average of 650 nM in 70 μM (1.0 mg/mL) protein, was detected. This trace amount of iron was enough to catalyze the lipid oxidation. We compared the POPC oxidation rates in the presence of Fe^{2+}, hemin, and α-synuclein with the results shown in Figure 7C. The iron content of α-synuclein was based on a concentration calculated from 650 nM iron/70 μM α-synuclein. The linear relationship of initial rate versus iron concentration for α-synuclein is identical to the one for free Fe^{2+} and also close to the values for hemin (Figure 7C). Thus, the iron bound to α-synuclein is a contributing factor in catalyzing the lipid oxidation and explains why higher concentrations of α-synuclein overcame the inhibitory effect with vesicles of POPG, POPA, and POPE.

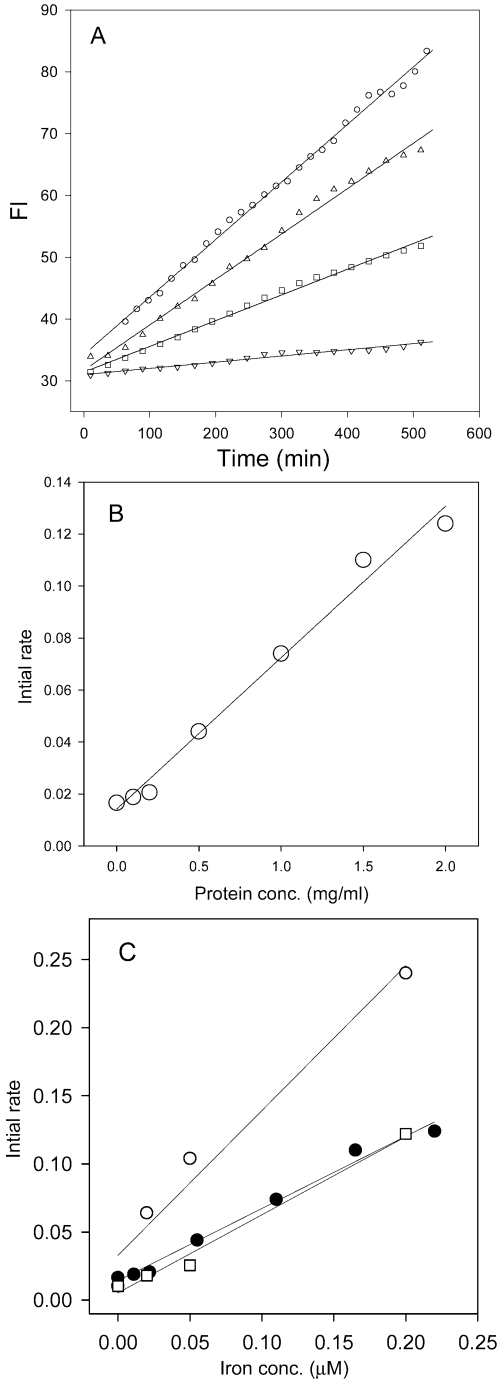

FIGURE 7: Oxidation of unsaturated POPC is stimulated by iron contained in α-synuclein. (A) Oxidation of POPC SUVs with 0 (inverted triangles), 0.5 (squares), 1.0 (triangles) and 1.5 (circles) mg/mL α-synuclein. (B) Initial oxidation rates vs α-synuclein concentration. (C) Initial rate as a function of iron concentration: hemin (open circles), Fe^{2+} (squares), Fe^{2+} in α-synuclein (filled circles). The data show the same oxidation rates for the equivalent iron concentrations from Fe^{2+} as a salt and associated with α-synuclein, confirming that the enhanced lipid oxidation with high concentrations of α-synuclein with POPG/POPC is due to the iron associated with α-synuclein.

The Inhibition of Lipid Oxidation by α-Synuclein Correlates with Its Binding Affinity for the Lipid. We next examined the binding of α-synuclein to vesicles containing unsaturated POPA, POPG, and POPC by collecting CD spectra (Figure 8A). Ellipticities at 222 nm plotted against protein to lipid ratio are shown in Figure 8B. As with saturated phospholipids (*16, 35*), increasing the mass ratio

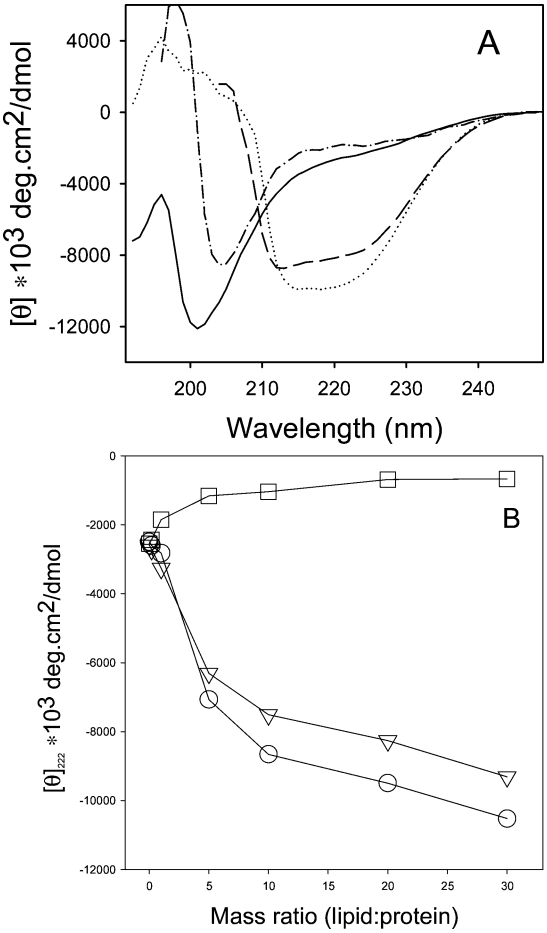

FIGURE 8: (A) CD spectra of α-synuclein (solid line) bound to POPA/POPC (dotted line), POPG/POPC (dash line), and POPC (dash−dot line) in the mass ratio 1:20 (protein to lipid) (1:1 for POPC, due to light scattering decreasing the signal below 210 nm). The phospholipid-induced helical structure may be responsible for its function as an inhibitor of lipid oxidation. (B) Plot of ellipticity at 222 nm versus mass ratio of lipid to protein: POPC, triangles; POPG/POPC, open circles; POPA/POPC, filled circles.

of POPA/POPC or POPG/POPC to protein led to an increase in helix content. In contrast, binding to POPC resulted in a decrease in ellipticity at 198 nm, but no helical structure was formed. From the results shown in Figure 8, the binding affinity of α-synuclein for unsaturated phospholipids is in the order of POPA > POPG >> POPC, the same order as for saturated phospholipids.

As discussed above, lipid oxidation was inhibited by POPA at all α-synuclein concentrations tested, only at lower α-synuclein concentrations for POPG, and not at all for POPC. Thus, the inhibition activity of α-synuclein toward unsaturated phospholipid oxidation is in the order of POPA > POPG >> POPC, the same as binding affinity, indicating that binding of α-synuclein to the vesicles is required for its antioxidant activity.

Lipid Oxidation Is Inhibited by Monomeric but Not Fibrillar α-Synuclein. α-Synuclein fibrils are a major component of Lewy bodies in PD. To determine if α-synuclein fibrils affect lipid oxidation, we added preformed mature fibrils to SUVs. Fibrils were formed by growing 140 μM α-synuclein in 100 mM NaCl and 20 mM Tris-HCl buffer (pH 7.4) at 37 °C for 36 h with agitation. At completion of the fibril formation, the solution was centrifuged to separate supernatant and pellet. The fibrils were

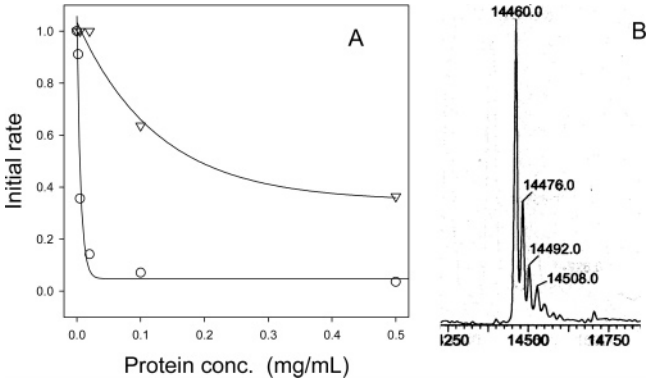

FIGURE 9: Monomeric, but not fibrillar, α-synuclein inhibits lipid oxidation and is oxidized at methionine. (A) The oxidation of POPA/POPC SUVs in the presence of monomeric (circles) and fibrillar (triangles) α-synuclein as a function of protein concentration. (B) Mass spectral analysis of α-synuclein after inhibition of lipid oxidation showing α-synuclein with one to four Met sulfoxides.

dispersed into a solution of POPA/POPC vesicles, and the rates of inhibition of oxidation were compared with those from α-synuclein monomers as a function of protein concentration. A plot of initial rates of oxidation versus protein concentrations is shown in Figure 9A. The fibrils exhibited >20-fold weaker inhibition activity of lipid oxidation compared to monomers, and it is possible that the observed activity came from adhering, nonfibrillar α-synuclein.

Characterization of α-Synuclein after Inhibition of Lipid Oxidation. If α-synuclein acts as an antioxidant, it is most likely through oxidation of its Met residues. To confirm this, we isolated the protein after 24 h of incubation with vesicles of POPG/POPE in the presence of 2 mM H_2O_2 and 50 μM hemin and analyzed it by ESI mass spectrometry. Under these conditions there is an ~100-fold molar excess double bond over α-synuclein. The MS spectrum (Figure 9B) shows that the protein is indeed oxidized through partial conversion of its Met residues to the corresponding sulfoxides and that 45% of the protein was oxidized at one or more Mets (Met-O_1, 23%; Met-O_2, 11%; Met-O_3, 8%; Met-O_4, 4%). There was no evidence for covalent modification by 4-hydroxynonenal.

Specificity of α-Synuclein as an Antioxidant in Lipid Peroxidation. To determine whether the antioxidant effect of α-synuclein is specific to α-synuclein or common to any lipid-binding protein, we also examined the effects of ApoE4 and β-synuclein. As expected, β-synuclein had an antioxidant effect essentially identical to that of α-synuclein (Figure 10A). However, apoE4 had the opposite effect, namely, significantly stimulating the rate of oxidation of POPA/POPC vesicles (Figure 10A). We also demonstrated that the antioxidant effect of α-synuclein is present in the absence of added H_2O_2; i.e., the spontaneous oxidation of unsaturated phospholipids in SUVs was inhibited by α-synuclein (Figure 10B). This was the case for both SUVs of SAPC alone and of SAPC/DPPA (1:10). The relative specificity of the antioxidant effect again showed that α- and β-synuclein had similar, strong antioxidant effects, whereas apoE4 had no effect (data not shown).

DISCUSSION

The selective neurodegeneration of dopaminergic neurons in the substantia nigra likely reflects the intrinsic oxidative

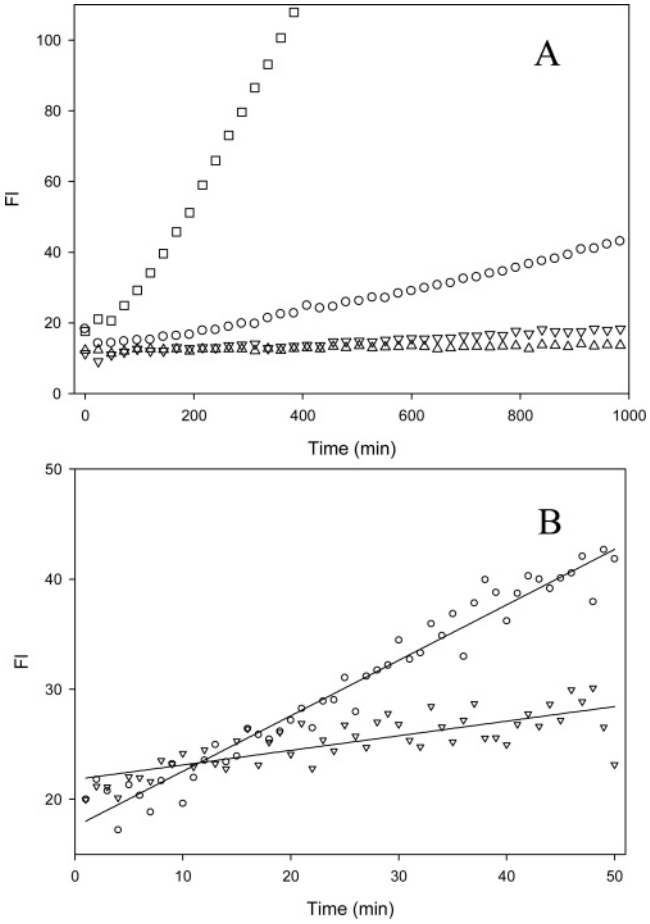

FIGURE 10: Specificity of α-synuclein antioxidant effect. (A) Oxidation of POPA/POPC vesicles by H_2O_2 in the absence of proteins (circles) and in the presence of α-synuclein (triangles), β-synuclein (inverted triangles), and apoE4 (squares). Conditions were 1.0 mg/mL SUVs (POPA/POPC, 1:1), 2 μM BODIPY, 20 mM Tris·HCl, pH 7.4, 100 mM NaCl, 37 °C, and 0.2 mg/mL protein. (B) Oxidation of SAPC/DPPA in the absence of H_2O_2: no protein (circles) and in the presence of α-synuclein (inverted triangles). Conditions were 0.5 mg/mL SUVs (SAPC:DPPA, 1:10), 2 μM BODIPY, 20 mM Tris·HCl, pH 7.4, 100 mM NaCl, 37 °C, and α-synuclein = 14 μM (0.2 mg/mL).

instability of dopamine; e.g., under physiological conditions dopamine undergoes spontaneous autoxidation in the presence of molecular oxygen, forming superoxide and H_2O_2· H_2O_2 is a major factor in lipid peroxidation leading to cytotoxicity. A number of observations implicate a functional role for α-synuclein in presynaptic vesicles, and α-synuclein has been observed to be associated with both the cytosolic surface and the lumen of vesicles (*20*). In this study we demonstrate that α-synuclein acts as an antioxidant in preventing lipid peroxidation.

The Binding of α-Synuclein to Acidic Lipids Is Responsible for the Inhibition of Lipid Oxidation. Under physiological-like experimental conditions α-synuclein, at concentrations as low as 350 nM, inhibited lipid oxidation of POPA in SUVs. Although the α-synuclein concentration in the presynaptic region of the dopaminergic neurons in the substantia nigra is unknown, it is likely to be in the hundred micromolar range, based on the total brain α-synuclein concentration (*36*). α-Synuclein also inhibited the lipid oxidation of POPG (an acidic lipid to which α-synuclein binds relatively strongly) at low protein concentrations but stimulated the lipid oxidation at high α-synuclein concentrations (100 μM).

The enhanced lipid oxidation by α-synuclein is attributed to the low concentration of Fe^{2+} bound to the protein. At low protein concentrations the levels of iron were too low to significantly catalyze the rate of oxidation, and lipid oxidation was inhibited. However, the metal-catalyzed oxidation became a major factor when the protein concentration reached 100 μM. Significantly, no inhibition of POPC oxidation by α-synuclein was observed; in contrast, a dose-dependent stimulation was observed and is attributed to the catalytic effect of the protein-bound iron. The results with POPC indicate that binding of α-synuclein is required for the inhibition of lipid oxidation (but not the Fe^{2+}-catalyzed oxidation), since α-synuclein does not bind significantly to POPC SUVs. The experiments with β-synuclein and apoE4 confirm that the antioxidant effect of α-synuclein is associated specifically with the synuclein structure, presumably the relatively disordered structure of the N- and C-terminal regions (where the Met residues are located) in the membrane-bound form of the protein.

Inhibition of Lipid Oxidation May Be a Normal Function of α*-Synuclein Monomer but Not Fibrils.* α-Synuclein has been shown to be neurotoxic as a result of aggregation mediated by association of partially folded structures. Our previous findings show that α-synuclein is protected from aggregation by binding to membranes in a helical conformation, although at low lipid concentrations relative to protein, fibrillation is accelerated (*16*). The current results indicate that the binding of monomeric α-synuclein to SUVs of unsaturated acidic lipids prevents the lipid from oxidation. Fibrillar α-synuclein, which is predominantly β-structure and cannot form the helical structure induced by lipid, does not significantly inhibit the lipid oxidation. Oxidation of unsaturated lipids in cell membranes is generally associated with cytotoxicity, probably due to the generation of reactive oxygen species, especially free radicals. Thus, the inhibition of unsaturated lipid oxidation by α-synuclein would be a neuroprotective effect. Since neurons have high levels of unsaturated lipids in their membranes, and recently α-synuclein has been shown to bind preferentially to vesicles enhanced in unsaturated fatty acids (*37*), this effect could be an important function of monomeric α-synuclein. Consequently, decreased levels of monomeric α-synuclein due to aggregation and fibrillation would contribute to increased lipid oxidation and potentially to Parkinson's disease.

How can α-synuclein inhibit lipid oxidation? Probably the simplest manner would be by acting directly as an antioxidant. It is known that the Met residues of α-synuclein are readily oxidized by H_2O_2 to the sulfoxide (*22*, *23*, *38*), and their significant solvent exposure, even in the bound helical conformation [since they are in regions that are not helical (*39*−*41*)], would make them susceptible to oxidation by peroxide to the sulfoxide. That this is indeed the likely mechanism for α-synuclein's antioxidant activity was confirmed by the presence of oxidized Met in α-synuclein after it inhibited lipid oxidation. Increased lipid peroxidation has been associated with Parkinson's disease (reviewed in ref *42*), and binding of polyunsaturated fatty acids to α-synuclein has been demonstrated, along with abnormal distribution in PD patients (*43*−*45*). A corollary of Met oxidation of α-synuclein is the formation of stable, soluble oligomers (*23*, *38*, *46*). Thus, if α-synuclein acts as an antioxidant, we would expect evidence of α-synuclein aggregation: this has been confirmed in vitro in that α-synuclein forms oligomers on exposure to membranes containing unsaturated fatty acids (*47*). That α-synuclein oxidation by unsaturated fatty acids may occur in vivo is supported by the observation of soluble oligomers of α-synuclein in normal brain supernatants, whose concentrations were increased in PD brains, and that polyunsaturated fatty acids enhanced the levels of α-synuclein oligomers in intact mesencephalic neuronal cells (*43*, *44*).

Unlike the oxidation of other amino acid residues (except cysteine, which is absent in α-synuclein), the oxidation of Met is readily reversed by the enzyme methionine sulfoxide reductase (MSR), which catalyzes the thioredoxin-dependent reduction of Met sulfoxide residues of proteins back to Met (*48*). We have shown (unpublished data) that MSR will catalyze the reduction of Met sulfoxides in α-synuclein back to Met. Thus, a simple cycle of α-synuclein oxidation followed by reduction by MSR could provide an important antioxidant mechanism in neurons. A decline in MSR activity has been reported in the brain of Alzheimer's disease patients (*49*), but no comparable information is available for PD.

Our results indicate that a potentially important function of α-synuclein is as an antioxidant to minimize lipid oxidation in the presynaptic PUFA-enriched membranes: the loss of this activity in fibrillar α-synuclein suggests one component of neurodegeneration in the synucleinopathies.

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